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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of GM-line FLO-40685-2 Carnation

European Union Reference Laboratory for Genetically Modified Food and Feed

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EURL European Union Reference Laboratory for GM Food & Feed

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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of GM-line FLO-40685-2 Carnation

Validation Report

28 October 2016

European Union Reference Laboratory for Genetically Modified Food and Feed

Executive Summary

Suntory Holdings Ltd has submitted an application for marketing (C/NL/13/02) of a genetically modified carnation line FLORIGENE® Moonvista™ (Unique identifier: FLO-40685-2). 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 FLO-40685-2.

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 250 and 1000 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 carnation line 123 (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 FLO-40685-2 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 reaction mix different from the one suggested.

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.

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Content

1. Introduction

Suntory Holdings Ltd has submitted an application for marketing (C/NL/13/02) of a genetically modified carnation line FLORIGENE® Moonvista™ (Unique identifier: FLO-40685-2). 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 detection and identification of the carnation GM line FLO-40685-2.

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 Commission Regulation (EC) No 503/2013 and according to its operational procedures ("Description of the EURL GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). 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 trial (step 4) was carried out.

2. Materials and Methods

2.1 Material received by the applicant

The EURL GMFF received from Suntory Holdings Ltd the positive and negative control samples as described in Table 1.

Table 1. Positive control (carnation line FLO-40685-2) and negative (parental) control samples

Sample name	**Line information**	**DNA Concentration (ng/µl)***	
		Nanodrop	Qubit 2.0
Negative control	Non GM carnation parental line 123	146.23	197.0
Positive control	FLO-40685-2	151.81	129.0

*concentration as determined by the applicant.

The positive control sample, carnation line FLO-40685-2, was declared by the applicant hemizygous for the inserted T-DNA.
Additional control DNA samples from genetically modified carnation lines (Table 2) were received and used by the EURL GMFF to test the specificity of the FLO-40685-2 detection method.

Table 2. Transgenic carnation lines tested by the EURL GMFF for verification of method specificity

Florigene Moonlite™
Florigene Moonaqua™
IFD-25958-3
IFD-26407-2
SHD-27531-4

In addition, 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 [123.8.8-2.1R, and RBF1a] targeting the GM line FLO-40685-2 with an expected amplicon size of 338 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 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 FLO-40685-2

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 1279 bp fragment of the *anthocyanidin synthase* carnation gene (ANS) and a 338 bp fragment of the inserted sequence. The ANS primers were included in the method as internal positive controls. For the detection of the FLO-40685-2 target, a reverse primer (123.8.8-2.1R) based on the carnation endogenous genomic DNA flanking sequence, and a forward primer complementary to the insertion sequence (RBF1a) were designed by the applicant (Table 3).

Table 3. Name, DNA sequence and length of primers used in the PCR test

Name	Oligonucleotide DNA Sequence (5′ to 3′)	Length (nt)
FLO-40685-2		
RBF1a	5′- TGG TGG ACC CTT GAG GAA ACT G-3′	22
123.8.8-2.1R	5′- ATA CAA TGG CTG ACT GAT GTA G -3′	22
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 carnation GM line FLO-40685-2 (positive control) according to the conditions described in Table 4 and Table 5. 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 RBF1a and the 123.8.8-2.1R primers targeting the GM line FLO-40685-2. PCR amplifications were performed in duplicate.

Table 4. Reaction mastermix for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (ANS) and the GM line FLO-40685-2

Reagent	Concentration stock	Final concentration/ amount	µL/reaction	Reference reagent
Primer RBF1a	10 µM	0.4 µM	1.0 µL	Eurofins
Primer 123.8.8-2.1R	10 µM	0.4 µM	1.0 µL	Eurofins
Primer ANS Forward	10 µM	0.4 µM	1.0 µL	Eurofins
Primer 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	100 ng	variable	-
Nuclease free water			to 25 µL	Promega (P119C)
Total volume µL			25 µL	

Table 5. Thermal profile for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (ANS) and the GM line FLO-40685-2

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, 27 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, detection and identification of event FLO-40685-2

PCR amplification products were generated by the EURL GMFF using as template the positive control sample and the primer sequences, and the amplification conditions described in Tables 3, 4 and 5. Two simplex reactions (instead of a duplex reaction) 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 (Thermo Scientific, cat # K0691), according to the manufacturer's instructions. The concentration of the DNA amplicons was measured via UV readings at 260 nm.

