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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of SHD-27531-4 GM Carnation

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of SHD-27531-4 GM Carnation

Validation Report

18 March 2016

European Union Reference Laboratory for Genetically Modified Food and Feed

Executive Summary

Suntory Holdings Ltd has submitted an application for marketing (coded: C/NL/13/01) of a genetically modified carnation line SHD-27531-4 (Unique identifier: SHD-27531-4). In this context, the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) was asked to carry out a single-laboratory validation of the performance of a polymerase chain reaction (PCR)-based method to detect and identify the carnation GM line SHD-27531-4.

This report describes the results of this validation, carried out by the EURL GMFF with control samples provided by the applicant. The method is a duplex end-point PCR, where a carnation (taxon) target sequence and a transgenic target sequence are simultaneously detected.

The limit of detection (LOD) of the GM assay was established to be between 25 and 5 copies, based on haploid genome copy number. The event-specificity of the method was assessed by the applicant as being sufficient. The EURL GMFF verified that the taxon-specific primers correctly detect the target designed on the endogenous gene sequence in genomic DNA of Cream Cinderella carnation line (conventional parental line) as well as in the genomic DNA of the GM carnation line; moreover, the GM target is only detected by the GM specific primers when genomic DNA of SHD-27531-4 line (positive control) is amplified according to the method described. Restriction analyses on the amplified products confirmed the identity of the reference- and GM-specific amplicons. The method is shown to tolerate the use of a different reaction mix.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

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Content

1. Introduction

Suntory Holdings Ltd has submitted an application for marketing (C/NL/13/01) of a genetically modified carnation line SHD-27531-4 (Unique identifier SHD-27531-4). In this context, the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) was asked to carry out a single-laboratory validation of the performance of a polymerase chain reaction (PCR)-based method for detecting and identifying the carnation GM line SHD-27531-4, in support to a notification submitted under Directive 2001/18/EC.

The EURL GMFF, following reception of the documentation and material, including control samples (step 1 of the validation process), carried out the scientific assessment of documentation and data (step 2) in accordance with the provisions of Commission Regulation (EC) No 641/200, as for applications submitted under Reg.(EC) No 1829/2003.
Subsequently, it established the method in its laboratory and validated its performance when applied to control materials submitted by the applicant (step 3) and prepared this report (step 5). No collaborative ring trial (step 4) was carried out.

2. Materials and Methods

2.1 Material received by the applicant

The EURL GMFF used the following DNA samples from Suntory Holdings Ltd (Table 1). The DNA was extracted by the applicant from GM and non-GM carnation leaf material.

Table 1. Positive (SHD-27531-4) and negative (Cream Cinderella) control samples

Sample name	**Line information**	**DNA Concentration***
Negative control	Conventional parental line "Cream Cinderella"	197.0 ng/µL
Positive control	SHD-27531-4	96.2 ng/µL

* DNA concentration was determined by the applicant

The positive control sample was declared by the applicant hemizygous for the inserted T-DNA.
In addition to the control samples the EURL GMFF received the following reagents from the applicant:

- Primer set 1 [non GM-line positive control ANS Forward (#1056), ANS Reverse (#1057)] targeting the *anthocyanidin synthase* carnation gene (*ANS*) with an expected amplicon size of 1279 bp.
- Primer set 2 [27531 LB Specific fw, and carLB-reverse] targeting the GM line 27531 with an expected amplicon size of 458 bp.

2.2 Estimation of DNA concentration by the EURL GMFF

The concentration of the DNA samples provided by the applicant was verified by the EURL GMFF, prior to use, by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Invitrogen, cat. No P7589). Each DNA extract was measured ten times, and the ten values were averaged. The DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/µL to 500 ng/µL using a Bio-Rad VersaFluor™ Fluorometer as fluorescence detector.

2.3 PCR-based detection method for identification of SHD-27531-4

The method developed and optimised by Suntory Holdings Ltd. is a duplex end-point PCR in which two targets are amplified in the same reaction; a 1,279 bp fragment of the *anthocyanidin synthase* carnation gene (*ANS*) and a 458 bp fragment of the inserted sequence. The *ANS* primers were included in the method as internal positive controls to show that no PCR inhibitors were present in the reaction. For the detection of the SHD-27531-4 target, a reverse primer (carLB-reverse) based on the carnation endogenous genomic DNA flanking sequence, and a forward primer complementary to the insertion sequence (27531 LB Specific fw) were designed by the applicant (Table 2).

