Title

Confirmation of the absence/presence of vector backbone sequences in *Glycine max* transformation event FG72

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Completed On

June 28th, 2010

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STUDY IDENTIFICATION PAGE

Study initiation date:	27/03/2009
Experimental start date:	01/04/2009
Experimental Termination date:	08/10/2009
Study Completion date	02/11/2009
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SUMMARY

Bayer Bioscience N.V. has introduced a *2mepsps* gene construct, encoding tolerance to glyphosate, and a *hppdPf W336* gene construct, encoding tolerance to isoxaflutole, in *Glycine max* by means of direct gene transfer.

This study was undertaken to confirm the absence of vector backbone in the *Glycine max* transformation event FG72.

For the purpose of this study, genomic DNA was isolated from transgenic FG72 plants. To assess the presence or absence of vector backbone, the isolated DNA samples were subjected to Southern blot analysis using two overlapping vector backbone probes covering the complete vector backbone of the pSF10 transformation vector. Since part of the sequence of both vector backbone probes is also present in the T-DNA sequences, fragments originating from inserted transgenic sequences hybridized with vector backbone probes.

No other than the expected fragments were obtained when hybridizing with vector backbone probes, confirming the absence of vector backbone in transformation event FG72. Re-hybridizing the membranes with a T-DNA probe, showed all expected FG72 hybridization fragments confirming that the experimental conditions were adequate for detecting integrated DNA fragments.

1. OBJECTIVE

Bayer BioScience N.V. has introduced a *2mepsps* gene construct, encoding tolerance to glyphosate, and a *hppdPf W336* gene construct, encoding tolerance to isoxaflutole, in *Glycine max* by means of direct gene transfer. In this study, the absence of vector backbone in the *Glycine max* transformation event FG72 was investigated by means of Southern blot analysis using overlapping vector backbone probes.

2. OVERVIEW OF EXPERIMENTAL DESIGN

Genomic DNA, prepared from transgenic FG72 plants and from wild type *Glycine max* plants (variety JACK), was digested with different restriction enzymes. The resulting DNA fragments were separated by agarose gel-electrophoresis, transferred to a membrane (blot) and sequentially hybridized with one of the two overlapping probes, covering the complete vector backbone of the transforming vector pSF10, and a T-DNA probe. The hybridization with the vector backbone probes demonstrates the absence of vector backbone, while the control hybridization assures that the experimental conditions for this Southern Blot analysis allowed an effective detection of the integrated DNA fragments in FG72.

3. TEST ITEM, REFERENCE ITEMS AND STANDARDS

3.1. Test item

The test item in this study was leaf material taken from plants of *Glycine max* transformation event FG72 (Test item IDs: T33-01 and T33-02).

The certified seed lot 32RRMM0497 (CoA n° MDA-09-002) was used to produce the FG72 leaf material. This seed lot was produced by Jan Aelvoet, reference material management, Bayer BioScience N.V..

The test items T33-01 and T33-02 were respectively produced by Hans Ronsse and Anja De Boever (Bayer BioScience N.V. – Research Station, Astene). FG72 plants were grown according to respectively greenhouse protocol 08BAGM0013 and 09BAGM0007. Harvested tissues were directly frozen in liquid nitrogen and transported on dry ice. Upon arrival at the Bayer BioScience N.V. test facility, the frozen leaf material was stored in the ultrafreezer until DNA preparation. Identification of the test item was performed by Molecular Diagnostics Applications – Bayer BioScience N.V.. The identification documents (CoA n° MDA-08-009b and MDA-09-010b) are presented in Appendix 3 and Appendix 4.

Test Item ID:	T33-01
Test Item Identity:	leaf material taken from plants of *Glycine max* transformation event FG72
Test Item generation:	T7
Batch n°:	08BAGM0013
Seed lot n°:	32RRMM0497
Expiry date:	November 7th, 2018

Test Item ID:	T33-02
Test Item Identity:	leaf material taken from plants of *Glycine max* transformation event FG72
Test Item generation:	T7
Batch n°:	09BAGM0007
Seed lot n°:	32RRMM0497
Expiry date:	May 18th, 2019

3.2. Reference items

During this study, two reference items are used:

3.2.1. Leaf material taken from non transgenic plants of *Glycine max* variety JACK

Non transgenic *Glycine max* plants variety JACK were grown and sampled in the Bayer BioScience N.V. greenhouse (BioScience N.V. – Research Station, Astene, Belgium) by Hans Ronsse according to greenhouse protocol 08BAGM0018. Harvested tissues were directly frozen in liquid nitrogen and transported on dry ice. Upon arrival at the Bayer BioScience N.V. test facility, the frozen leaf material was stored in the ultrafreezer until DNA preparation. Identification of this reference item was performed according to quality standard SQ-STD-GM902 by Molecular Diagnostics Applications – Bayer BioScience N.V..

Reference item ID: R17-01
Reference item Identity: Leaf material taken from non-transgenic *Glycine max* plants, variety JACK
Batch n°: 08BAGM0018
Seed lot n°: 32CON0521
Expiry date: November 7th, 2018

3.2.2. *E. coli* cells containing pSF10 plasmid DNA (Figure 1)

E. coli cells strain DH5α containing the pSF10 plasmid (Reference item ID: R18-01) were supplied by Kristel D'hont (Bayer BioScience N.V. – Research, Trait Biology, Gent). Five ml liquid LB medium containing 100 µg/mL *triacillin* was inoculated with one colony of these *E. coli* cells. The cell culture was grown overnight at 37°C while shaking at ca. 200 rpm.

Two glycerol cell stocks were prepared by combining 500 µL of the cell suspension with 500 µL sterile 80% glycerol. One tube was used for archiving; the other was used as the working stock. Both stocks were stored in the ultrafreezer.

The identity of the *E. coli* cells containing the pSF10 plasmid was confirmed by performing 13 different PCR reactions according to SOP BBS 07/21/03 with a reaction mixture and thermocycling profile as described in Appendix 1.

Table 1 gives an overview of the primer combinations used, the primer sequences, the position in the pSF10 plasmid and the expected and obtained amplicon sizes.

Reference item ID: R18-01
Reference item identity: *E. coli* cells containing pSF10 plasmid DNA
Batch number: E11026-1
Expiry date: April 7th, 2019

3.3. Standards

3.3.1. Probe templates

The two overlapping probes covering the vector backbone of pSF10 (PT056-1 and PT057-1) and the T-DNA probe (PT058-1 to PT058-6) used to perform the hybridizations (Figure 2) are standards. All information about the probe templates is listed in Table 3.

3.3.2. λ DNA digested with *Pst*I or with *Hin*dIII.

When genomic DNA of the bacteriophage Lambda is digested with the restriction enzyme *Pst*I or *Hin*dIII, restriction fragments of known size are obtained which are used as molecular weight markers.

