

# Use of pJANUS<sup>TM</sup>-02-001 as a calibrator plasmid for Roundup Ready soybean event GTS-40-3-2 detection: an interlaboratory trial assessment

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**Abstract** Owing to the labelling requirements of food and feed products containing materials derived from genetically modified organisms, quantitative detection methods have to be developed for this purpose, including the necessary certified reference materials and calibrator standards. To date, for most genetically modified organisms authorized in the European Union, certified reference materials derived from seed powders are being developed. Here, an assessment has been made on the feasibility of using plasmid DNA as an alternative calibrator for the quantitative detection of genetically modified organisms. For this, a dual-target plasmid, designated as pJANUS<sup>TM</sup>-02-001, comprising part of a junction region of genetically modified soybean event GTS-40-3-2 and the endogenous soybean-specific lectin gene was constructed. The dynamic range, efficiency and limit of detection for the soybean event GTS-40-3-2 real-time quantitative polymerase chain reaction (Q-PCR) system described by Terry et al. (*J AOAC Int* 85(4):938–944, 2002) were shown to be similar for in house produced homozygous genomic DNA from leaf tissue of soybean event GTS-40-3-2 and for plasmid

pJANUS<sup>TM</sup>-02-001 DNA backgrounds. The performance of this real-time Q-PCR system using both types of DNA templates as calibrator standards in quantitative DNA analysis was further assessed in an interlaboratory trial. Statistical analysis and fuzzy-logic-based interpretation were performed on critical method parameters (as defined by the European Network of GMO Laboratories and the Community Reference Laboratory for GM Food and Feed guidelines) and demonstrated that the plasmid pJANUS<sup>TM</sup>-02-001 DNA represents a valuable alternative to genomic DNA as a calibrator for the quantification of soybean event GTS-40-3-2 in food and feed products.

**Keywords** Fuzzy logic · Genetically modified organisms · pJANUS · Real-time quantitative polymerase chain reaction · Validation

## Introduction

Genetically modified (GM) crops have become a reality in agriculture and the food/feed market [1, 2]. In many countries (e.g. in those of the European Union), to provide consumer information, threshold levels for labelling food and feed products containing materials derived from GM organisms (GMO) have been established [3, 4]. The detection, the identification and the quantification of the GMOs in certified seed lots and during the food/feed production chain is thus essential to properly fulfil downstream labelling and traceability requirements.

The European Union legislation and similar jurisprudence in a number of other countries (such as Japan and Brazil) [5, 6] foresees a labelling requirement on a threshold basis (e.g. labelling obligatory above 0.9%

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GMO in the European Union). Such labelling necessitates quantifying the GMO content on an ingredient-based regime, requiring compatible detection methods for an endogenous species marker on the one hand and a GMO-specific DNA sequence on the other hand [7]. To assess measurement uncertainties during the sample analysis process (e.g. from shipload to analytical report), any reference calibrator material applied for the purpose of quantifying a GMO in a product should introduce the lowest possible level of bias with respect to such quantitative analysis.

The key technology applied in GMO analysis is the polymerase chain reaction (PCR) [8] with its quantitative variant designated as “real-time quantitative polymerase chain reaction” [9] (here abbreviated as Q-PCR). PCR methods target and amplify a specific DNA sequence, rendering low levels of target DNA detectable and quantifiable by means of fluorescence. By carefully choosing the target DNA sequences and the appropriate PCR conditions, one can achieve generic or specific detection of GMOs (for a review see Holst-Jensen and Berdal [10]). With respect to GMO quantification, the Community Reference Laboratory for GM Food and Feed of the Institute of Health and Consumer Protection at the European Commission Joint Research Centre (Ispra, Italy) has to date organized the largest number of interlaboratory trials for validating such Q-PCR GMO detection methods [11]. In these interlaboratory trials, GMO quantification is performed by applying “calibration curves” of serial diluted standards consisting of genomic DNA containing the endogenous and transgene targets. A number of controls with a determined amount of GMO are then used to assess the feasibility of the method to be used in quantitative GMO analysis [7].