Table 2. Name, DNA sequence and length of primers used in the PCR test

Name	Oligonucleotide DNA Sequence (5′ to 3′)	Length (nt)
SHD-27531-4		
27531 LB Specific fw	5′- CGA GTA AAT TCA AGC ATG CCC -3′	21
carLB-reverse	5′- CCA TAT TGA CCA TCA TAC TCA TTG C -3′	25
ANS		
ANS Forward (#1056)	5′-CTA GAT CGG AGG TCA CCA TAC C-3′	22
ANS Reverse (#1057)	5′-GAA ACC GTG ACC ATG GTC TCG-3′	21

The method described above was applied by the EURL GMFF to genomic DNA of the conventional parental carnation line (negative control) and of the GM line SHD-27531-4 (positive control) according to the conditions described in Table 3 and Table 4. The EURL GMFF conducted the tests using the duplex configuration as specified by the method developer, with the ANS forward and reverse primers targeting the *anthocyanidin synthase* carnation gene (*ANS*) and the 27531 LB Specific fw and the carLB-reverse primers targeting the GM line SHD-27531-4. The PCR was performed in duplicate.

Table 3. Reaction mix for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (*ANS*) and the GM line SHD-27531-4

Reagent	Concentration stock	Final concentration/ amount	µL/reaction	Reference reagent
27531 LB Specific fw	10 µM	0.4 µM	1.0 µL	Eurofins
carLB-reverse	10 µM	0.4 µM	1.0 µL	Eurofins
ANS Forward	10 µM	0.4 µM	1.0 µL	Eurofins
ANS Reverse	10 µM	0.4 µM	1.0 µL	Eurofins
AmpliTaq Gold 360 MasterMix	2x	1x	12.5 µL	Applied Biosystems (4398881)
Genomic DNA template	variable	50 ng	variable	-
Nuclease free water			to 25 µL	Promega (P119C)
Total volume µL			25 µL	

Table 4. Thermal profile for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (*ANS*) and the GM line SHD-27531-4

Step		Temperature	Time
1	Activation/Initial Denaturation	95°C	10 min
2	Denaturation	95°C	30 sec
3	Annealing	55°C	30 sec
4	Extension, 30 cycles from Step 2	72°C	1 min
5	Final extension	72°C	7 min
6	Hold	4°C	

2.4 Purification of PCR products and restriction analysis

PCR amplification products were generated by the EURL GMFF using as template the SHD-27531-4 DNA, in accordance with the primer sequences and amplification conditions described in Tables 2, 3 and 4. Two simplex reactions were assembled in order to obtain separate amplicons for the restriction analyses. After gel excision, the PCR products were purified with the GeneJET Gel Extraction Kit (cat # K0691), according to the manufacturer's instructions. The concentration of the DNA amplicon was measured by the EURL GMFF via UV readings at 260 nm.

Bioinformatics analyses were also carried out to identify the appropriate restriction sites and confirm the identity of the amplification products. Results of the digestion were visualised in 1% (w/v) agarose gel electrophoresis.

2.5 Limit of Detection (LOD) estimated by the applicant

Suntory Holdings Ltd determined the limit of detection (LOD) on the following concentrations, expressed as haploid genome copies per reaction (ten replicates per reaction): 7500 (4725 pg genomic DNA), 5000 (3150 pg genomic DNA), 2500 (1575 pg genomic DNA), 1000 (630 pg genomic DNA), 500 (315 pg genomic DNA), 160 (100 pg genomic DNA), 80 (50 pg genomic DNA), and 0 (no DNA). One copy of the carnation haploid genome is assumed to correspond to 0.63 pg according to the Royal Botanic Garden[1]. After amplification, the fragments were resolved on a 1.5% (w/v) agarose gel electrophoresis using GelRed and were visualized under UV light.
However, given the hemizygous nature of the GM-locus SHD-27531-4 (§ 2.1), only half of the nominal genome copies per each tested level contained the modified carnation locus and presumably the number of GM copies per reaction in the LOD testing of the applicant should be divided by a factor 2 (see § 3.2).

2.6 Estimation of the sample size in the determination of the Limit of Detection by the EURL GMFF

The optimal sample size to assess the LOD was determined by estimating the number *n* of replicates per GM level that would generate a 95% confidence interval around the proportion of GM-negative samples with an upper boundary not exceeding 5%.

For an accurate estimate of the 95% confidence interval (depending on the degrees of freedom used to compute p), the F-distribution was used based on the relationship between such distribution and the binomial distribution [2]. This method, derived from Bliss [3] and recently re-proposed by Zar [4], leads to an estimate of n = 100. Additionally, the standard approach based on the normal approximation was also considered, as suggested by Cochran [5]. This alternative method returns an estimate of n = 60. Computational details are given in Annex 1.

Given the experimental design for a LOD study where it is required to test a large number of replicates in each sample characterized by defined analyte content (DNA copy number content) over a linearly decreasing series of concentrations, the Cochran approach was accepted as the most feasible. Hence, every sample (GM-concentration level) was tested in 60 replicates [8].