The λ DNA is supplied by Promega. λ-*Pst*I digested and λ-*Hin*dIII digested are prepared in the Bayer BioScience N.V. Test Facility. The stock solutions and the working solutions of λ-*Pst*I digested and of λ-*Hin*dIII digested are stored in respectively the freezer and the refrigerator.

Concentration of λ-*Pst*I digested: 100 ng/µL
Concentration of λ-*Hin*dIII digested: 100 ng/µL
Batch numbers of λ-*Pst*I digested used in this study: λ-*Pst*I-27 → λ-*Pst*I-32
Batch numbers of λ-*Hin*dIII digested used in this study: λ-*Hin*dIII-04 → λ-*Hin*dIII-06

4. EXPERIMENTAL DESIGN

4.1. Preparation and concentration determination of pSF10 plasmid DNA

Two batches of pSF10 plasmid DNA (PL007-1 and PL007-2) were prepared according to SOP BBS 07/18/03. The identity of these plasmid preparations was confirmed by performing 7 restriction digestions according to SOP BBS 07/20/03. The identity of the pSF10 plasmid DNA was confirmed if in at least 5 restriction digests the fragments of expected sizes are obtained, or unexpected fragments can be explained as being partial digestions, based on their sizes. Aliquots of the 7 restriction digestions were loaded together with undigested plasmid DNA and 100 bp ladder (GE Healthcare) and λ DNA digested with *Pst*I as molecular weight markers on a 1% TAE agarose gel according to SOP BBS 07/25/02. Determination of fragment sizes was done by visually comparing with the known fragments of the molecular weight markers.

To determine the concentration of the plasmid preparations, the concentration of one of the restriction digests was measured according to SOP BBS 07/19/03.

Table 2 shows the plasmid preparations, their identity confirmation results, the concentration and the expiry dates.

4.2. Preparation and concentration determination of genomic DNA

Total genomic DNA isolation from FG72 and from the wild type JACK leaf material, DNA quality control and DNA concentration determination was performed according to SOP BBS 07/16/03. Some genomic DNA samples were pooled and the concentration of these pooled DNA samples was determined mathematically, based on the concentration and the volume of the individual samples that are pooled. An overview of the DNA isolations prepared in this study and DNA preparations of study BBS09-005 that were used in this study as well is presented in Table 4.

4.3. Preparation of probe templates

Probe templates PT056-1 and PT057-1 were prepared by means of PCR amplification according to SOP BBS 07/21/03 with a reaction mixture and thermocycling profile as described in Appendix 1. Information on the primers used to prepare these probe templates is given in Table 3. In order to prepare a sufficient amount of probe template, at least 30 identical PCR reactions were performed for each probe template preparation.

After amplification, all identical PCR reactions were pooled and the volume of this pool was reduced by means of evaporation (SOP BBS 04/29/02). The pooled and evaporated samples were loaded on an agarose gel according to SOP BBS 07/25/02 and the fragments of the correct size for each probe were cut out of the gel and purified according to SOP BBS 07/27/04.

Six batches of the T-DNA probe template PT058 (PT058-1 to PT058-6) were prepared by double restriction digestion of the pSF10 plasmid with *Sac*I and *Sma*I according to SOP BBS 07/20/03. Some samples were pooled after incubation. An aliquot of the (pooled) digest was loaded on a 1% TAE agarose gel (SOP BBS 07/25/02) to check if digestion is complete. To purify the probe template, the complete reaction mixture of the restriction digestion was loaded on a 1% TAE agarose gel (SOP BBS 07/25/02), the fragment of 7204 bp was cut out of the gel and the probe template was isolated from the gel slice according to SOP BBS 07/27/04 (PT058-1 and PT058-2) or according to SOP BBS 07/27/05 (PT058-3 to PT058-6). For some

preparations an additional unexpected fragment was seen after double restriction digestion. To make sure the correct fragment was cut out of gel, the purified probe templates were checked for identity by restriction digestion of an appropriate amount with *Alw*NI (SOP BBS 07/20/03). The complete digest was checked on a 1% TAE submarine gel (SOP BBS 07/25/02) next to undigested probe template, undigested pSF10 and λ-*Pst*I as molecular weight marker. Two fragments of 1713 bp and 5491 bp were observed, which confirmed the identity of probe templates PT058-3, PT058-4 and PT058-6.

To verify the quality and the concentration of the prepared probe templates, appropriate amounts were loaded on a 1% TAE agarose gel next to a High and/or Low DNA Mass Ladder (Invitrogen) according to SOP BBS 07/25/02. The concentration of the probes was determined using the GeneTools Software (SOP BBS 07/42/02). The fragment sizes were determined by comparing with the known fragment sizes of the High and/or Low DNA Mass Ladder.

The concentration of probe templates PT058-1, PT058-2 and PT058-5 was too low for hybridization. These probe templates were not used. All information on the used probe templates is listed in Table 3.

4.4. Restriction digestion of total genomic DNA

Genomic DNA prepared from FG72 plants was digested with 2 different restriction enzymes: *Hind*III and *Hinc*II. Genomic DNA prepared from wild type plants was digested with the *Hind*III restriction enzyme. All restriction digests were performed according to SOP BBS 07/20/03.

4.5. Restriction digestion of pSF10 plasmid DNA

Restriction digestions of pSF10 DNA (PL007-1) with *Hind*III were performed according to SOP BBS 07/20/03 in this study and in study BBS09-005. Completion of the restriction digestion was confirmed by loading an appropriate amount on a 1% TAE agarose gel (SOP BBS 07/25/02).

4.6. Concentration determination of the digested samples

The concentration of each digested genomic DNA sample and of the digested pSF10 plasmid DNA was determined by means of the PicoGreen® method according to SOP BBS 07/19/03. The concentrations of all samples that are loaded on the same gel (FG72 genomic DNA, wild type genomic DNA and digested pSF10 plasmid DNA) were determined in the same measurement.

4.7. Non-submarine agarose gel electrophoresis

Seven 1% TAE agarose gels were prepared according to SOP BBS 07/26/06.

Each gel used for the Southern blot analysis contained:

- FG72 genomic DNA digested with *Hinc*II or digested with *Hin*dIII;
- A DNA negative control: genomic DNA of wild type plants digested with *Hind*III. The negative control was used to confirm the absence of background hybridization;
- DNA positive controls: genomic DNA of wild type plants digested with *Hind*III and supplemented with a 0.05x and a 0.5x or a 0.1x and a 1x the equimolar amount of pSF10 plasmid DNA digested with *Hind*III. This control was used to demonstrate that the hybridizations were performed under conditions allowing hybridization of the probes with the target sequences;
- λ-*Pst*I digested and λ-*Hin*dIII digested as molecular weight markers. These molecular weight markers covered the appropriate size range of the hybridization fragments that were expected to be detected by means of Southern blot analysis.