Bioinformatics analyses were also carried out in order to identify appropriate restriction sites in order to confirm the correctness of the fragment(s) size and therefore the specificity of the amplification products. Results of the digestions were visualised in 1.5% (w/v) agarose gel electrophoresis.

2.5 Limit of Detection (LOD) estimated by the applicant

Suntory Holdings Ltd determined the limit of detection (LOD) of the method by applying it to the following concentrations, expressed in 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 Bennett and Leitch[1]. After amplification, the fragments were resolved on a 1.5% (w/v) agarose gel electrophoresis and were visualized under UV light.

However, given the hemizygous nature of the GM-locus FLO-40685-2, it is considered that half of the genomes loaded per each level contained the modified carnation locus and presumably the number of GM copies per reaction should then be divided by a factor 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 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 characterised 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.

For the estimation of the LOD_{abs}, 5000, 2000, 500 and 50 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 (see paragraph 4.1.3), the LOD study was only focused on the detection of the GM-target FLO-40685-2.

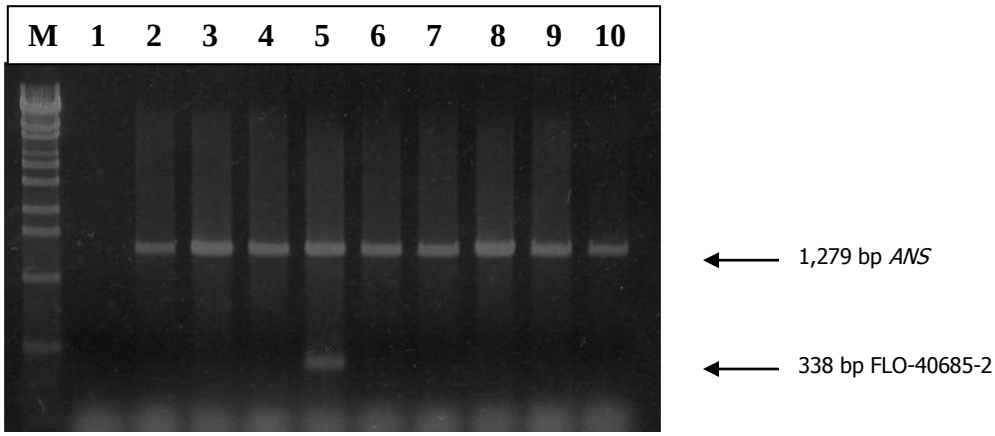
3. Results of tests conducted by the applicant

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 the specificity and stability of the FLO-40685-2 method. The duplex assay was tested on genomic DNA extracted from conventional carnation lines (parental line 123, line 145 and line 5) and on genomic DNA of transgenic carnation lines (Florigene Moonvista™ (line FLO-40685-2), Moonaqua™, Moondust™, Moonlite™, Moonshadow™, Moonshade™. 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 FLO-40685-2 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 FLO-40685-2 was indicated as 338 bp. According to Figure 1, the latter band was only present when the method amplified the FLO-40685-2 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 = No template control; Lane 2 = Line 123; Lane 3 = Line 145; Lane 4 = Line 5; Lane 5 = FLORIGENE Moonvista; Lane 6 = FLORIGENE Moonshade; Lane 7 = FLORIGENE Moonlite; Lane 8 = FLORIGENE Moonaqua; Lane 9 = FLORIGENE Moonshadow; Lane 10 = FLORIGENE Moondust.

3.2. Determination of the limit of detection (LOD)

According to Suntory Holdings Ltd the LOD of the method for detection of the FLO-40685-2 was determined to be 500 copies. However, the applicant declared that FLO-40685-2 was hemizygous (paragraph 2.1) hence, following the considerations expressed at paragraph 2.5, the LOD level is expected to contain 250 copies of the GM target.

4. Results of tests conducted by the EURL GMFF

The data reported here 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 (FLO-40685-2 and line 123, positive and negative controls respectively) was measured and the following values were observed (Table 6).