For the estimation of the LOD_{abs}, 25 and 5 copies per reaction were tested. Each GM-level was tested in 60 replicates following the statistical model outlined above. Positive controls and no template controls were included.

After amplification, the fragments were resolved on a 1.0% (w/v) agarose gel electrophoresis using ethidium bromide staining and were visualized under UV light. Given the uncertainty on the copy

number of the *ANS* endogenous reference gene (paragraph 4-1-3), the LOD study was only focused on the detection of the GM target SHD-27531-4.

3. Results of tests conducted by the applicant

The data reported here below were produced by the applicant as a part of the notification dossier.

3.1. Identification and specificity

Suntory Holdings Ltd provided information on specificity and stability of the SHD-27531-4 method. The duplex assay was tested on genomic DNA extracted from conventional carnation lines (Cream Cinderella, Piccola, Cerise Westpearl) and on genomic DNA of transgenic carnation lines (Florigene Moonvista™, Moonaqua™, Moondust™, Moonlite™, Moonshadow™, Moonshade™, and SHD-27531-4). The method amplified a band corresponding to the expected *ANS* amplicon in all carnation lines and a band corresponding to the GM amplicon only in the SHD-27531-4 line (Figure 1). The resulting amplicons were compared by agarose gel electrophoresis to a ladder marker of known molecular weight. The size of the *ANS* amplicon was indicated by the applicant as 1,279 bp while the one of SHD-27531-4 was indicated by the applicant as 456 bp (for bioinformatics analysis on the amplicon length see 4.1.3). According to the results shown in Figure 1, the latter band was only present when the method amplified the SHD-27531-4 control sample.

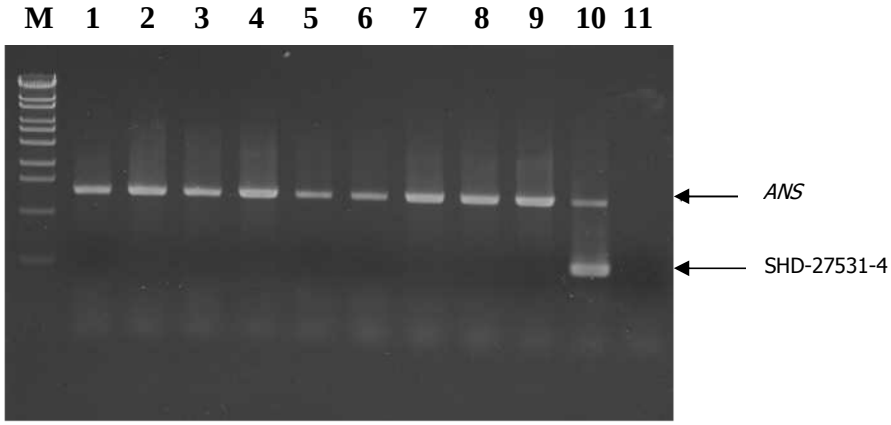

Figure 1. Agarose gel electrophoresis of the PCR products obtained with the duplex method. Lanes M = λ*Eco*T14 I digest (19.3, 7.7, 6.2, 4.3, 3.5, 2.7, 1.9, 1.5, 0.9 and 0.4 kb fragments); Lane 1 = Cream Cinderella; Lane 2 = Piccola; Lane 3 = Cerise Westpearl; Lane 4 = FLORIGENE Moonvista™; Lane 5 = FLORIGENE Moonaqua™; Lane 6 = FLORIGENE Moonshadow™; Lane 7 = FLORIGENE Moondust™; Lane 8= FLORIGENE Moonlite™; Lane 9 =FLORIGENE Moonshade™; Lane 10 = SHD-27531-4; Lane 11 = no template control.

3.2. Determination of the limit of detection (LOD)

According to Suntory Holdings Ltd the LOD of the method for detection of the SHD-27531-4 was determined to be 80 copies. However, the applicant declared that SHD-27531-4 was hemizygous (paragraph 2.1) hence, following the considerations expressed in paragraph 2.5, the LOD is expected to be 40 copies of the GM target.

4. Results of tests conducted by the EURL GMFF

The data reported below were produced by the EURL GMFF during the single-laboratory validation of the method provided by the applicant.

4.1. Quality checks on the control samples

4.1.1. DNA concentration

The concentration of the DNA of the control samples (SHD-27531-4 and Cream Cinderella, positive and negative controls respectively) was measured and the following values were observed (Table 5).

Table 5. DNA concentrations of the negative (conventional Cream Cinderella) and the positive (SHD-27531-4) control samples

Carnation line	**Concentration (ng/μL)**
Conventional line (Cream Cinderella)	142.3
GM line (SHD-27531-4)	111.4

4.1.2. DNA integrity

The DNA integrity of the control samples was evaluated by agarose gel electrophoresis; 223 ng and 213 ng of the positive (SHD-27531-4) and negative (conventional Cream Cinderella) control samples respectively, were analysed in a 1.0% (w/v) agarose gel run at 80 volts for 40 minutes (Figure 2).