To prepare the DNA positive control, an appropriate dilution of the *Hin*dIII digested pSF10 plasmid DNA was made and added to *Hin*dIII digested wild type genomic DNA. With a single copy integration of the transgene into the *Glycine max* genome, ten µg of genomic heterozygous DNA (*Glycine max* genome size: 2.2 x 10^9 bp (Arumuganathan K., 1991)) corresponds to approximately 47.3 pg of pSF10 plasmid DNA (pSF10 size: 10398 bp).

Electrophoresis was performed according to SOP BBS 07/26/06.

4.8. Loading sequence of the gel

Gel IDs: NG/09-003/01, NG/09-003/02, NG/09-003/03 (*)

Lane 1:	λ-*Pst*I digested
Lane 2:	λ-*Hind*III digested
Lane 3:	*Glycine max* transformation event FG72 – *Hind*III digested
Lane 4:	*Glycine max* transformation event FG72 – *Hinc*II digested
Lane 5 (7):	*Glycine max* wild type variety JACK – *Hind*III digested
Lane 6 (5):	*Glycine max* wild type variety JACK – *Hind*III digested + a ca. 0.05 equimolar amount of pSF10 – *Hind*III digested
Lane 7 (6):	*Glycine max* wild type variety JACK – *Hind*III digested + a ca. 0.5 equimolar amount of pSF10 – *Hind*III digested
Lane 8:	λ-*Hind*III digested
Lane 9:	λ-*Pst*I digested

(*) The lane numbers for NG/09-003/03 is mentioned between brackets

NG/09-003/04 to NG/09-003/07

Lane 1:	λ-*Pst*I digested
Lane 2:	λ-*Hind*III digested
Lane 3:	*Glycine max* transformation event FG72 – *Hind*III digested
Lane 4:	*Glycine max* transformation event FG72 – *Hinc*II digested
Lane 5:	*Glycine max* wild type variety JACK – *Hind*III digested
Lane 6:	*Glycine max* wild type variety JACK – *Hind*III digested + a ca. 0.1 equimolar amount of pSF10 – *Hind*III digested
Lane 7:	*Glycine max* wild type variety JACK – *Hind*III digested + a ca. 1 equimolar amount of pSF10 – *Hind*III digested
Lane 8:	λ-*Hind*III digested
Lane 9:	λ-*Pst*I digested

After completion of the agarose gel electrophoresis, photographs were taken from each gel according to SOP BBS 04/77/01.

4.9. Blotting

Transfer of the separated DNA fragments from the agarose gel to a membrane (GE Healthcare) was performed according to SOP BBS 07/30/02 (Alkali Blotting, Hybond-XL membrane, M/09-003/01 till M/09-003/03) or according to SOP BBS 07/32/00 (Neutral Blotting, Hybond N^+ membrane, M/09-003/04 till M/09-003/07).

A photograph was taken from the agarose gels after blotting to check whether the DNA fragments were completely transferred to the membranes (SOP BBS 04/77/01).

4.10. Hybridization

Probe templates were labeled with [α-^{32}P]-dCTP according to SOP BBS 07/33/03.

Hybridization and washing steps were performed according to SOP BBS 07/34/03.

Visualization of the hybridizing fragments is achieved after exposure of a BioMax MR film, according to SOP BBS 07/35/02. Films were developed using the Fuji Medical Film Processor FPM100A (SOP BBS 04/73/01).

Probes were removed from the membranes by stripping (BBS 07/36/02).

4.11. Determination of hybridization fragment sizes

The size of the hybridization fragments obtained with the membranes M/09-003/06 and M/09-003/07 were determined using the GeneTools software, according to SOP BBS 07/42/02. In order to validate this method, the length of obtained fragment(s) of the pSF10 plasmid digested with *Hin*dIII were determined at least once. The data are acceptable in case the determined fragment size of these fragments differs by less than 10% from the expected value.

If the size of the obtained FG72 fragments differed less than 10% from that of the expected fragment sizes, the expected fragment size was reported. Size determination for fragments of more than 14 Kb is not possible with this experimental setup. These fragments are noted as '>14kb'.

5. RESULTS AND DISCUSSION

To demonstrate the absence of vector backbone DNA in the transformation event FG72, Southern blot analysis was performed. Because of poor quality, results of the hybridizations performed on membranes M/09-003/01 till M/09-003/05 are not presented in this report.

Membrane M/09-003/06 was sequentially hybridized with vector backbone probe PT056-1 and with T-DNA probe PT058-6. Membrane M/09-003/07 was sequentially hybridized with vector backbone probe PT057-1 and with T-DNA probe PT058-6. The obtained hybridization results are presented in Figure 3 and Figure 4. Expected and obtained hybridization fragments are listed in Table 5. A number of fragments did run atypically on the agarose gel. This is probably caused by secondary DNA structures.

Since both vector backbone probes contain a number of regions also present in the T-DNA sequence, several fragments originating from inserted transgenic DNA were hybridizing with vector backbone probes. Only the expected fragments, based on sequence homology, were obtained when hybridizing with vector backbone probes. This demonstrates the absence of vector backbone.

After hybridization with the T-DNA probe, the expected internal 947 bp *Hin*dIII fragment was visible for the genomic FG72 DNA samples, while this fragment was not visible in the positive control. Also the 0.1x equimolar amount of *Hin*dIII digested pSF10 plasmid DNA could not be visualized after hybridization with the T-DNA probe while it could be visualised after hybridization with the vector backbone probes. However, these results have no influence on the final interpretation of the Southern blot analyses.

The expected Southern blot profile was obtained in the FG72 samples after hybridization with the T-DNA probe. This demonstrates that an adequate amount of a sufficient quality of FG72 genomic DNA is loaded on the gels to be able to detect the eventual presence of vector backbone sequences in the FG72 event.

6. CONCLUSION

To demonstrate the absence/presence of vector backbone in the *Glycine max* transformation event FG72, Southern blot analyses were performed. pSF10 backbone hybridization signal was absent in the FG72 genomic DNA samples. In the DNA positive controls and FG72 genomic DNA samples, expected hybridization fragments were obtained after hybridization with the different vector backbone probes. These results demonstrate the absence of vector backbone in the *Glycine max* transformation event FG72.

7. ARCHIVING

The study plan, the amendments and deviations, the original final report and the raw data will be archived under the study number BBS09-003 in the GLP Test Facility Archives of Bayer BioScience N.V.

Samples of the test items T33-01 and T33-02 and of the reference items R17-01 and R18-01 were archived in ultrafreezer of the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium.