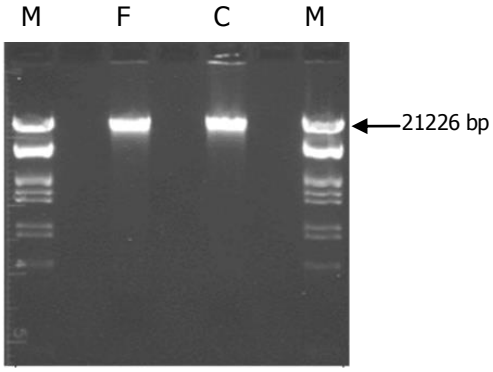
Table 6. Measured concentrations for the negative (conventional line 123) and the positive (FLO-40685-2) control samples

Carnation line	**Concentration (ng/μL)**
Conventional line 123	150.1
GM line (FLO-40685-2)	142.6

4.1.2. DNA integrity

The DNA integrity of the control samples was evaluated by agarose gel electrophoresis; 250 ng of the GM-line FLO-40685-2 and of the non-GM parental carnation line 123 (respectively, positive and negative control samples submitted for the method validation) were analysed in a 1.0% (w/v) agarose gel run at 100 Volts for 45 minutes (Figure 2).

Figure 2. Agarose gel electrophoresis of DNA from FLO-40685-2 carnation line and conventional line 123



C= Non-GM carnation line 123; F = FLO-40685-2 carnation line; M= Molecular Weight Marker (DNA λ*Eco*RI+*Hind*III: 21226, 5148-3530, *ca.* 2000, 1584, 1375, 947, 831, and 564 bp).

The control samples appeared as high molecular weight DNA bands. DNA samples did not show signs of significant degradation, thus indicating satisfactory DNA integrity.

4.1.3. Bioinformatics analyses

FLO-40685-2 method

Bioinformatics analyses, based on the sequence information provided by the applicant indicate that FLO-40685-2 is characterized by four inserts, one being the main insert containing the whole transgenic cassette.

The detection and identification method is built on the second insert which is not expected to express any of the genes of the original transgenic vector. The detection method reported spans the junction between the transgenic insert and the 3' genomic region of the second insertion site.

The RBF1a (forward-) primer binds to the insert, in a region that corresponds to the T-DNA region from *A. tumefaciens* containing the Right Border sequence used for transfer of the DNA. The *"123.8.8-2.1R"* (reverse-) primer binding site was found in the genomic border adjacent to the insertion. The method is a duplex end-point PCR; hence there is no probe for this method.

The EURL GMFF addressed to the applicant a request for clarifications concerning the choice of the detection method built on an insertion site not expected to contribute and therefore not under positive pressure for selection of the desired trait of the genetically modified plant. The applicant replied that carnation, including FLO-40685-2, was vegetatively propagated and there was no possibility for loss of locus 2 due to recombination and that the event would not be used for breeding. The applicant also provided a stability test on DNA extracted from FLO-40685-2 plants maintained for a long period of time at a different location (Suntory glasshouse, Japan).

The amplicon size is expected to be 338 bp, consistent to what is reported by the applicant. The sequence of the amplicon was analysed by BLAST[6] 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, Zea mays, etc.*), and no significant similarity was found with any other published sequence except the first 204 bp that correspond to the *A. tumefaciens* T-DNA border sequence that is common in many vector sequences and for the genomic region (GenBank: DF341379.1: 251-338 annotated as *"Dianthus caryophyllus DNA, scaffold: scaffold516, whole genome shotgun sequence"*).

In addition, the primers were tested against the sequences of the other GMO events present in the CCSIS, Central Core Sequence Information System of the JRC, (https://ec.europa.eu/jrc/en/scientific-tool/central-core-dna-sequence-information-system-ccsis) as well as the whole genomes of more than 80 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum, Zea mays, etc.*) using the e-PCR prediction tool (NCBI[7,8]), and two other potential amplicons were identified by ePCR when using only the *"RBF1a"* (forward-) primer that binds in the insert, in the very common T-DNA region.