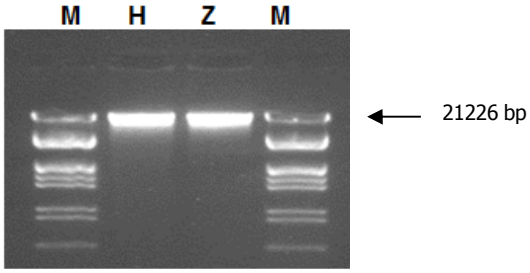

Figure 2. Agarose gel electrophoresis of DNA from positive (SHD-27531-4) and negative (conventional Cream Cinderella) control samples.
Z= Negative control (Cream Cinderella), 1.5 µL; H = Positive control (SHD-27531-4) 2 µL; M= Molecular Weight Marker (DNA λ*Eco*RI+*Hind*III: 21226, 5148-3530, ca. 2000, 1584, 1375, 947, 831, and 564bp).

The samples appeared as high molecular weight DNA bands. DNA samples did not show signs of significant degradation.

4.1.3. Bioinformatics analysis

SHD-27531-4 method
Bioinformatics analyses, based on similarity searches, confirmed that the detection method spans the junction between the transgenic insert and the 5' genomic region and would not cross-react with any other GM-event contained in the CCSIS (Central Core Sequence Information System) of the EURL GMFF. However, little information on the genomic sequences of *Dianthus caryophyllus* (carnation) is available in public databases. Therefore, initially that aspect of the event-specificity of the method for the detection of SHD-27531-4 GM event was based only on the documentation and data provided by the applicant.

Recently, the sequence of *D. caryophyllus* cultivar *"Francesco"* was determined and published [6] and similarity searches by BLAST [7] on the *Francesco* cultivar genome with the event-specific primer sequences as query were performed on the CarnationDB website (http://carnation.kazusa.or.jp/, coming with the above cited [6]). The *"27531 LB specific FW"* primer binding site was found in the genomic border adjacent to the insertion, matching a region of about 1,000 bp upstream a gene predicted to be encoding a putative 3-ketodihydrosphingosine reductase-like protein.

The *"carLB-Reverse"* primer binds in the insert, in a region that corresponds to the T-DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the DNA. The amplicon produced by the described primers on the submitted sequence is predicted to be 458 bp, and not 456 bp as described by the applicant in the "SHD-27531-4 JRC dossier Suntory 130204.pdf" document (see also § 3.1).

The sequence of the event-specific amplicon has been analysed by BLAST (NCBI, 1) against local copies of the "nt" and "patents" databases, as well as the whole genomes of more than 80 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum* and *Zea mays, etc.*), and no significant similarity was found with any other published sequence (except for the above mentioned carnation genomic region (GenBank: DF342181.1: 1-185 and 258-195, annotated as *"Dianthus caryophyllus DNA, scaffold: scaffold1330, whole genome shotgun sequence"*, genome version Dianthus_caryophyllus_DCA_r1.0_genomic) and the last 67 bases corresponding to the *A. tumefaciens* T-DNA border sequence that is common to many vector sequences. In addition, the primers were tested against the sequences of the other GM events present in the Central Core Sequence Information System of the JRC, as well as against the whole genomes of more than 80 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum* and *Zea mays, etc.*) using the e-PCR prediction tool (NCBI, 2, 3), and no potential amplicon was identified.

ANS carnation method

A sequence comparison of both primer sequences for the carnation *ANS* assay with the nr NCBI database revealed the existence of a sequence named *D. caryophyllus genomic DNA, anthocyanidin synthase promoter region* (GenBank: AB727362, last update 29 August 2012) that can be referred to as the carnation *ANS* gene. This sequence initially (2012) did not contain any annotation about the *ANS* gene, but contains the 1,279 bp amplicon sequence provided by the applicant. Moreover, the provided primers are able to amplify a region of 1,279 bp, located upstream the predicted promoter region of the *ANS* gene. With the availability of the *Francesco* cultivar genome, similarity search by BLAST with the AB727362 sequence as query (that contains the amplicon sequence provided by the applicant) was performed. Alignments revealed the presence of two genomic regions that are very similar to the *ANS* amplicon submitted by the applicant:

1. Region 167,545-168,823 of scaffold233, 99% identity
2. Region 11,240-12,503 of scaffold11560, 93% identity

The primers provided by the applicant are predicted to amplify both regions producing two amplicons of 1,279 bp and 1,264 bp, respectively. Those regions are both annotated as part of two singular predicted histone deacetylase complex subunit SAP18 genes and not as part of the carnation *ANS* gene. Moreover, according to the *Francesco* cultivar annotation, only one *ANS* gene is annotated on the genome as a two-exon gene mapped on region scaffold233:169751-170973, i.e. downstream the region scaffold233:167545-168823 corresponding to the *ANS* amplicon submitted by the applicant.