Most commonly used calibrators for quantitative GMO analysis are DNA solutions extracted from certified reference powders obtained from admixed GM grain samples. These powders are available (e.g. from the Institute of Reference Materials and Measurements, European Commission Joint Research Centre, Geel, Belgium [12]), and are certified for a certain weight percentage of a specific GM event, hence their name “certified reference materials” (CRMs). A number of drawbacks are, however, connected with the use of such CRMs. Firstly, their production from raw materials (mostly grains) necessitates a guaranteed permanent supply of adequate plant material from the GMO developer/notifier. Secondly, their manufacturing is tedious and expensive. Guaranteed supplies may be difficult for newly authorized GMOs and for GMOs that are being withdrawn from the commercial market. To date, only a limited working range (0–5%) of CRMs is available for only a restricted number of GM events [12]. Another critical issue connected with grain-derived CRM powders is the weight-percentage-based

certification. In hybrid crops (such as maize and cotton), the application of the recommended GMO unit, the so-called haploid genome equivalent (being the percentage of GM target sequences per taxon specific sequence) (EC decision 2004/787 [13]), may be biased in ploidy of the transgene according to the parental donor of the transgene copies (of either male or female origin). Finally, the use of CRMs as a source of calibrator standards represents a considerable cost in quantitative GMO analysis for the laboratories.

Next to seed powders, plasmid DNA containing a specific target DNA sequence has been shown to be suitable for calibration purposes [14–17]. Plasmids have several advantages over genomic DNA isolated from CRMs as calibrators: (1) plasmids are relatively easy to construct, (2) they can be rapidly propagated, upscaled and purified and (3), when frozen, they can be stored stably over long periods of time. Owing to the relatively low molecular weight of plasmids, manipulating them at high concentrations may pose a risk of laboratory contamination through aerosol formation. However, strict isolation of the production and manipulation of high-titre plasmid stock solutions and common good laboratory practices when handling plasmid solutions at low concentration (e.g. below 1,000 copies per microlitre) should allow the use of plasmids as a source of a suitable DNA template in PCR analysis, even in routine analysis.

The use of plasmids as calibrators in quantitative GMO detection has been described for different GM events in maize [17–19] and soybean [16]. Taverniers et al. [17] and Burns et al. [16] documented the use of single-target plasmids in quantifying GM soybean, based on the event-specific junction region isolated by Windels et al. [20]. The use of construct-specific GM soybean targets in combination with an endogenous marker was applied by Kuribara et al. [15]. Recently, substantial work was performed on the metronomic implications of the use of plasmids as quantitative reference calibrators [18, 19]. Together, these studies indicate that plasmids may well represent a suitable calibrator for quantitative GMO detection, but that ploidy remains an important issue.

In this study, the use of dual-target plasmids as calibrators in Q-PCR GMO analysis was further evaluated by an interlaboratory trial. For this purpose, a specific dual-target plasmid pJANUS™-02-001, suitable for quantifying Roundup Ready soybean event GTS-40-3-2, was developed. The Taqman Q-PCR system of Terry et al. [21] was chosen as the Q-PCR system. The description of the plasmid, the test materials, the calibrators and the outcome of the interlaboratory study is presented. On the basis of statistical analysis and fuzzy-logic interpretation, the commutability of genomic DNA or dual-target plasmid DNA as a quantifier calibrator is documented. Future research for

establishing dual-target plasmids as calibrators in Q-PCR GMO analysis in general is addressed.

## Materials and methods

DNA extraction, quantification, Q-PCR amplification, DNA sequencing and chemicals used

Genomic DNA was extracted using a standard cetyltrimethylammonium bromide (CTAB)-based protocol [22] and the DNA concentration was determined both spectrophotometrically (Bio-Rad Smartspec) and fluorimetrically by PicoGreen (Bio-Rad Versafluor).

Standard and real-time Q-PCR was carried out with a Bio-Rad iCycler using Amplitaq Gold (Applied Biosystems) and the buffers supplied and using GMO-detect master mix (Diagenode) or supermix (Bio-Rad), respectively.

