Development and Validation of *gmgba2*, a Novel Soybean Endogenous Reference Assay for Qualitative and Quantitative PCR Detection

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Objective

A novel soybean (*Glycine max*) endogenous references assay, *gmgba2*, was developed and validated using qualitative gel-based and quantitative real-time PCR methods. The assay was evaluated for sensitivity, specificity, and assay performance to meet the criteria proposed and described in the European Network of GMO Laboratories technical report regarding analytical methods (1) while taking into account country-specific requirements for reference assays used in GMO testing. The minimum acceptance criteria to meet both sets of guidelines includes:

- Sensitivity (dynamic range): ≤ 10 and ≥ 56,000 haploid genome copies based on the haploid genome weight of 1.13 pg (2)
- Limit of Detection (LOD): ≤ 10 copies with a level of confidence of 95 %
- Specificity: no amplification in samples that do not contain soybean DNA
- Efficiency and Fit: Average slope is in the range of -3.1 ≥ slope ≥ -3.6 with PCR efficiencies of 110 % to 90 % and R^2 value ≥ 0.98
- Precision: RSD_r of ≤ 25 % over the whole dynamic range
- Trueness (BIAS): ± 25 % of the reference value over the dynamic range

Material and Methods

DNA Extraction. Genomic DNA was extracted from seeds or leaf tissue of soybean and 14 additional species using a Fast ID Extraction kit (Genetic ID).

Identification of candidate sequences for soybean assay development. ~ 230 sequences of the internally developed soybean single copy SNP markers compared to the soybean reference genome at Phytozome v10 (3). An RNA polymerase II transcription subunit (hereafter, designated as *gmgba2*; XM_014765306) was identified and used for assay development.

Detection methods. Primers and probes were designed using Vector NTI v. 11 (Thermo Fisher Scientific) or Primer 3.0 software (Life Technologies). PCR was performed using a HotStar Taq PCR Master Mix Kit (Qiagen) (qualitative PCR) and a LightCycler® 480 real-time PCR instrument (Roche) using Probes Master reaction mix (Roche) (quantitative PCR).

Specificity and Sensitivity of internal control assay
Soybean DNA diluted from 100 ng to 0.01 ng (88496 to 9 copies) was used for sensitivity testing. To evaluate specificity, genomic DNA of soybean and 14 additional species were use as template. For LOD determination, 60 replicates with 9 copies as template were used in the quantitative assay.

Assay Performance. Calibration samples ranging from 88496 to 9 soybean copies were run in triplicate in four independent runs to create a reference curve. Reference standards diluted from 88496 to 44 copies were tested in triplicate, with four replicates per plate, and four independent runs. Ct values were converted to copy number using the linear equation in Figure 5.

Further Testing

In addition to the outlined methods, additional testing will be done to fully validate the *gmgba2* endogenous reference assay:

- Allelic specificity testing by qualitative gel-based PCR, quantitative real-time PCR, and Southern blot analysis using 14 soybean lines
- Robustness evaluation using a multifactorial experimental design entailing 16 total combinations of deviation
- Biplex performance using spiked GMO samples

Sensitivity and Limit of Detection

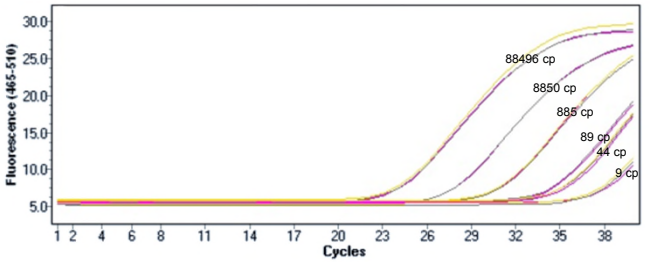

Figure 1. Sensitivity of the quantitative *gmgba2* assay using soybean DNA diluted from 88496 to 9 haploid genome copies (100 to 0.01 ng) per reaction

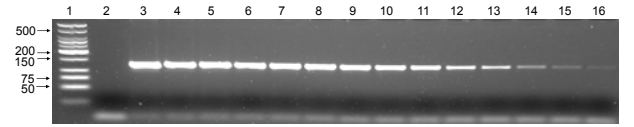

Figure 2. Sensitivity of the qualitative *gmgba2* assay using soybean DNA serially diluted from 88496 to 10 copies (100 ng to 0.0122 ng) per reaction.

9 cp (LOD)	34.7	34.4	34.0	34.3	34.4	34.4	34.3	35.0	34.5	33.3	all positive (Ct ≤ 40 in all reactions)
	33.9	34.4	33.0	33.9	32.5	34.9	34.6	34.5	33.9	34.5	
	33.6	33.6	33.6	33.6	34.6	34.6	34.6	33.9	34.8	34.9	
	33.8	34.3	33.5	33.5	34.0	34.0	33.5	33	33.5	33.6	
	33.8	33.6	33.7	33.9	34.0	33.8	33.6	33.8	34.5	33.7	
	32.7	33.9	33.6	33.5	34.6	33.8	33.5	33.5	34.2	32.0	

Table 1. Ct values from 60 replicates of soybean DNA diluted to 9 copies (0.01 ng).