Additionally, ePCR tests with the primer set - for 100% without any mismatch - predicts an amplicon of 99.77% sequence similarity in the GM insert sequence of GMO Event IFD-26407-2 carnation as provided by Suntory that itself contains the "*Promoter of the carnation anthocyanidin synthase gene (pDcANS)*" as genetic element. The applicant used the region of the carnation genome targeted by the ANS method as an element (promoter) in the transgenic cassettes for the

carnation GM event [IFD-26407-2], meaning that the *ANS* method cannot be considered anymore taxon-specific.

According to these findings, we conclude that even if the assay is referred to an endogenous gene (*ANS*) that is present in single copy in the carnation genome, we cannot exclude that it is able to produce two different amplicons, since two potential targets were identified in a close cultivar of carnation. In conclusion, the *ANS* assay can be used to detect carnation DNA, and therefore it is fit only for the purpose of qualitative detection of carnation. Due to the above mentioned uncertainties the assay is not recommended for quantitative purposes[a].

4.2. Identification of SHD-27531-4 carnation

4.2.1. PCR amplification

The protocol provided by the method developer for identification of SHD-27531-4 was applied by the EURL GMFF according to the described amplification conditions (§ 2.3) on the control samples submitted (§ 2.1). The PCR was performed using an Applied Biosystems PCR apparatus (GeneAmp PCR System 9700).

The assay was run in duplicate on the positive (SHD-27531-4) and negative (conventional Cream Cinderella) control samples, using the recommended DNA amount of 50 ng per reaction.
The amplification products were separated by agarose gel electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light (Figure 3).

[a] Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of Florigene™ 26407 GM Carnation, paragraph 4.1.3. at http://gmo-crl.jrc.ec.europa.eu/valid-2001-18.htm

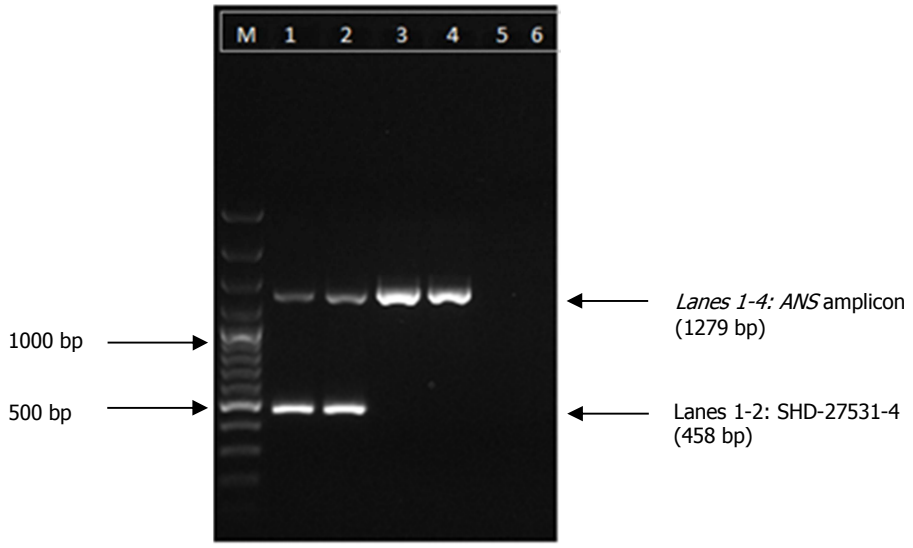

Figure 3. Agarose gel electrophoresis of PCR products obtained by the EURL GMFF by PCR amplification of genomic DNA of the positive control (SHD-27531-4) and the negative (conventional Cream Cinderella) control.

M= Molecular Weight Marker (Fermentas SM0324) bp: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. Lanes 1-2 = Positive control (SHD-27531-4, 50 ng genomic DNA in reaction); lanes 3-4 = Negative control (Cream Cinderella, 50 ng genomic DNA in reaction); lanes 5-6 = No template control;

Both samples (SHD-27531-4 and negative control) reacted with the *ANS* amplification system, resulting in the amplification of a fragment of the expected size (1,279 bp) for the target sequence of the carnation *anthocyanidin synthase* (*ANS*) gene, as compared to molecular weight marker.
Only the GM target line SHD-27531-4 reacted with the event-specific amplification system yielding a fragment of the expected size (458 bp) for the SHD-27531-4 amplicon, as compared to molecular weight marker.