Table 1: Information on primers used for confirmation of *E. Coli* cells containing pSF10

Primer ID	Primer sequence (5' → 3')*	Position in pSF10	Expected amplicon size (bp)	Amplicon obtained (Y/N)
STV063 MLD149	**Critical Confidential Information removed**	bp 6425 → bp 6443 bp 7445 ← bp 7464	1040	Y
STV281 STV283	**Critical Confidential Information removed**	bp 6408 → bp 6426 bp 7419 ← bp 7439	1032	Y
MLD148 MLD071	**Critical Confidential Information removed**	bp 7446 → bp 7465 bp 8455 ← bp 8474	1029	Y
SMP084 SMP071	**Critical Confidential Information removed**	bp 8309 → bp 8329 bp 9601 ← bp 9620	1312	Y
SMP100 MVW019	**Critical Confidential Information removed**	bp 9347 → bp 9374 bp 69 ← bp 87	1139	Y
DPA308 YTP232	**Critical Confidential Information removed**	bp 10296 → bp 10315 bp 1141 ← bp 1159	1262	Y
YTP007 YTP019	**Critical Confidential Information removed**	bp 979 → bp 999 bp 2123 ← bp 2143	1165	Y
MDB124 STV038	**Critical Confidential Information removed**	bp 2094 → bp 2113 bp 3075 ← bp 3094	1001	Y
MDB070 SMP104	**Critical Confidential Information removed**	bp 2916 → bp 2934 bp 3913 ← bp 3932	1017	Y
SMP083 WAA037	**Critical Confidential Information removed**	bp 3558 → bp 3581 bp 4481 ← bp 4502	945	Y
SMP105 STV129	**Critical Confidential Information removed**	bp 4187 → bp 4206 bp 5086 ← bp 5109	923	Y
WAA038 STV090	**Critical Confidential Information removed**	bp 4419 → bp 4438 bp 5618 ← bp 5637	1219	Y
STV284 STV282	**Critical Confidential Information removed**	bp 5083 →bp 5102 bp 6466 ← bp 6483	1401	Y

* A lowercase 'g' is used to avoid confusion between 'G' and 'C'

Table 2: Overview of the plasmid DNA preparations and plasmid DNA confirmation.

Restriction enzyme	Expected restriction fragments	PL007-1 (Expiry date 17/04/2019)			PL007-2 (Expiry date 26/05/2019)		
		Obtained	Identity Confirmed	Conc. (ng/µL)*	Obtained	Identity Confirmed	Conc. (ng/µL)*
*Alw*NI	7632 bp and 2766 bp	Yes	Yes	220	Yes	Yes	530
*Bsa*I	4343 bp, 2616 bp, 2563 bp, 685 bp and 191 bp	Yes			Yes		
*Bsp*HI	7864 bp, 1526 bp and 1008 bp	Yes			Yes		
*Hinc*II	3010 bp, 2908 bp; 2476 bp, 1093 bp, 713 bp and 198 bp	Yes			Yes		
*Hind*III	3420 bp, 3070 bp, 2961 bp and 947 bp	Yes			Yes		
*Kpn*I	5914 bp, 3074 bp and 1410 bp	Yes			Yes		
*Sca*I	6888 bp and 3510 bp	No			Yes		

* Concentrations are rounded to the nearest tens

Table 3: Information on the used probe templates.

Probe template ID	Description	Primer pair / Restriction digest	Primer sequence (5' → 3')*	Position in pSF10	Size probe template	Concentration (ng/µL)	Expiry date
PT056-1	Vector backbone probe	KM033	Critical Confidential Information removed	bp 10356 → bp10373	1730 bp	30	21/04/2019
		DPA010	Critical Confidential Information removed	bp 1666 ← bp 1687			
PT057-1	Vector backbone probe	VH055	Critical Confidential Information removed	bp 1115 → bp 1134	1982 bp	100	21/04/2019
		STV039	Critical Confidential Information removed	bp 3077 ← bp 3096			
PT058-3	T-DNA probe	*Sac*I/*Sma*I	/	bp 3142 → bp 10345	7204 bp	30	04/06/2019
PT058-4	T-DNA probe	*Sac*I/*Sma*I	/	bp 3142 → bp 10345	7204 bp	34	15/07/2019
PT058-6	T-DNA probe	*Sac*I/*Sma*I	/	bp 3142 → bp 10345	7204 bp	25	10/08/2019

* A lowercase 'g' is used to avoid confusion between 'G' and 'C'

Table 4: Overview of the genomic DNA preparations prepared and/or used in BBS09-003.

DNA ID	Description	Conc. (ng/µL)*	Expiry date
DNA/09-003/01	FG72 genomic DNA	590	03/04/2019
DNA/09-003/02	FG72 genomic DNA	1550	03/04/2019
DNA/09-003/03	FG72 genomic DNA	640	03/04/2019
DNA/09-003/17	FG72 genomic DNA	970	03/04/2019
DNA/09-003/18	FG72 genomic DNA	350	03/04/2019
DNA/09-005/64	FG72 genomic DNA	790	08/07/2019
DNA/09-003/19	*Glycine max* variety JACK	500	03/04/2019
DNA/09-003/10	*Glycine max* variety JACK	1210	03/04/2019
DNA/09-003/20	*Glycine max* variety JACK	250	03/04/2019
DNA/09-005/45	*Glycine max* variety JACK	1020	24/06/2019

* Concentrations rounded to the nearest tens

Table 5: Expected and obtained hybridization fragments of the presented hybridizations.

Sample	Expected T-DNA or plasmid fragment sizes	Fragment description	M/09-003/06				M/09-003/07			
			PT056-1 Vector backbone probe		PT058-6 T-DNA probe		PT057-1 Vector backbone probe		PT058-6 T-DNA probe	
			Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
FG72 - *Hin*dIII	9550 bp [c]	5' integration fr.	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	947 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	No	Yes	Yes
	6333 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	5500 bp [c]	3' integration fr.	No	No	Yes	Yes	No	No	Yes	Yes
	1480 bp [c]	3' junction translocation	No	No	Yes [b]	No	No	No	Yes [b]	No
FG72 – *Hinc*II	5250 bp [c]	5' integration fr.	Yes [d]	No	Yes [b]	No	No	No	Yes [b]	No
	3010 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	713 bp	internal fragment	Yes [d]	Yes	Yes	Yes, weak	Yes [d]	No	Yes	Yes, weak
	2476 bp	internal fragment	No	No	Yes	Yes	No	No	Yes	Yes
	4091 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	1130 bp [c]	3' integration fr.	No	No	Yes	Yes	No	No	Yes	Yes
	1300 bp [c]	3' junction translocation	No	No	Yes [b]	No	No	No	Yes [b]	No
WT - *Hin*dIII	/	/	/	/	/	/	/	/	/	/
WT - *Hin*dIII + 0.1 equimolar amount pSF10 - *Hin*dIII	3420 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [b]	No	Yes	No [a]
	947 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [d]	No	Yes	No [a]
	2961 bp	positive control	Yes [b]	No	Yes	No [a]	No	No	Yes	No [a]
	3070 bp	positive control	Yes	Yes	Yes [b]	No	Yes	Yes	Yes [b]	No
WT - *Hin*dIII + 1 equimolar amount pSF10 - *Hin*dIII	3420 bp	positive control	Yes [d]	No	Yes	Yes	Yes [b]	No	Yes	Yes
	947 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [d]	No	Yes	No [a]
	2961 bp	positive control	Yes [b]	No	Yes	Yes	No	No	Yes	Yes
	3070 bp	positive control	Yes	Yes	Yes [b]	No	Yes	Yes	Yes [b]	No
		Hybridization ID	H1/09-003/06-F2		H3/09-003/06-F1		H1/09-003/07-F1		H3/09-003/07-F1	