Calf thymus DNA (Invitrogen) was used as carrier DNA for plasmid dilution. pUC18 plasmid was purchased from Invitrogen. All primers were purchased from Eurogentec (Oligold oligonucleotides).

DNase- and RNase-free water was purchased from both Agros Organics and Fluka. Calf intestine alkaline phosphatase was purchased from Sigma. EZ Load HT molecular weight markers (100–2,000 bp) were purchased from Bio-Rad. CTAB was obtained from Fluka.

Preparation of plant material for isogenic wild-type and GTS-40-3-2 leaf tissue and preparation of the unknown samples

Plants from isogenic wild-type and GTS-40-3-2 soybean seeds (also indicated as Roundup Ready soybean) were cultivated in a growth chamber under standard conditions (25°C, 16 h/8 h day/night regime, 80% humidity, 20,000 lux).

Leaf tissue from both GTS-40-3-2 and non-transgenic plants was subjected to CTAB extraction to obtain genomic DNA [22]. Preparation of plasmid DNA was performed using a Bio-Rad Quantum miniprep kit and purified plasmid DNA was quantified spectrophotometrically (high concentration, i.e. falling outside the PicoGreen working range) or by PicoGreen (low concentration). The genomic DNA stock concentration was adjusted to 20 ng $\mu$ l<sup>-1</sup> with Milli-Q water and the solution was stored at -20°C.

Description of the pJANUS™-02-001 dual-target plasmid

The dual-target plasmid, pJANUS™-02-001, was constructed in house as a pUC18 derivative into which two PCR target sites, separated by a short linker, were inserted at position 399 (*Hind*III site), bringing the total size of the

plasmid to 3,191 bp. One target site consists of a 360-bp fragment of the p35S/plant border of Roundup Ready soybean event GTS-40-3-2; the other target sequence consists of 125 bp of the soybean lectin gene (*Le1*) (accession numbers are given in “Appendix”). The Roundup Ready GTS-40-3-2 soybean junction amplicon matches the junction fragment described by Windels et al. [20]. The DNA sequence representing the soybean lectin gene target was chosen in house. The plasmid was propagated in TOP10F' cells (Invitrogen) and grown in liquid Luria–Bertani medium (Miller, Merck) containing 100 $\mu$ g ml<sup>-1</sup> ampicillin (Sigma). The plasmid was extracted using a Quantum midiprep kit according to the manufacturer's protocol (Bio-Rad) and was stored at -20°C.

Preparation of plasmid and genomic DNA stock solutions and calibrator standards

During the first steps, purified plasmid DNA was diluted in RNase- and DNase-free water in the absence of carrier DNA: the midiprep plasmid DNA (referred to as superior master stock) was quantified spectrophotometrically (Bio-Rad Smartspec) prior to dilution and stored at -80°C. The superior master stock was diluted firstly to a concentration of 50 pg $\mu$ l<sup>-1</sup> in Milli-Q water (master stock) in two steps (intermediate dilution to 5 ng $\mu$ l<sup>-1</sup>). The master stock was requantified by PicoGreen and the latter measured quantity of DNA applied as the starting concentration for the preparation of the working stock. The working stock was prepared by a two-step dilution of the master stock in Milli-Q water containing 4 ng $\mu$ l<sup>-1</sup> calf thymus DNA (final plasmid concentration at 5 fg $\mu$ l<sup>-1</sup> in a final working stock volume of 10 ml) (intermediate dilution to 5 pg $\mu$ l<sup>-1</sup>). The master stock and the working stock were prepared freshly and stored at 6°C. The stability of the plasmid stock solutions was verified by Q-PCR analysis and was found to be constant under the above-mentioned conditions for at least 2 weeks (standard deviation of the Ct value less than 0.25 Ct for 10,000 plasmid copies).

The standard with the highest plasmid copy number concentration (S1) was prepared by dilution of the working stock in RNase- and DNase-free water (containing 4 ng $\mu$ l<sup>-1</sup> of the carrier DNA). All subsequent standards (S2–S6) were prepared by serial dilution of standard S1 in RNase- and DNase-free water (containing 4 ng $\mu$ l<sup>-1</sup> of the carrier DNA).