This analysis demonstrates that the application of the proposed duplex PCR assay allowed amplifying: *i*) a fragment corresponding to the expected molecular size for the endogenous reference gene, the carnation *anthocyanidin synthase* (*ANS*) in the carnation samples (conventional carnation and SHD-27531-4) and *ii*) a fragment corresponding to the expected molecular size for the GM specific amplification product, only in the positive control SHD-27531-4 carnation line.

4.2.2. Confirmation of PCR products

Bioinformatics analyses carried out at the MBG Unit identified a unique restriction site for the restriction enzyme *Taq*I in the 458 bp PCR product of the GM amplicon. Two fragments of respectively 197 and 261 bp were expected in order to confirm the identity of the 458 bp amplicon of the SHD-27531-4 target fragment. The 1,279 bp *ANS* amplicon was expected to result in two fragments of 824 and 455 bp, respectively when digested with *Spe*I.

The EURL GMFF measured the concentration of the purified amplicons via UV readings at 260nm; 360 ng of the GM amplicon were digested with 10 Units of *Taq*I and were incubated at 65°C overnight. The *ANS* purified amplicon (288.5 ng), was digested with *Spe*I and incubated at 37°C overnight as well. Lambda DNA (Invitrogen, cat. No P7589) was used as a restriction control. Results of the digestion were visualised in 1% (w/v) agarose gel electrophoresis (Figure 4).

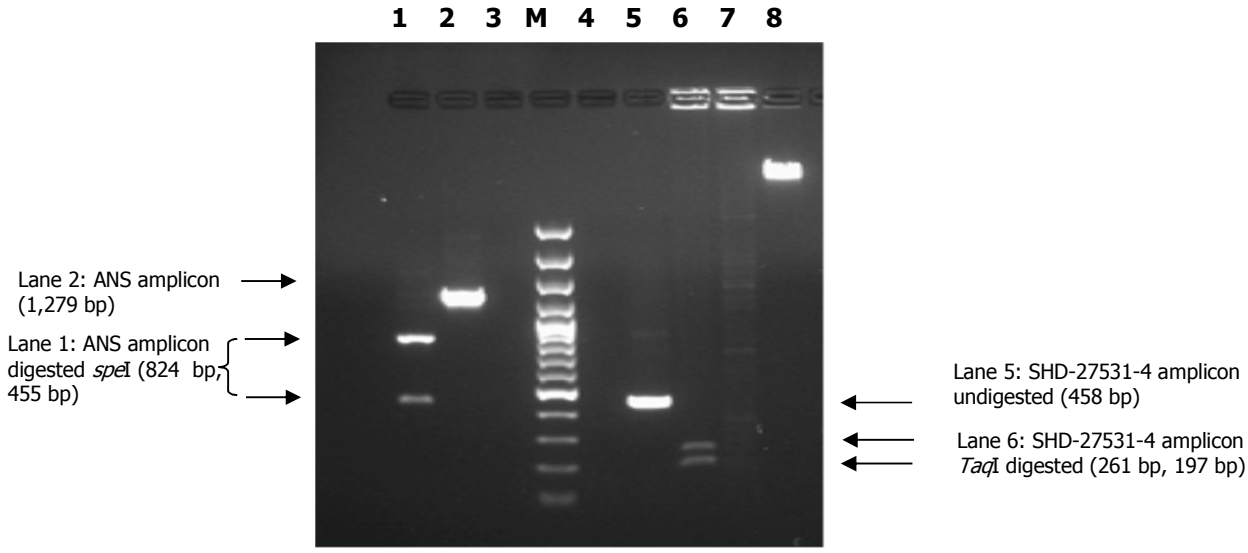

Figure 4. Restriction analysis of the 458 bp SHD-27531-4 and the 1,279 bp *ANS* amplicon
Lane 1 = ANS amplicon *Spe*I digested; lane 2 = ANS purified amplicon; lane 3 = Negative control *Spe*I digest; M = Molecular Weight Marker 100bp (Thermo Scientific #SM0323): 100-1000 in 100s, 1200, 1500, 2000, 3000; lane 4 = Negative control *Taq*I digest; lane 5 = SHD-27531-4 amplicon purified; lane 6 = SHD-27531-4 amplicon after digestion with *Taq*I (197+ 261 bp); lane 7 =positive control lambda DNA after *Taq*I digestion ; lane 8 = lambda DNA;

The amplicon size generated for simultaneous detection of SHD-27531-4 and *ANS* carnation genes were in agreement with the expected size determined via bioinformatics analysis. Hence, the identity of the amplicons generated by the duplex PCR assay for detection and identification of carnation event SHD-27531-4 and the carnation endogenous reference gene *ANS* was confirmed.

4.3. Robustness

The robustness of the method was tested by changing the PCR reaction components; the original method submitted by the applicant was based on the use of AmpliTaq Gold360 Master Mix (Applied Biosystems). For the robustness test, HotStar Taq DNA polymerase, 10x reaction buffer and dNTPs from Qiagen were used. The reactions were carried out according to the amplification conditions described in Table 5. The reaction mixture was prepared as described in Table 6.