[a] .These fragments of the positive control could not or very weakly be visualized after hybridization with the T-DNA probe. This has no impact on the interpretation of the results.
[b] The overlap between the probe and the fragment can be too small to visualize this fragment.
[c] Expected fragment sizes as determined in the detailed insert characterization study (Verhaeghe S.; 2010).
[d] Since part of the sequence of both vector backbone probes is as well present in the T-DNA sequences, fragments originating from inserted transgenic sequences are hybridizing with vector backbone probes.

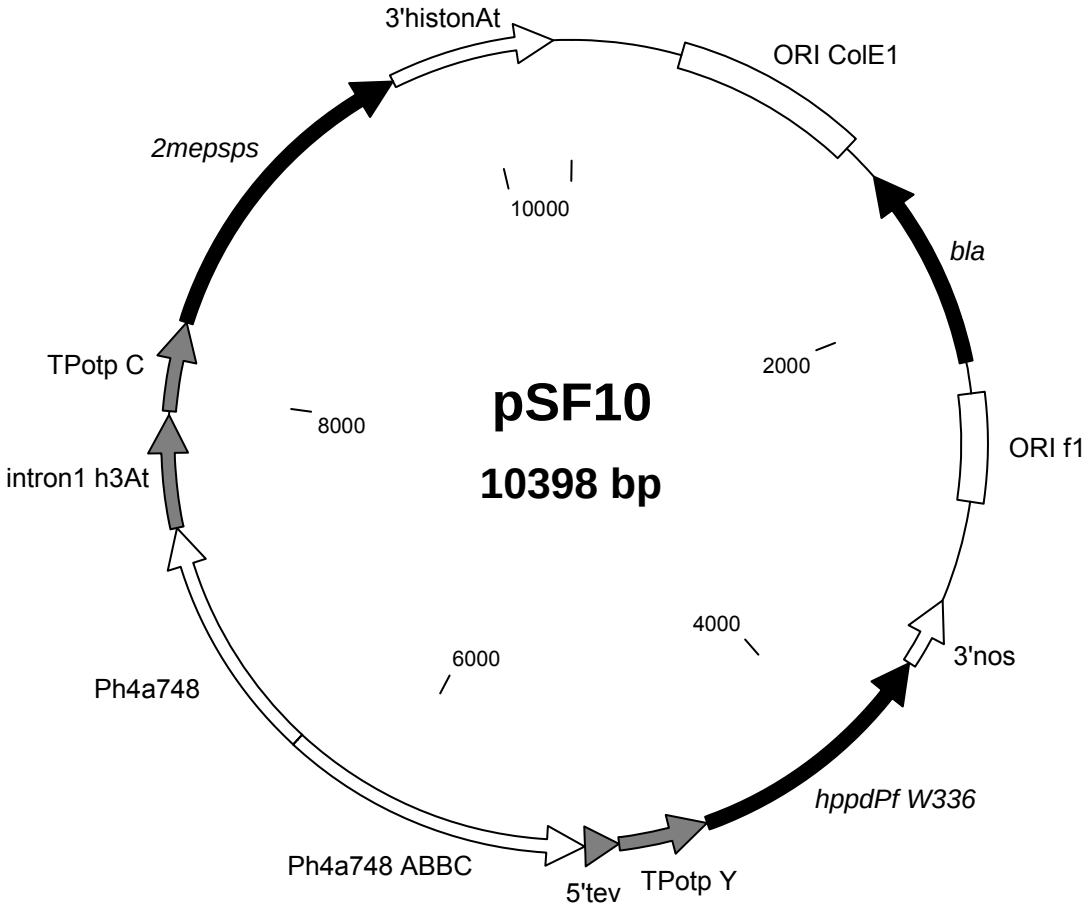


Figure 1: Map of vector pSF10.