Due to the specific palindromic structure of these target sequences, ePCR tool predicts that with 100% similarity without any mismatch, an amplicon of 414 bp of GMO event Florigene Moonvista carnation (FLO-40685-2) third insertion site is generated by the primer FLO-40685-2_RBF1a. Additionally, an amplicon is predicted of 353 bp at position 10576 – 10928 of the CCSIS record JRC50022 of the unauthorised GMO Event 1345-4 Tomato, T-DNA insert without flanking genomic sequences, based on Sequence 1 from patent US 5952546A[a].

[a] https://www.lens.org/lens/patent/US_5952546_A

Moonberry, Moonvelvet and Moonaqua detection methods

Results of bioinformatics analyses aimed at verifying whether previously validated methods for detection of carnation GM lines could cross react with Moonvista GM line revealed that the methods for detection of events Moonaqua (FLO-040689-6), Moonberry (IFD-25958-3) and Moonvelvet (IFD-26407-2) may target a palindromic structure in GM event Florigene Moonvista carnation (FLO-40685-2). The bioinformatics analyses predict that a single primer from each of these published methods (http://gmo-crl.jrc.ec.europa.eu/valid-2001-18.htm) would anneal on two sites of the third insert of the DNA from Moonvista carnation line and prime amplification (Table 7).

Table 7. Predicted amplicons generated on Moonvista carnation DNA and comparison with length of the event-specific amplicons

Commercial name (unique identifier)	**Primer Name[b]**	**Location of target in Moonvista according to ePCR**	**Predicted amplicon length (bp) on Moonvista DNA**	**Length of event-specific amplicons (bp)**
Moonaqua (FLO-040689-6)	rRB_7-26	Moonvista 3rd insert	1246	754
Moonberry (IFD-25958-3)	RB forward (1333)	Moonvista 3rd insert	408	482
Moonvelvet (IFD-26407-2)	RB forward (1334)	Moonvista 3rd insert	292	491

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 (in 2012) did not contain any annotation about the ANS gene, but contains the 1,279 bp amplicon sequence provided by the applicant. Moreover, the 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[c] 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 similar to the ANS amplicon submitted by the applicant:

[b] Primer sequences are reported in Annex 2
[c] Available at http://carnation.kazusa.or.jp/

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. These 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[d] 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[e].

In consideration of these findings, it is concluded that although the assay is referred to an endogenous gene (ANS) that is present in single copy in the carnation genome, it cannot be excluded that two different amplicons are produced, since two potential targets were identified in a close cultivar of carnation. Therefore, 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.

4.2. Detection and identification of FLO-40685-2 carnation

4.2.1. PCR amplification

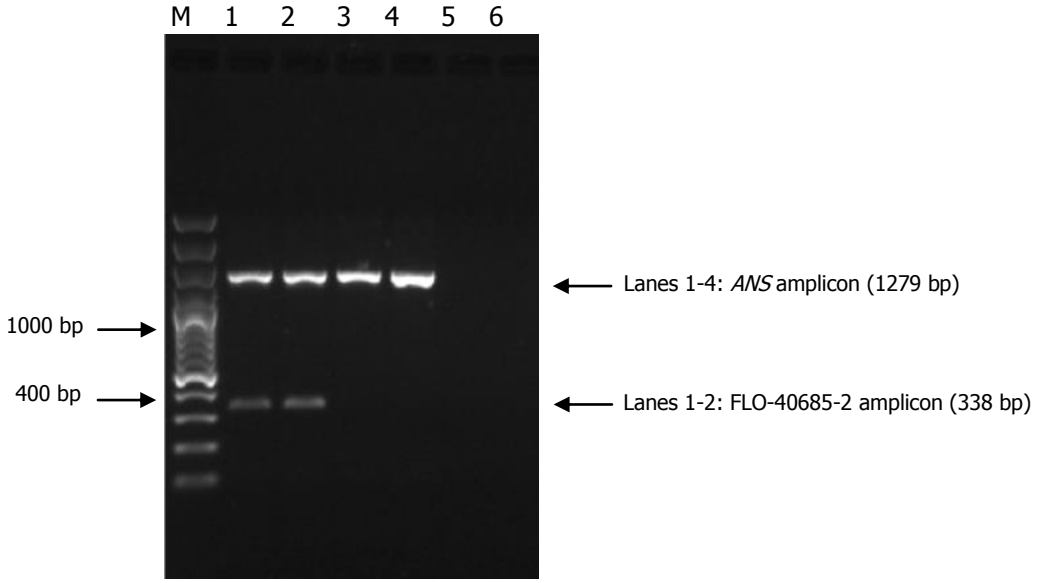
The protocol provided by the method developer for specific identification of FLO-40685-2 was applied by the EURL GMFF according to the described amplification conditions (paragraph 2.3) on the control samples submitted (paragraph 2.1). The PCR was performed using an Applied Biosystems PCR apparatus (GeneAmp PCR System 9700).