Table 6. Reaction mix with Qiagen reagents for the duplex PCR targeting *anthocyanidin synthase* carnation gene (*ANS*) and the carnation GM event SHD-27531-4

Reagent	**Concentration stock**	**Concentration/ amount final**	**µL/reaction**	**Reference reagent**
10 x PCR Buffer	10 x	1 x	2.5 µL	Qiagen (cat 203205)
dNTPs mix	10 mM each	0.2 mM each	0.5 µL	Qiagen (cat 203205)
27531 LB Specific fw	10 µM	0.4 µM	1.0 µL	Eurofins (lot# 16153263)
carLB-reverse	10 µM	0.4 µM	1.0 µL	Eurofins (lot#16153264)
ANS Forward	10 µM	0.4 µM	1.0 µL	Eurofins (lot#14249102)
ANS Reverse	10 µM	0.4 µM	1.0 µL	Eurofins (lot#14249103)
HotStar Taq	5 units/µL	2.5 units	0.5 µL	Qiagen (cat 203205)
Genomic DNA template	variable	50 ng	variable	
Nuclease free water			up to 25 µL	Promega, P119C
Total volume µL	25 µL			

The amplification products were separated by agarose gel electrophoresis in a 1.0% (w/v) gel (Figure 5).

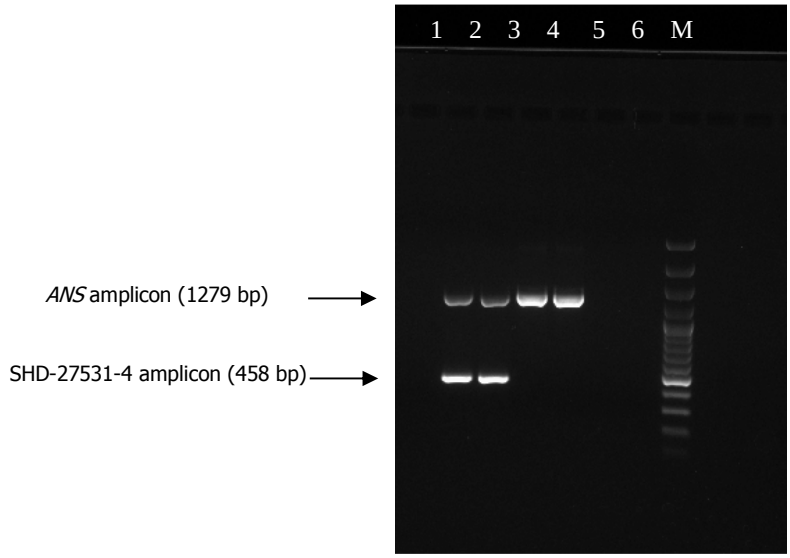

Figure 5. Agarose gel electrophoresis of PCR products obtained from amplification of genomic DNA of the positive and the negative control with Qiagen reagents.
Lanes 1-2 = positive control (SHD-27531-4, 50 ng genomic DNA in reaction); lanes 3-4 = negative control (conventional Cream Cinderella, 50 ng genomic DNA in reaction) M= Molecular Weight Marker 100bp (Fermentas SM0323): 100-1000 in 100s, 1200, 1500, 2000, 3000.

The results of the amplification are in line with the results obtained using the Applied Biosystems components: the PCR amplification generated *i)* a fragment of a size corresponding to the endogenous carnation *anthocyanidin synthase* (*ANS*) gene fragment in the carnation samples (Cream Cinderella and SHD-27531-4) and *ii*) a fragment corresponding to the GM specific amplification product only in the positive control SHD-27531-4 carnation GM-line.

Therefore, the robustness test showed that the proposed detection method tolerates the described changes in the reaction components.

4.4. Limit of detection (LOD)

Based on the statistical assumptions outlined in paragraph 2.6 and in Annex 1, the experimental absolute LOD is set at 59 positive tests over 60 replicates. Genomic DNA from carnation line SHD-27531-4 was amplified at defined copy numbers in the described conditions (Tables 3 and 4).

The weight of one copy of carnation haploid genome is considered to correspond to 0.63 pg [1]. The zygosity of the positive control sample (hemizygous for the inserted T-DNA as declared by the applicant) was also taken into consideration in order to calculate the target copies per reaction (Table 8). Amplification results were evaluated by gel electrophoresis. A PCR volume of 25 µL was

loaded on a 1% (w/v) agarose gel and run at 70 volts for 1 hour. Two different GM-levels were tested. Results are shown in Table 7.