The genomic GTS-40-3-2 soybean DNA standards were prepared in a similar fashion: the highest concentration standard (S1) was prepared directly from the genomic DNA stock, whereas subsequent standards (S2–S6) were prepared by serial dilution of standard S1. No carrier DNA was used for preparation of the genomic standards.

Three unknowns at 0.1, 1 and 5% GTS-40-3-2 soybean (percent haploid genome equivalents) were prepared by

admixing GTS-40-3-2 soybean genomic DNA with genomic DNA from non-transgenic soybean plants. The unknowns were prepared as a single large batch, mixed overnight at 4°C under constant agitation. Thirty aliquots of 200 µl of each unknown were prepared as test samples for the interlaboratory trial. The remainder of each unknown was stored separately and used to determine the “true values” of each unknown. Throughout the validation of the different materials received (master mix, primers, etc.) several quantifications of the unknowns were performed (Table 1) and the GMO value for each unknown (assigned value) was determined as the average of the different measurements (within run and between runs).

#### Performance of the interlaboratory GTS-40-3-2 trial

Twelve laboratories were provided with the test materials, as described above. A set of genomic DNA samples with unknown GMO content (U1–U3) were included. In addition, each laboratory received a 7-ml vial of 2× universal Taqman PCR master mix (Diagenode), nuclease-free water and the GMO-specific primers and probe (targeting the 35S/plant border region) and the soybean-specific primers and probe (targeting the lectin *Le1* gene). Primers and probes were provided lyophilized. All primers and probes were manufactured by Eurogentec (Oligold primer/probe quality) from a single batch and subsequently split into 12 vials. All materials were shipped on dry ice (DHL, same-day shipping). An Excel-based data report format was provided, allowing retrieval of all analytical data from all analyses.

All experimental analyses were performed between October 2007 and January 2008. All laboratories (but one) sent back their results including the raw data.

The GMO content of a sample was determined by the participating laboratories using real-time PCR relative to the endogenous reference. As a consequence, two calibration curves, transgenic and endogenous, were set up for each DNA calibrator of this relative quantification. The slope and the

efficiency ( $\epsilon$ ) of the PCR are related to each other by the equation based on conventional real-time PCR theory [23]:

$$\epsilon = \left( 10^{-\frac{1}{\text{slope}}} - 1 \right) \times 100.$$

#### Statistical evaluation

The similarity of the transgenic and the endogenous calibration curves obtained with each type of calibrator was investigated by comparing (means and variances) the efficiencies of the PCR and the linearity ( $R^2$ ) of the two calibration curves.  $F$  ratio and Student  $t$  tests were performed to investigate if variances and means between groups were similar. When the  $F$  test revealed significant differences ( $p < 0.05$ ), a Student  $t$  test assuming unequal variances was performed, using the Welch–Satterthwaite equation [24, 25] to calculate an approximation to the effective degrees of freedom. Otherwise, two-sided Student  $t$  tests assuming equal variances were used to investigate if the differences between groups were significant.

Percent genetic modification data of unknown samples were processed using procedures in SAS (version 9, SAS Institute, Cary, NC, USA). PROC UNIVARIATE provided an array of tests (Shapiro–Wilk, Kolmogorov–Smirnov, Cramer–von Mises and Anderson–Darling) for departure of the data distribution from normality, and graphic tools (box plot, stem-and-leaf representation) to detect outlying data. An ISO procedure [26] based on use of the Grubbs test and the Cochran test was run by the software program AMPE [27] to identify and discard outlying data prior to running analysis of variance (ANOVA). ANOVA is a technique for analysing experimental data in which one or more response (or dependent) variables are measured under various conditions identified by one or more classification factors. The variation in the response is separated into variation attributable to differences between the classification factors and variation attributable to random errors. An ANOVA constructs tests to determine the significance of the classification effects. A typical goal is to compare means of the response variables for various combinations of the classification factors. In this study, two-factor ANOVA was undertaken in SAS (PROC GLM) to investigate the effect on GMO values (response variable) of two classification factors—laboratory (1–12), source of the calibrator DNA (genomic, plasmid)—and their interaction. The two factors were arranged in a fully crossed experimental design. The Levene test was used to assess departure from homogeneous variances.