The method was run in duplicate on the positive (FLO-40685-2) and negative (conventional line 123) control samples, using the recommended DNA amount of 100 ng per reaction.

[d] Electronic PCR, a computation procedure that is used to search DNA sequences for PCR amplicons, given a specific primer pair
[e] 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

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).

Figure 3. Agarose gel electrophoresis of PCR products obtained by the EURL GMFF from PCR amplification of genomic DNA of the positive control (FLO-40685-2) and the negative (conventional line 123) control



Lane 1-2 = amplification of FLO-40685-2; lane 3-4= amplification of conventional carnation line 123; lane 5-6 = No template control; M= Molecular Weight Marker (Fermentas SM0323, Generuler 100 bp DNA ladder)

Both samples (FLO-40685-2 and conventional line 123) reacted with the *ANS* amplification system, resulting in the amplification of a fragment in the range of the expected size (1279 bp) for the target sequence of the carnation *anthocyanidin synthase* (ANS) gene, as compared to the molecular weight marker.

Only the GM target line FLO-40685-2 reacted with the event-specific amplification system yielding a fragment in the range of the expected size (338 bp) for the FLO-40685-2 amplicon, as compared to molecular weight marker. No additional band at the predicted length of 414 is visible in the FLO-40685-2 control sample (§ 4.1.3. Bioinformatics analyses. *FLO-40685-2 method*). Suntory Holdings Ltd verified experimentally that the primer RBF1a of Moonvista does not produce a second amplicon of 414 bp with Moonvista DNA (Annex 2).

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 FLO-40685-2) and ii) a fragment corresponding to the expected molecular size for the GM specific amplification product, only in the positive control FLO-40685-2 carnation line.