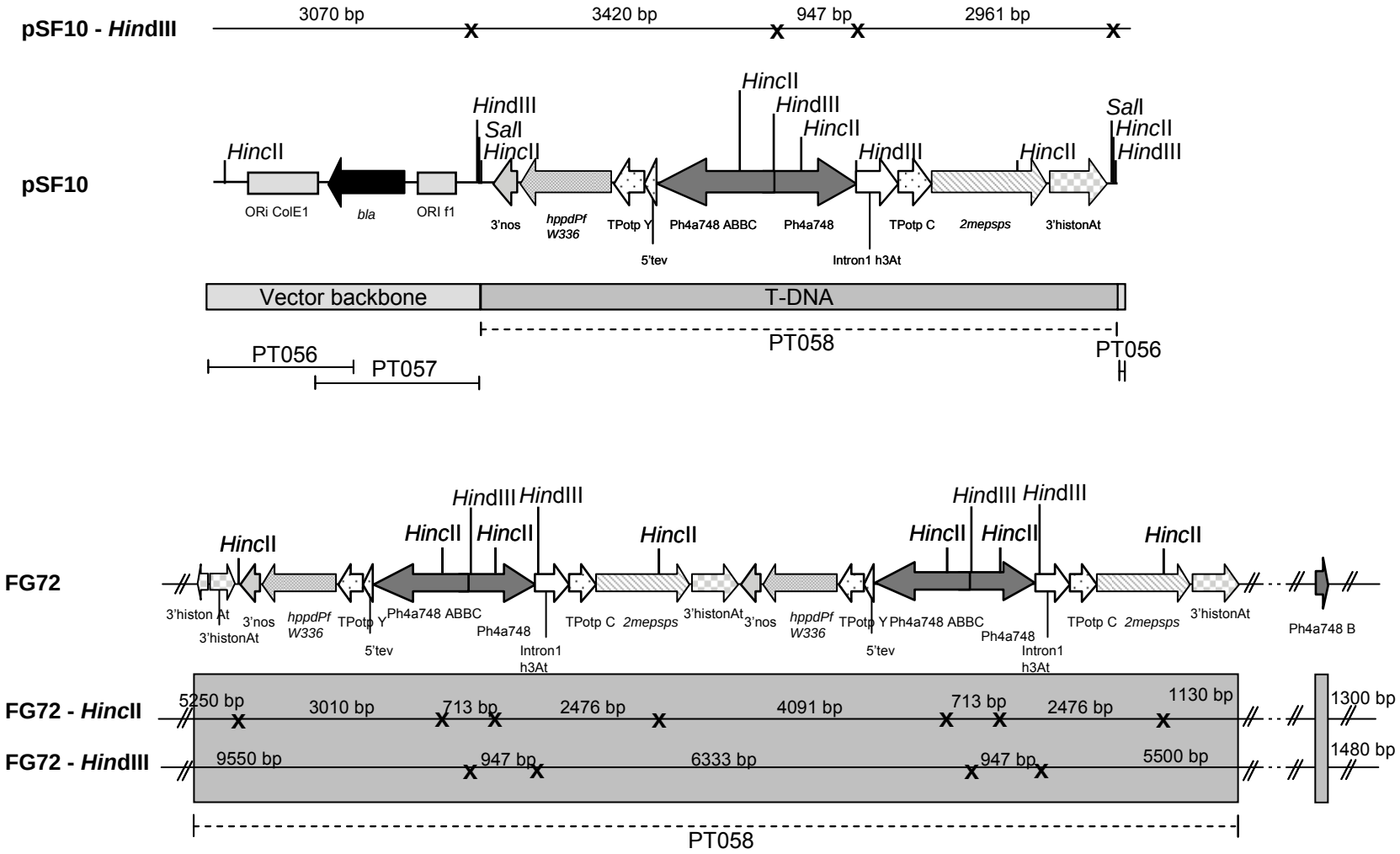
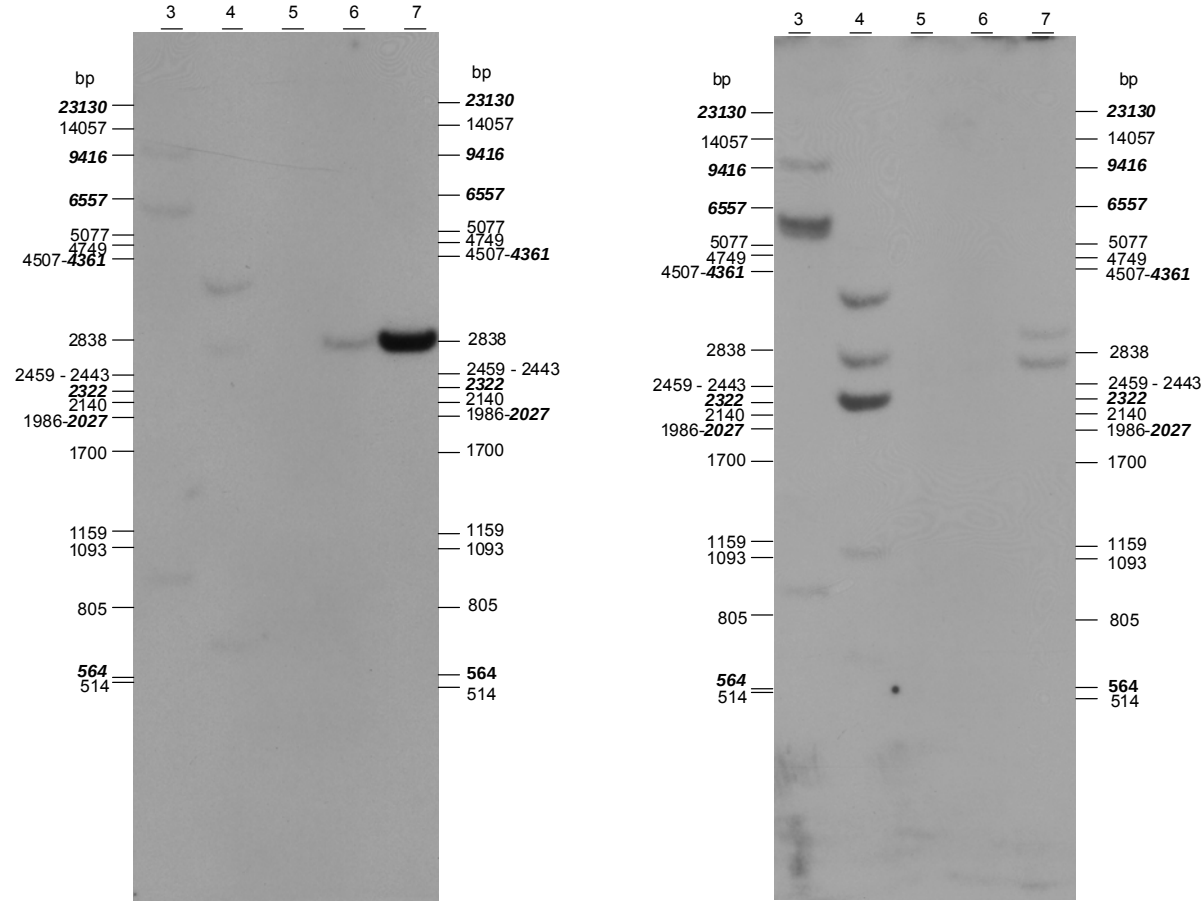


Figure 2: Schematic drawing of pSF10 with indication of the relevant restriction sites and the position of the used probe templates.