Specificity

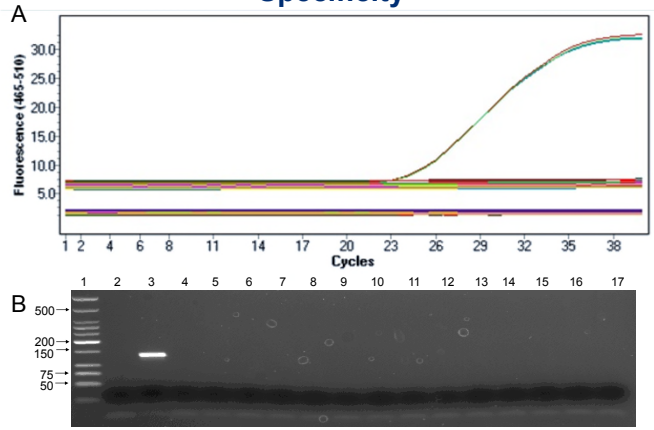

Figure 3. Soybean specificity was performed using the quantitative (A) and qualitative (B) *gmgba2* assays against *Glycine max* (soybean), *Arabidopsis thalania*, *Arachis hypogaea* (peanut), *Brassica napus* (canola), *Cicer arietinum* (garbanzo bean), *Gossypium hirsutum* (cotton), *Hordeum vulgare* (barley), *Lens culinaris* (lentil), *Medicago sativa* (alfalfa), *Nicotiana tabacum* (Tobacco), *Oryza sativa* (rice), *Phaseolus lunatus* (lima bean), *Pisum sativum* (pea), *Triticum aestivum* (wheat), and *Zea mays* (corn). Soybean showed positive results as expected. No amplification was obtained in closely related or non-related species.

Assay Performance

	Slope	Intercept	R^2	Efficiency
Run 1	-3.35	37.7	1.00	0.99
Run 2	-3.27	37.4	1.00	1.02
Run 3	-3.34	37.5	1.00	0.99
Run 4	-3.35	37.4	1.00	0.99
MEAN	-3.3275	37.5	1.00	1.00

Table 2. Results from four independent qPCR runs using soybean DNA diluted from 88496 to 9 haploid genome copies

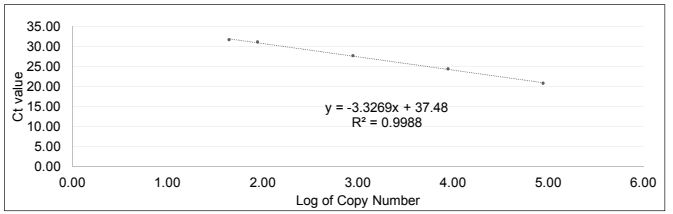

Figure 5. Mean Ct values from four independent runs plotted against the logarithm of the haploid genome copy number

Soybean haploid genome cp	Run 1	Run 2	Run 3	Run 4	Mean (cp)	BIAS (%)	STDEV (cp)	RSD_r (%)
88496	87809	98773	105122	103676	94747	7%	12319.0	13.0%
	82127	87003	84432	87003				
	68602	89033	99230	112915				
	87809	109074	110850	102487				
8850	7808	8252	7404	8157	8944	1%	1755.9	19.6%
	7665	7438	8157	9833				
	7935	7971	8139	9902				
	8803	12645	9788	13212				
885	831	812	893	866	892	1%	108.7	12.2%
	808	1167	1011	1000				
	807	823	939	990				
	751	772	899	899				
89	86	86	92	95	101	13%	25.0	24.7%
	110	79	108	117				
	70	70	85	104				
	115	113	121	172				
44	44	50	48	46	44	0%	5.9	13.5%
	41	42	46	57				
	37	37	41	42				
	35	46	44	54				

Table 1. Soybean DNA diluted from 88496 to 44 haploid genome copies (100 ng to 0.05 ng) was tested in triplicate, with four replicates on each plate, and four independent runs. Ct values were converted to copy number using the linear equation generated in Figure 5. The copy number, bias, standard deviation, and RSD_r were calculated and met the acceptance criteria.

Summary

The *gmgba2* qualitative gel-based and quantitative real-time PCR assays were found to be highly sensitive and specific with assay performance in compliance with the stringent requirements of regulatory agencies globally. Additional testing will confirm the *gmgba2* target and assays as a reliable method to complement assays used for the detection of GMO crops.

References

(1) European Network of GMO Laboratories (ENGL), 2015. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. Ispra, Italy. European Commission Joint Centre.
(2) Arumuganathan, K., Earle, E.D., 1991. Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9: 208–218.
(3) Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., and Rokhsar DS, 2012. Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 2012 40 (D1): D1178-D1186