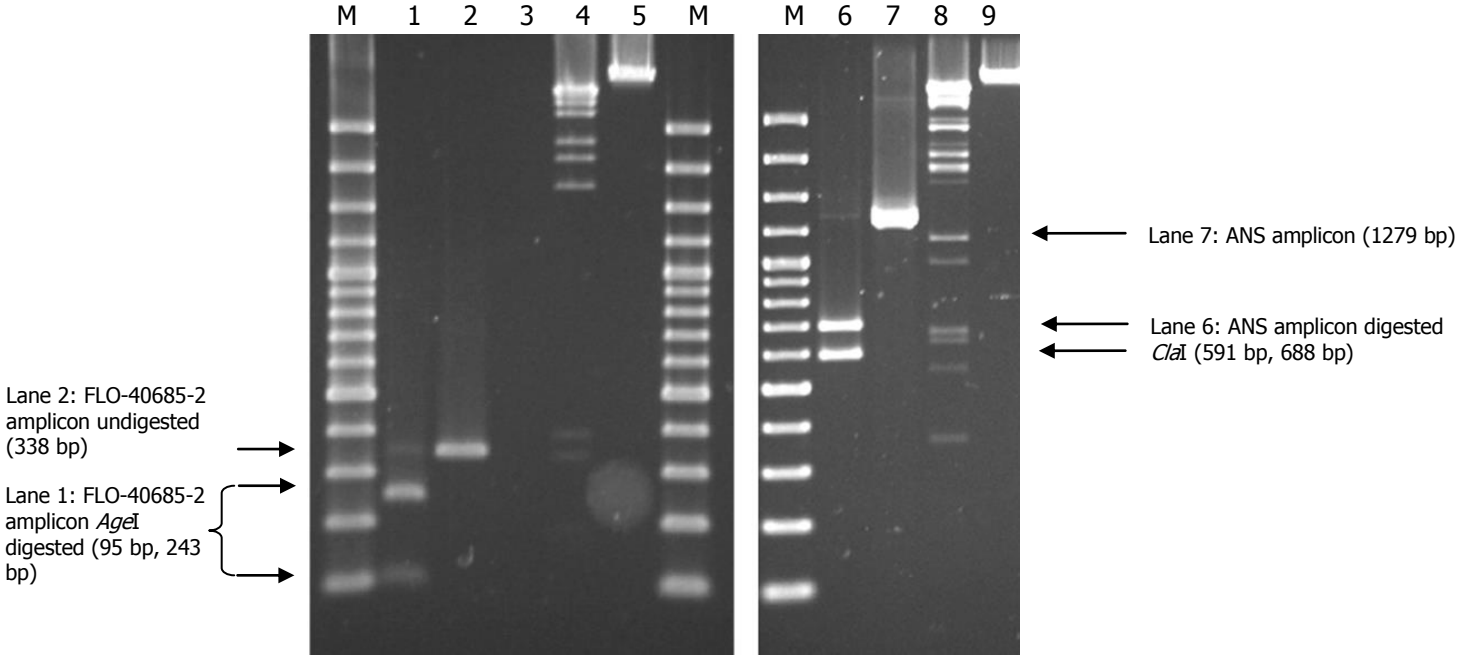
4.2.2. Confirmation of PCR products

Bioinformatics analyses carried out by the EURL GMFF identified a unique restriction site for the restriction enzyme *AgeI* in the 338 bp PCR product of the GM amplicon. Two fragments of respectively 95 and 243 bp were expected in order to confirm the identity of the 338 bp amplicon with the FLO-40685-2 event amplicon. The 1279 bp ANS amplicon was digested with *Cla*I which was unique and expected to result in two fragments of 591 and 688 bp, respectively to confirm the identity of the reference amplicon.

Both the FLO-40685-2 and the ANS amplicons were excised from an agarose gel and purified (paragraph 2.4).

The EURL GMFF estimated the concentration of the purified amplicons via UV readings; an amount of GM amplicon corresponding to 219 ng was digested with 5 Units of *Age*I (Promega R7251) and was incubated at 37°C overnight. An amount of ANS amplicon corresponding to 219 ng was digested with 10 Units of *Cla*I and incubated at 30°C overnight. Lambda DNA (Invitrogen, cat. No P7589) digested respectively with *AgeI* and *ClaI* was used as a restriction control. Results of the digestion were visualised in agarose gel electrophoresis (Figure 4a and 4b).

Figure 4a and 4b. Restriction analysis of the 338 bp FLO-40685-2 and the 1279 bp ANS amplicon



M = Molecular Weight Marker (Fermentas SM0323, Generuler 100 bp DNA ladder); Lane 1 = FLO-40685-2 amplicon *Age*I digested (95 bp, 243 bp); lane 2 = FLO-40685-2 amplicon undigested; lane 3 = Negative

extraction from agarose gel; lane 4 = λDNA AgeI digested; lane 5 = λDNA undigested; lane 6 = ANS amplicon ClaI digested (591 bp, 688 bp); lane 7 = ANS amplicon undigested; lane 8 = λDNA *Cla*I digested; lane 9 = λDNA undigested

The amplicon sizes generated for detection of FLO-40685-2 and ANS carnation genes were in agreement with the expected size determined by the bioinformatics analysis. It is noted that the lowest molecular size fragment generated by *Age*I cleavage in FLO-40865-2 (95 bp) migrates slightly slower than the molecular size marker corresponding to 100 bp.