#### Interlaboratory validation

Three ISO validation measures [26] were computed: average bias ( $B$ , %), relative standard deviation of repeat-

**Table 1** Overview of datasets in the “true value” calculation for samples U1, U2 and U3 (expressed as % Haploid Genome Equivalents)

		U1	U2	U3
Plasmid DNA	No. of Ct values	78	162	78
	No. of runs	14	15	14
	Standard deviation (%)	0.94	0.95	0.16
	True values (%)	4.40	1.94	0.29
Genomic DNA	No. of Ct values	81	162	87
	No. of runs	14	15	14
	Standard deviation (%)	1.20	0.95	0.17
	True values (%)	4.60	1.94	0.29

ability ( $RSD_r$ , %) and relative standard deviation of reproducibility ( $RSD_R$ , %). The larger these values, the less well the method performs.

For assessing method performance, (1) efficiency measures from the PCR were considered ( $E_1$ , average PCR efficiency for the endogenous target;  $E_2$ , average PCR efficiency for the transgenic target) and (2) the number of reagents used to run the PCR (indicated as “score”) was used as a measure of the practicability of the methods.

Both validation and PCR measures were combined into groups of similar measures (accuracy, efficiency and practicability) and then aggregated to an indicator of method validity, based on an implementation of the fuzzy-logic-based principle for method validation (software program AMPE [27]) and using the design and settings described in Bellocchi et al. [28]. Accuracy derives from combining the three validation measures; efficiency is the combination of the two efficiency measures; practicability includes the score. The three groups of measures are combined into the final indicator. Both fuzzy-logic-based groups and indicators range between 0 (best performance) and 1 (worst performance).

## Results and discussion

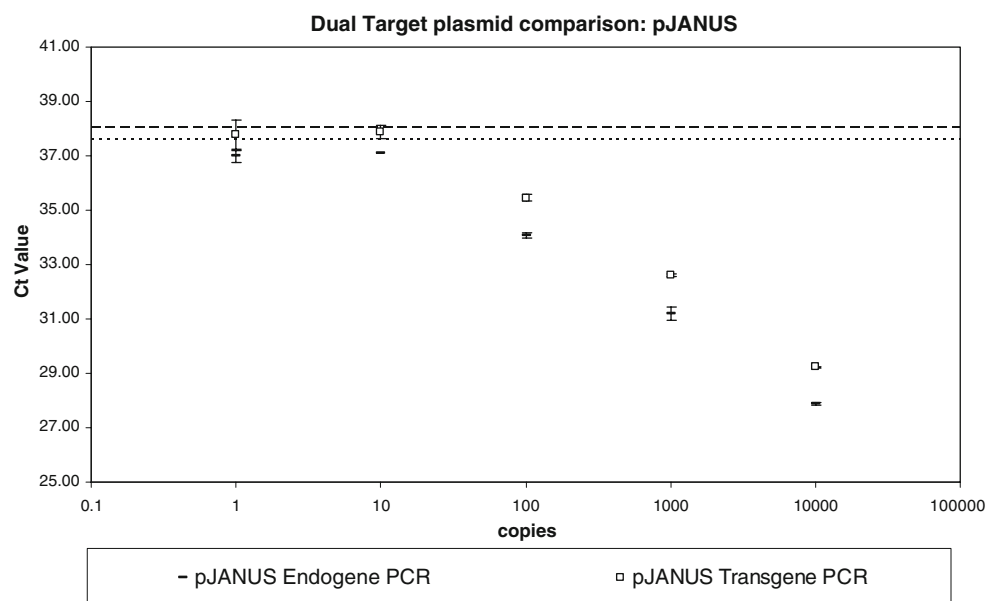
Preparation and assessment of pJANUS™-02-001 plasmid DNA and GTS-40-3-2 genomic DNA as Q-PCR calibrators and/or templates

A dual-target plasmid was constructed wherein a part of the endogenous lectin gene and a region spanning one of the insert junction regions of GTS-40-3-2 soybean are integrated