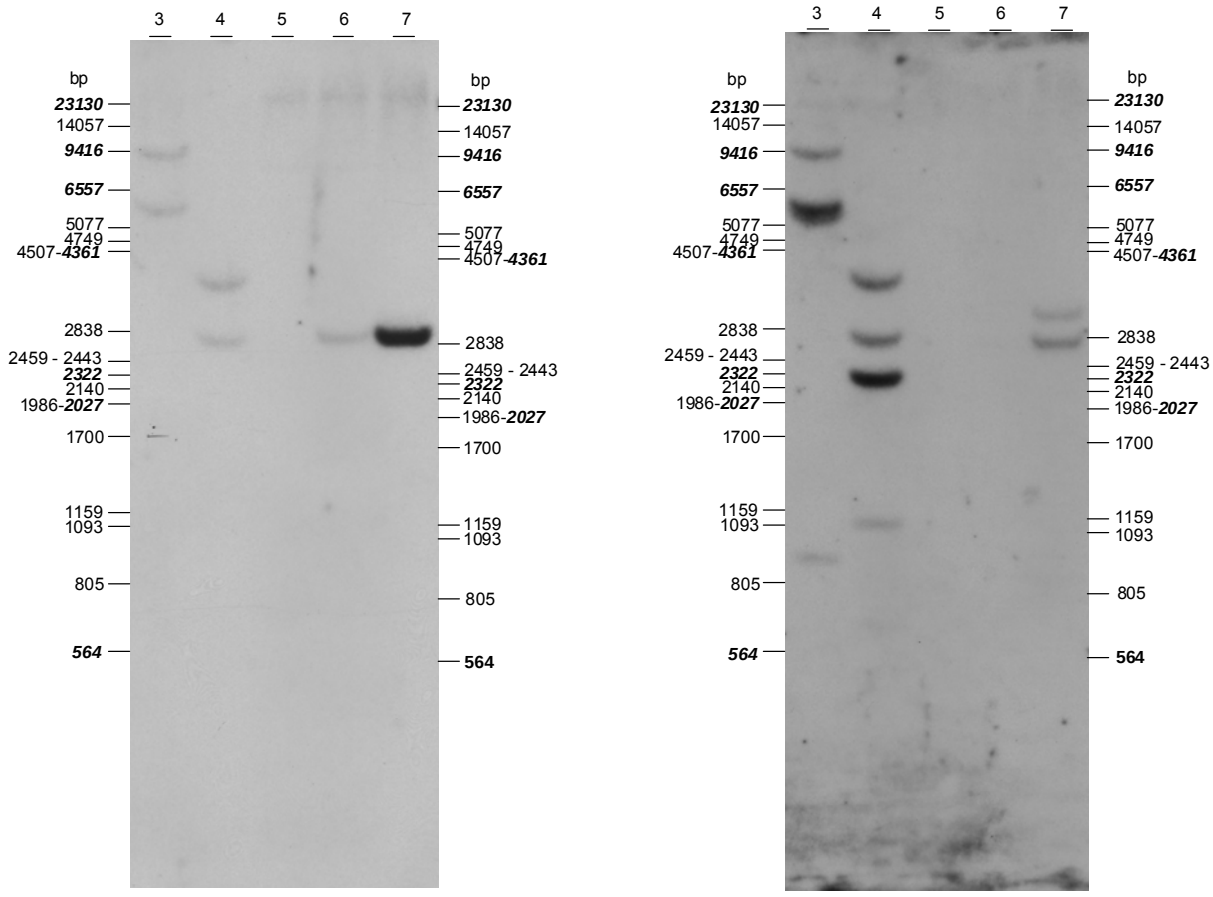
Panel A: Film ID: H1/09-003/06-F2
Probe template ID: PT056-1

Panel B: Film ID: H3/09-003/06-F1
Probe template ID: PT058-6

Figure 3: Southern blot analysis of FG72 - H1/09-003/06 and H3/09-003/06.

Total genomic DNA was isolated from *Glycine max* transformation event FG72 and from the non-transgenic counterpart JACK. The DNA samples (10 µg) were digested with different restriction enzymes and hybridized sequentially with a vector backbone probe (PT056-1: 1730 bp, KM033 – DPA010 fragment of pSF10) and with the T-DNA probe (PT058-6: 7204 bp, *Sac*I/*Sma*I fragment of pSF10).

Lane 1: λ-*Pst*I digested
Lane 2: λ-*Hin*dIII digested
Lane 3: *Glycine max* transformation event FG72 – *Hin*dIII digested
Lane 4: *Glycine max* transformation event FG72 – *Hin*cII digested
Lane 5: *Glycine max* wild type variety JACK – *Hin*dIII digested
Lane 6: *Glycine max* wild type variety JACK – *Hin*dIII digested + a 10-fold dilution of an equimolar amount of pSF10 – *Hin*dIII digested
Lane 7: *Glycine max* wild type variety JACK – *Hin*dIII digested + an equimolar amount of pSF10 – *Hin*dIII digested
Lane 8: λ-*Hin*dIII digested
Lane 9: λ-*Pst*I digested



Panel A: Film ID: H1/09-003/07-F1
Probe template ID: PT057-1

Panel B: Film ID: H3/09-003/07-F1
Probe template ID: PT058-6

Figure 4: Southern blot analysis of FG72 - H1/09-003/07 and H3/09-003/07.

Total genomic DNA was isolated from *Glycine max* transformation event FG72 and from the non-transgenic counterpart JACK. The DNA samples (10 µg) were digested with different restriction enzymes and hybridized sequentially with a vector backbone probe (PT056-1: 1982 bp, VH055 – STV039 fragment of pSF10) and with the T-DNA probe (PT058-6: 7204 bp, *Sac*I/*Sma*I fragment of pSF10).

Lane 1: λ-*Pst*I digested
Lane 2: λ-*Hin*dIII digested
Lane 3: *Glycine max* transformation event FG72 – *Hin*dIII digested
Lane 4: *Glycine max* transformation event FG72 – *Hin*cII digested
Lane 5: *Glycine max* wild type variety JACK – *Hin*dIII digested
Lane 6: *Glycine max* wild type variety JACK – *Hin*dIII digested + a 10-fold dilution of an equimolar amount of pSF10 – *Hin*dIII digested
Lane 7: *Glycine max* wild type variety JACK – *Hin*dIII digested + an equimolar amount of pSF10 – *Hin*dIII digested
Lane 8: λ-*Hin*dIII digested
Lane 9: λ-*Pst*I digested

REFERENCES

No	Doc No	Report No	Author(s), year, title, source, edition, pages
1.	M-232805-01-1		Dellaporta S., Wood J. and Hicks J. (1983). A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1(4), 19-21.
2.	- - - - - - -		Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) - Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3.	M-257571-01-1		Arumuganathan K. and Earle E.D. (1991). Nuclear DNA Content of Some Important Plant Species. Plant Molecular Biology Reporter, 9 (3), 208-218.
4.			Verhaeghe S. (2010) – Detailed insert characterization of *Glycine max* transformation event FG72 by Southern blot analysis.

Appendix 1: **PCR conditions used to confirm *E. Coli* cells containing pSF10 plasmid DNA and to prepare probe templates PT056-1 and PT057-1**

Amplitaq® Gold DNA polymerase with GeneAmp® 10X PCR Buffer:
Components for a 20 µL reaction:

1 µL pre-culture (*E. Coli* confirmation) or 100 pg pSF10 plasmid-DNA (probe template preparation)
2 µL 10x PCR Gold Buffer without $MgCl_2$
0.4 µL 10 mM dNTPs
1 µL forward primer (10 pmol/µL)
1 µL reverse primer (10 pmol/µL)
4 µL Betain (5M)
1.2 µL $MgCl_2$ (5 mM)
0.2 µL Amplitaq® Gold DNA polymerase (5U/µL)
MQ water up to 20 µL

Thermocycling profile:

14 min at 94°C
followed by: 1 min at 94°C
1 min at 60°C (56°C for primer combination STV284-STV282 in *E. Coli* confirmation)
2 min at 72°C
for 5 cycles
followed by: 30 sec at 94°C
30 sec at 60°C (56°C for primer combination STV284-STV282 in *E. Coli* confirmation)
1 min at 72°C
for 30 cycles
followed by: 10 min at 72°C
followed by: 5 min at 4°C
followed by: 10°C forever

Appendix 2: Used analytical methods.