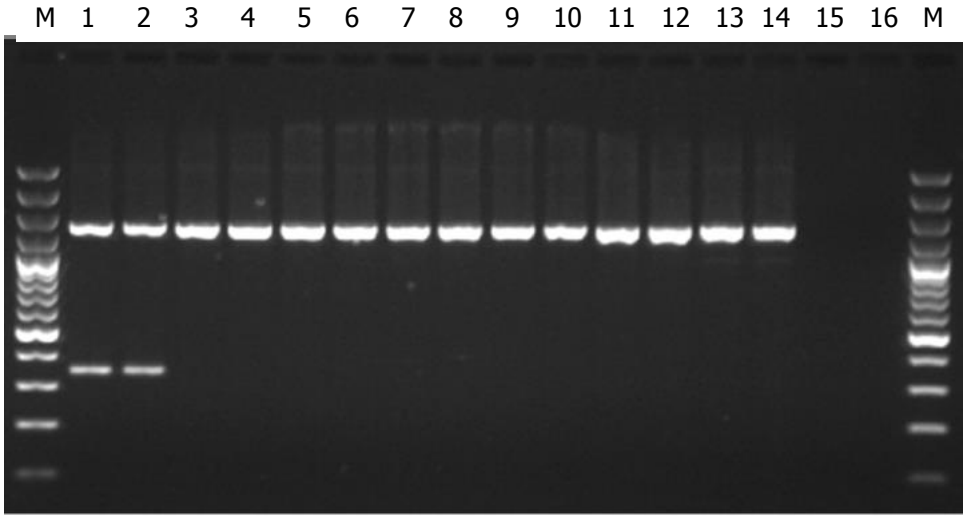
The identity of the amplicons generated by the duplex PCR assay for detection and identification of carnation event FLO-40685-2 and the carnation endogenous reference gene ANS was confirmed.

4.3. Method specificity

4.3.1. Testing the Moonvista method on GM carnation lines

PCR amplification products were generated by the EURL GMFF using as template the positive control sample FLO-40685-2 and DNA from GM carnation lines indicated in Table 2 with the primer sequences, and amplification conditions described in Tables 3, 4 and 5 (Figure 5).

Figure 5. Agarose gel electrophoresis of PCR products obtained by the EURL GMFF from PCR amplification of genomic DNA of carnation GM lines and conventional carnation line 123 with the FLO-40685-2 method



M = Molecular Weight Marker (Fermentas SM0323, Generuler 100 bp DNA ladder) ; Lane 1-2 = amplification of FLO-40685-2; lane 3-4 = amplification of conventional carnation line 123; lane 5-6 = amplification of Moonlite™; lane 7-8 = amplification of Moonaqua™ FLO-40689-6; lane 9-10 = amplification of Moonberry; lane 11-12 = amplification of Moonvelvet; lane 13-14 = amplification of SHD-27531-4; lane 15-16 = NTC

Results showed that the method amplifies the ANS reference target in all the carnation lines conventional line 123, Moonvista, Moonlite, Moonaqua, Moonberry, Moonvelvet, SHD-27531-4. The method also produces the lower size molecular fragment corresponding to the range of the expected 338 bp event-specific amplicon only on the FLO-40685-2 control sample.

4.3.2. Moonberry, Moonvelvet and Moonaqua detection methods

Suntory Holdings Ltd verified that the primers indicated in Table 7 do not cross-react with Moonvista DNA (see Annex 2); according to the notifier the inverted repeat structure in the insertion site of Moonvista hampers amplification by PCR. Therefore, based the currently available information, the methods for detection of Moonaqua, Mooonberry and Moonvelvet are still considered event-specific.

4.4. Robustness

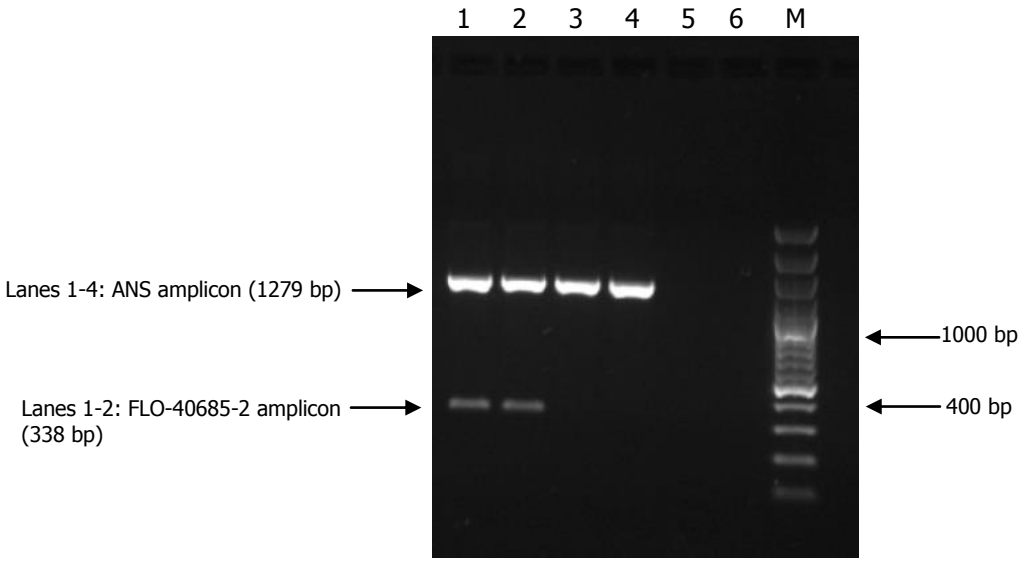
The robustness of the method was tested by modifying the PCR reaction components; the original method submitted by the applicant was based on the use of AmpliTaq Gold360 Master Mix (Applied Biosystems). For the robustness test, HotStarTaq DNA polymerase, 10x reaction buffer and dNTPs from Qiagen substituted the previously used reagents. The reactions were carried out according to the amplification conditions described in Table 5. The reaction mixture was prepared as described in Table 8.