(see “Materials and methods”). An in-house Q-PCR efficiency estimation with the GTS-40-3-2 detection system described by Terry et al. [21] was performed on a linear dilution series spanning from about one to 100,000 plasmid copies (Fig. 1). The results show an acceptable linear correlation between 10 and 100,000 copies (endogene  $R^2=98.8$ ; transgene  $R^2=99.3$ ). The PCR efficiencies determined were within the limits as proposed by the European Network of GMO Laboratories (ENGL) guidelines (see Table 2). It was concluded that the pJANUS™-02-001 plasmid performs adequately for use as a calibrator template DNA in combination with the GTS-40-3-2 Q-PCR system of Terry et al. [21]. Note that this pJANUS™-02-001 is a versatile tool, allowing the use of other Q-PCR methods [29–31] for event-specific and endogene detection.

To limit bias in the Q-PCR determination, the reference material must fulfil the following characteristics: (1) the zygosity of the GM material and the wild-type material with respect to the applied targets has to be well established or homozygous by nature, (2) the GM material and the wild-type material should preferentially be isogenic and (3) the DNA should not be degraded to prevent bias by extensive loss of target copies. In most plants, leaf tissue obtained from isogenic GM and wild-type soybean plants would fulfil the first two requirements. For this, plants from both isogenic wild-type and Roundup Ready soybean were grown and leaf tissue was used to isolate the genomic DNA. CTAB-based methods are generally recognized as suitable for extracting soybean DNA for GMO quantification purposes [22, 32]. Indeed, agarose gel electrophoresis showed that the CTAB-purified genomic DNA from both wild-type and Roundup Ready soybean leaves migrates as a high molecular weight band (estimated at about 20 kb), without the presence of major degradation (visible as

**Fig. 1** The dynamic range of quantitative polymerase chain reaction (PCR) system of Terry et al. [21] with pJANUS™-02-001 plasmid as template DNA. Ct results from a dilution series spanning four log values are plotted against the number of copies involved in the reactions. The *dashed line* represents the endogene no template control (NTC) reaction, whereas the *dotted line* represents the transgene NTC reaction



**Table 2** Homozygosis assessment of GTS-40-3-2 soybean genomic DNA by polymerase chain reaction (PCR) analysis. Both for plasmid pJANUS<sup>TM</sup>-02-001 and GTS-40-3-2 soybean genomic DNA, different quantitative PCR (Q-PCR) analyses were performed to assess deviations

in measured Ct values. Neither the SYBR<sup>®</sup> Green/Taqman Q-PCR analysis of equal genomic DNA amounts nor the Taqman Q-PCR analysis of equal estimated haploid genome equivalents of plasmid and genomic DNA could demonstrate deviations from a 1:1 ratio

Primer	Target	SYBR <sup>®</sup> Green Q-PCR 50ng genomic DNA	Taqman Q-PCR			Efficiency (%)	
			50ng genomic DNA	HGE (gDNA)	10,000 plasmid copies	Genomic DNA	Plasmid
Sltm 1/2	Endogene	20.61±0.03	22.1±0.13	23.91±0.04	27.87±0.04	101	112
LHRRfor/RHRRrev	Transgene	21.87±0.29	24.5±0.39	26.27±0.30	31.1±0.12	96	90

All measurement were performed in duplicate.

background smears) (data not shown). The homozygosis of the GM soybean material with respect to the transgene was tested by Q-PCR analysis with both plasmid and genomic DNA as a template (Table 2). At an expected 1:1 ratio and assuming equimolar binding of SYBR<sup>®</sup> Green molecules to both amplicons, the  $\Delta C_t$  between endogene and transgene in plasmid and genomic DNA templates should be similar, taking into account PCR efficiencies for the different systems. The results of the Q-PCR analysis with the Q-PCR system of Terry et al. [21] point out that there is a 1:1 ratio between transgene and endogene targets in the Roundup Ready soybean leaf tissue genomic DNA.