Table 7. Results of the determination of the limit of detection (LOD) on SHD-27531-4

GM Copy number/ reaction	**Number of replicates**	**Positive results GM (SHD-27531-4)**	**Negative results GM (SHD-27531-4)**	**Positive results reference (*ANS*)**	**Negative results reference (*ANS*)**
25	60	60	0	60	0
5	60	0	60	42	18

The results indicate that the absolute LOD of the method is between 25 and 5 copies of GM target SHD-27531-4.
If it is assumed that one copy of the reference target is present per carnation genome in the variety Cream Cinderella, the LOD_{abs} of the method for the target should be at least 25 copies, but we cannot exclude the presence of a second copy, as in the cultivar Francesco of *D. cariophyllus* (paragraph 4.1.3.). For this reason the LOD_{abs} of the method is not reported for the *ANS* target.

5. Conclusions

The single-laboratory validation study conducted by the EURL GMFF demonstrates that the duplex end-point PCR method proposed by Suntory Holdings Ltd for the identification of SHD-27531-4 and of the carnation target *ANS* is applicable for the detection and identification of the GM event. The method is not suitable for quantification, due to uncertainties on the copy number of the *anthocyanidin synthase* (*ANS*) reference gene. The sizes of the amplicons are 1,279 bp for the carnation *anthocyanidin synthase* (*ANS*) reference gene and 458 bp for the GM event SHD-27531-4, respectively. The two DNA fragments generated by the restriction analysis of the 458 bp amplicon correspond to the expected pattern. The method is robust, i.e. it functions with different reaction mixes and should therefore be widely applicable. The LOD_{abs} of the method for the GM target SHD-27531-4 is 25 copies.

6. References

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Annex 1. Determination of the limit of detection

The EURL GMFF carried out tests to estimate the limit of detection (LOD) of the assay for detection of the SHD-27531-4 GM target. The optimal sample size (number of replicates *n* per assayed GM level) is defined as the sample size that is needed to determine the limit of detection (LOD), defined as the GM level (*p*) detected at least 95% of the times and hence ensuring ≤5% false negative results, with a 0.95 confidence level. According to the method from Bliss and Zar, in a sample of (n) data, (X) of which showing the character of interest, confidence limits (L_1: lower limit, L_2: upper limit) of a proportion (p) are computed as follows:

$$L_1 = \frac{X}{X + (n - X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}$$

$$L_2 = \frac{(X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}{n - X + (X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}$$

where the degrees of freedom v1 and v2 are:

$$\nu1 = 2 \cdot (n - X + 1)$$

$$\nu2 = 2 \cdot X$$

and the degrees of freedom ‘v1 and ‘v2 are:

$$'\nu1 = \nu2 + 2$$

$$'\nu2 = \nu1 - 2$$

Based on this method, with X = 1, α= 0.05, and L_2 = 0.05, (n) is equal to 100.

According to Cochran the simplest approach to estimate the confidence interval of a sample proportion (p), is the use of the normal distribution (z) and its standard deviation p (1 - p):

$$L_1 = p - z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1 - p)}{n - 1}}$$

$$L_2 = p + z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1-p)}{n-1}}$$

Based on this simplified approach, with X = 1 and α= 0.05, L2 = 0.05 (n) would be equal to 60, thus resulting for determining the absolute LOD in an experimental set at 59 positive tests (n - X) over 60 replicates.

Bliss C.I. (1967) Statistical biology, Vol. 1. McGraw-Hill, New York, USA, 558 pp.
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European Commission
Joint Research Centre – Institute for Health and Consumer Protection

Title: **Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of SHD-27531-4 GM Carnation**

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Abstract

Suntory Holdings Ltd has submitted an application for marketing (coded: C/NL/13/01) of a genetically modified carnation line SHD-27531-4 (Unique identifier: SHD-27531-4). In this context, the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) was asked to carry out a single-laboratory validation of the performance of a polymerase chain reaction (PCR)-based method to detect and identify the carnation GM line SHD-27531-4.

This report describes the results of this validation, carried out by the EURL GMFF with control samples provided by the applicant. The method is a duplex end-point PCR, where a carnation (taxon) target sequence and a transgenic target sequence are simultaneously detected.

The limit of detection (LOD) of the GM assay was established to be between 25 and 5 copies, based on haploid genome copy number. The event-specificity of the method was assessed by the applicant as being sufficient. The EURL GMFF verified that the taxon-specific primers correctly detect the target designed on the endogenous gene sequence in genomic DNA of Cream Cinderella carnation line (conventional parental line) as well as in the genomic DNA of the GM carnation line; moreover, the GM target is only detected by the GM specific primers when genomic DNA of SHD-27531-4 line (positive control) is amplified according to the method described. Restriction analyses on the amplified products confirmed the identity of the reference- and GM-specific amplicons. The method is shown to tolerate the use of a different reaction mix.

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