Table 8. Reaction mastermix with Qiagen reagents for the duplex PCR targeting *anthocyanidin synthase* carnation gene (*ANS*) and the carnation GM event FLO-40685-2

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 201900)
RBF1a	10 µM	0.4 µM	1.0 µL	Eurofins
123.8.8-2.1R	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
HotStarTaq	5 units/µL	2.5 units	0.5 µL	Qiagen (cat 203205)
Genomic DNA template	variable	100 ng	variable	
Nuclease free water			up to 25 µL	Promega (cat P119C)
Total volume µL	25 µL			

The amplification products were separated by agarose gel electrophoresis in a 1.0% (w/v) gel (Figure 6).

Figure 6. Agarose gel electrophoresis of PCR products obtained from amplification of genomic DNA of the positive and the negative control with Qiagen reagents



Lane 1-2 = amplification of FLO-40685-2; lane 3-4= amplification of conventional carnation line 123; lane 5-6 = No template control; M= Molecular Weight Marker (Fermentas SM0323, Generuler 100 bp DNA ladder)

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 (line 123 and FLO-40685-2) and *ii*) a fragment corresponding to the GM specific amplification product only in the positive control FLO-40685-2 carnation GM-line. No additional band at a predicted length of 414 is visible in the FLO-40685-2 control sample (§ 4.1.3. Bioinformatics analyses. *FLO-40685-2 method*).

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

4.5. Limit of detection (LOD)

Based on statistical assumptions outlined in paragraph 2.6 and Annex 1, the absolute LOD is set at 59 positive tests over 60 replicates. Genomic DNA from carnation line FLO-40685-2 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 90 volts for 40 minutes. Results are shown in Table 9.

Table 9. Results of the determination of the limit of detection (LOD) on FLO-40685-2

GM Copy number/ reaction	**Number of replicates**	**Positive results GM (FLO-40685-2)**	**Negative results GM (FLO-40685-2)**	**Positive results reference (*ANS*)**	**Negative results reference (*ANS*)**
2500	60	60	0	60	0
1000	60	60	0	60	0
250	60	0	60	60	0
25	60	0	60	0	60

The results indicate that the absolute LOD of the method is between 250 and 1000 copies of GM target FLO-40685-2.

If it is assumed that one copy of the reference target is present per carnation genome variety line 123, the LOD_{abs} of the method for ANS target should be between 250 and 1000 copies, but the presence of a second copy cannot be excluded, as detailed in 4.1.3. For this reason the LOD_{abs} of the method is not reported for the ANS target.

5. Conclusions

The results presented demonstrate that the duplex end-point PCR method proposed by Suntory Holdings Ltd for the identification of FLO-40685-2 and of the carnation target *ANS* is applicable for the detection and identification of the GM event.

The sizes of the amplicons are 1279 bp for the carnation *anthocyanidin synthase* (*ANS*) reference gene and 338 bp for the GM event FLO-40685-2, respectively. The two DNA fragments generated by the restriction analysis of the 338 bp amplicon correspond to the expected pattern.

The method is robust, i.e. it functions with different master mixes and should therefore be widely applicable. The LOD_{abs} of the method for the GM target FLO-40685-2 is between 250 and 1000 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 FLO-40685-2 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 [3] [4], 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 [5] 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.

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