Description of the commutability interlaboratory trial applying homozygous Roundup Ready soybean leaf tissue DNA and pJANUS<sup>TM</sup>-02-001 dual-target plasmid as calibrators

In total, 12 laboratories participated in the Roundup Ready soybean trial. Each laboratory ran two 96-well plate analyses with both transgene and endogene reactions; in one analysis 100% Roundup Ready soybean isogenic genomic DNA was used for construction of a calibration curve, whereas in the other analysis the pJANUS<sup>TM</sup>-02-001

plasmid DNA was used in a carrier DNA background. Standard curves consisted of six-point dilution series (non-linear, spanning from 15,600 copies down to about eight). Both analyses contained the same set of three unknown samples (at 4.5, 1.94 and 0.29% measured true values, see “Materials and methods”).

Comparison of PCR efficiencies and linearity of the calibrators in the interlaboratory trial

When genomic DNA was used as the calibrator, the estimated PCR efficiencies for both endogenous and transgenic targets were relatively low (Table 3). However, compared with the genomic DNA calibrator, the plasmid calibrator did not yield significantly higher efficiencies [ $p(t)>0.05$ ] for both the endogenous and the transgenic targets. With the endogenous target, PCR efficiencies from the genomic DNA showed a significantly larger variance [ $p(F)<0.05$ ] than those obtained by using the plasmid DNA.

The coefficient of determination ( $R^2$ ) provides information about the fitting of data to a linear calibration curve. Comparison of  $R^2$  values of the plasmid and genomic DNA calibration curves did not show significant differences for both endogenous and transgenic targets.

**Table 3** Comparison of the PCR efficiencies and linearity of the calibration curves of the genomic and plasmid DNA calibrators by means of a two-sided Student  $t$  test (comparison of means) and the  $F$  ratio test (comparison of variances) for endogenous and transgenic targets

Target sequence	Calibrator	Mean ± Standard error	Variance	$p(t)$ for difference in means	$p(F)$ for difference in variances
PCR efficiency (%)					
Endogenous	Genomic DNA	98.7±2.9	89.9	0.57	0.04
	Plasmid DNA	100.7±1.6	28.0		
Transgenic	Genomic DNA	91.5±2.3	57.4	0.35	0.24
	Plasmid DNA	95.0±2.9	91.9		
Linearity of the calibration curve ( $R^2$ )					
Endogenous	Genomic DNA	0.97±0.01	$1.3 \times 10^{-3}$	0.16	~0.00
	Plasmid DNA	0.99±~0.00	$7.6 \times 10^{-5}$		
Transgenic	Genomic DNA	0.97±0.01	$4.7 \times 10^{-4}$	0.27	0.89
	Plasmid DNA	0.98±~0.00	$2.1 \times 10^{-4}$		

**Table 4** Genetically modified organism (GMO) content from unknown samples: two-factor PROC GLM analysis of variance table

Source of variation	Degrees of freedom	Variance	<i>F</i>	<i>p</i> ( <i>F</i> )
Total	182	3.73		
Laboratory ( <i>L</i> )	10	1.85	0.50	0.89
DNA source ( <i>S</i> )	1	~0.00	~0.00	0.98
<i>L</i> × <i>S</i>	10	6.83	1.85	0.06
Error	161	3.70		

Also in this case, significantly larger variability [ $p(F)<0.05$ ] was observed with the genomic calibrator for endogenous DNA.

#### Comparison of GMO contents

Outliers were identified at four data sets and were discarded. The remaining GMO values obtained from the unknown samples were in general normally distributed, although in two cases a small deviation from normality ( $p<0.05$ ) was observed. Violations of the assumption of equal variances were not recognized. For this reason, a conventional parametric approach was applied in the fully crossed two-factor ANOVA. Table 4 shows the breakdown of the total variance into contributing components to perform statistical inferences for experimental factors (laboratory and DNA source) and their interactions. On the basis of the

data in Table 4, there are only random differences ( $p>0.05$ ) associated with either the laboratory or the DNA source. Also, non-significant interactive effects between the laboratory and the DNA source ( $p>0.05$  in Table 4) prove the stability of the use of both genomic DNA and plasmid DNA as a calibrator under different laboratory conditions.