SOP number	Title
BBS 07/16/03	Preparation of genomic DNA according to Dellaporta *et al.*
BBS 07/18/03	Preparation of plasmid DNA from *E. coli* bacterial cells
BBS 07/19/03	Quantification of double stranded DNA - PicoGreen® method
BBS 07/20/03	Restriction digestion
BBS 07/21/03	PCR – *Taq* DNA polymerase
BBS 07/25/02	Agarose gel electrophoresis – horizontal submarine gel system
BBS 07/26/06	Agarose gel electrophoresis – non submarine gel system
BBS 07/27/04 BBS 07/27/05	DNA gel extraction
BBS 07/30/02	Southern blot analysis – Alkali blotting of DNA
BBS 07/32/00	Southern blot analysis – Neutral blotting of DNA
BBS 07/33/03	Southern blot analysis – [α-^{32}P] Labeling of DNA probes
BBS 07/34/04	Southern blot analysis – Hybridization
BBS 07/35/02	Southern blot Hybridization – Autoradiography
BBS 07/36/02	Southern blot Hybridization – Stripping of membranes
BBS 07/42/02	Fragment analysis using the GeneTools software

Appendix 3: Certificate of Analysis of T33-01

CONFIDENTIAL

Bayer CropScience
BioScience

Certificate of Analysis No. MDA-08-009b

October 29th, 2009
Bayer BioScience N.V.
Technologiepark 38
B-9052 Gent
Belgium
www.bayercropscience.com

***Glycine max* plants,**
FG72 – Batchnumber 08BAGM0013

Outline
For GLP purposes, analysis was performed to confirm the identity of FG72 *Glycine max* plants – Batchnumber 08BAGM0013, according to MDA request 2008-DY-001.

Stability
This certificate is valid until October 29th 2019 on condition that leaf samples have remained stored properly in the ultra freezer and not have been thawed during this period. This validity may be extended, as further evidence of DNA stability becomes available.

Designation of the Material

MDA ref. n°:	2008-DY-001
Material:	Leaf samples taken from FG72 *Glycine max* plants
Plant numbers	1 -> 50
Batch numbers	08BAGM0013
Greenhouse protocol	08BAGM0013

Production Facility
Bayer BioScience N.V. -Research Station, Nazarethsesteenweg 77, B-9800 Astene (Deinze), Belgium.

The leaf material was provided by Jan Aelvoet, Bayer BioScience N.V. Nazarethsesteenweg 77, B-9800 Astene (Deinze), Belgium.

Control Facility
Molecular Diagnostics Applications (MDA), Bayer BioScience N.V., Technologiepark 38, B-9052 Zwijnaarde (Gent), Belgium.

Production Process for Source Leaf material
According to greenhouse protocol 08BAGM0013.

Date: 30/10/09

Marijke Vantyghem
Technician

Date: 30/10/09

Tom van Acker
Team leader

CONFIDENTIAL

Bayer CropScience
BioScience

October 29th, 2009
Bayer BioScience N.V.
Technologiepark 38
B-9052 Gent
Belgium
www.bayercropscience.com

***Glycine max* plants,**
FG72 – Batchnumber 08BAGM0013

Quality Control Certification

This document certifies that the genomic DNA sample(s) extracted from leave(s) of plant(s) have been analyzed using the methods and quality standards described below. This analysis confirms the identity of the indicated event using the methods and quality standards described below, for the samples with a confirmation in table 1.

This document replaces CoA MDA-08-009 (dated December 10th 2008), which became obsolete.

Quality Standards Applied to the Plant (Leaf) Material
The following standards were applied:

zPCR FG72 (MDP0726_01)

PCR confirmation 3'junction translocated region FG72 (MDP1034_01)

Result:

Information about the performed analyses can be found in Laboratory notebook: n° 01326, pages 105 till 106.
The identity of all samples is confirmed: all samples are homozygous for FG72.

Date: 30/10/09

Marijke Vantyghem
Technician

Date: 30/10/09

Tom van Acker
Team leader

Appendix 4: Certificate of Analysis of T33-02

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Bayer CropScience
BioScience

Certificate of Analysis No. MDA-09-010b

October 29th, 2009
Bayer BioScience N.V.
Technologiepark 38
B-9052 Gent
Belgium
www.bayercropscience.com

***Glycine max* plants,**
FG72 – Batchnumber 09BAGM0007

Outline
For GLP purposes, analysis was performed to confirm the identity of FG72 *Glycine max* plants – Batchnumber 09BAGM0007, according to MDA request 2009-ES-003.

Stability
This certificate is valid until October 29th 2019 on condition that leaf samples have remained stored properly in the ultra freezer and not have been thawed during this period. This validity may be extended, as further evidence of DNA stability becomes available.

Designation of the Material

MDA ref. n°:	2009-ES-003
Material:	Leaf samples taken from FG72 *Glycine max* plants
Plant numbers	1 -> 50
Batch numbers	09BAGM0007
Greenhouse protocol	09BAGM0007

Production Facility
Bayer BioScience N.V. -Research Station, Nazarethsesteenweg 77, B-9800 Astene (Deinze), Belgium.

The leaf material was provided by Jan Aelvoet, Bayer BioScience N.V. Nazarethsesteenweg 77, B-9800 Astene (Deinze), Belgium.

Control Facility
Molecular Diagnostics Applications (MDA), Bayer BioScience N.V., Technologiepark 38, B-9052 Zwijnaarde (Gent), Belgium.

Production Process for Source Leaf material
According to greenhouse protocol 09BAGM0007.

Date: 30/10/2009 Date: 3/11/09

Sandy Neirynck
Technician

Tom van Acker
Team leader

CONFIDENTIAL

Bayer CropScience
BioScience

October 29th, 2009
Bayer BioScience N.V.
Technologiepark 38
B-9052 Gent
Belgium
www.bayercropscience.com

Glycine max plants,
FG72 – Batchnumber 09BAGM0007

Quality Control Certification

This document certifies that the genomic DNA sample(s) extracted from leave(s) of plant(s) have been analyzed using the methods and quality standards described below. This analysis confirms the identity of the indicated event using the methods and quality standards described below, for the samples with a confirmation in table 1.

This document replaces CoA MDA-09-010 (dated May 28th 2009), which became obsolete.

Quality Standards Applied to the Plant (Leaf) Material
The following standards were applied:

zPCR FG72 (MDP0726_01)

PCR confirmation 3'junction translocated region FG72 (MDP1034_01)

Result:

Information about the performed analyses can be found in Laboratory notebook: n° 01452, page 39.
The identity of all samples is confirmed: all samples are homozygous for FG72.

Date: 30/10/2009 Date: 3/11/09

Sandy Neirynck
Technician

Tom van Acker
Team leader