#### Interlaboratory validation

A set of performance measures are reported in Table 5 as computed from the interlaboratory trial using estimated assigned values as true GMO contents. The precision measures are similar for genomic and plasmid DNA, indicating quite large variability ( $RSD_r >25\%$  and  $RSD_R >35\%$ , indicating precision measures exceeding the minimum performance requirements set by the ENGL [7]).

**Table 5** Basic and fuzzy-logic-based aggregated performance metrics for genomic and plasmid calibrator DNA, evaluated at three GMO levels

Validation/efficiency measures (and fuzzy-logic-based groups and indicator)		Assigned GMO level		
Genomic		0.28%	2.00%	4.32%
Basic measures	Average bias (%)	16.2	7.8	0.5
	Relative standard deviation repeatability ( $RSD_r$ (%))	32.5	26.1	31.3
	Reproducibility standard deviation repeatability ( $RSD_R$ (%))	50.3	53.2	36.4
	Score	6		
	Reference gene efficiency (%)	98.7		
	Target gene efficiency (%)	91.5		
Fuzzy-logic-based aggregated measures	Accuracy	0.757	0.750	0.750
	Practicability	0.000		
	Efficiency	0.465		
	Indicator	0.575	0.569	0.569
Plasmid		0.23%	1.84%	3.67%
Basic measures	Average bias (%)	16.2	18.1	6.0
	Relative standard deviation repeatability ( $RSD_r$ (%))	34.2	25.9	32.8
	Reproducibility standard deviation repeatability ( $RSD_R$ (%))	44.2	78.4	37.3
	Score	6		
	Reference gene efficiency (%)	100.7		
	Target gene efficiency (%)	95.0		
Fuzzy-logic-based aggregated measures	Accuracy	0.757	0.798	0.750
	Practicability	0.000		
	Efficiency	0.141		
	Indicator	0.527	0.547	0.523

Whether the average bias tends to be higher when the plasmid DNA is used as a calibrator (especially for higher GMO contents), the values for the accuracy are similar. Higher PCR efficiencies yielded by the plasmid DNA are reflected in better values for the efficiency (0.141 against 0.465). However, for the target gene, the efficiencies are less than the 0.98 limit as set by the ENGL as the acceptance criterion.

Overall, the final values of the aggregated indicator are more than 0.500, with the plasmid calibrator performing only marginally better (0.523–0.547) than the genomic DNA (0.569–0.575).

## Conclusions

The dual-target plasmid pJANUS<sup>TM</sup>-02-001 was shown in an interlaboratory study to be equally suitable as genomic DNA as a calibrator for quantitative analysis of Roundup Ready soybean event GTS-40-3-2. Two-factor ANOVA revealed a homogeneous pattern of responses (no significant differences) between genomic and plasmid DNA sources and when both types of DNA are applied in a variety of laboratories as quantitative calibrators. Fuzzy logic interpretation of the outcomes, a robust approach for uncertain and imprecise data incorporating expert decisions, was applied to complement the statistical analysis with expert judgement on the method performance (via synthetic indicators) for different calibrators and GMO concentrations. On the basis of both statistical results and synthetic indicators, this dual-target plasmid can be considered as a calibrator that performs equally as well as genomic DNA.

As indicated before, general use of such dual-target plasmids, wherein a 1:1 ratio between the endogene and transgene targets is *de facto* established, will depend on the determination of the measurement uncertainty in crops where the zygosity between the two types of standards is not constant (e.g. maize). A recent approach described by Corbisier et al. [33], applying digital PCR to precisely determine copy numbers of DNA templates (both plasmid and genomic DNA) could greatly facilitate such measurements and allow for the definition of an average correlation factor between the crop genomic DNA and the plasmid dual-target marker.

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## Appendix

Accession numbers (EMBL/GENBANK):

- AJ308514 (synthetic construct for p35S promotor/plant junction region), position 1-359 for pJANUS<sup>TM</sup>-02-001
- K00821 [soybean lectin (*Le1*) gene, complete cds], position 1208-1332 for pJANUS<sup>TM</sup>-02-001

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