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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of Florigene™ 26407 GM Carnation

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Joint Research Centre

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Report on the Single-laboratory Validation of a PCR-based Detection Method for Identification of Florigene™ 26407 GM Carnation

Validation Report

6 November 2013

European Union Reference Laboratory for Genetically Modified Food and Feed

Executive Summary

Suntory Holdings Ltd has submitted an application for marketing (C/NL/09/02) of a genetically modified carnation line 26407 (Unique identifier: IFD-26407-2). In this context, the European Union Reference Laboratory for GM Food and Feed (EU-RL 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 26407, developed by the applicant.

This report describes the results of this validation, carried out by the EU-RL GMFF with control samples provided by the applicant.

The method is a duplex end-point PCR, where a carnation (taxon) target and a transgenic sequence are detected simultaneously.

The limit of detection (LOD) of the method has been established to be at least 10 copies for the taxon-specific target and between 50 and 10 copies for the GM target, based on haploid genome copy number. The event-specificity of the method was assessed by the applicant as being sufficient.

The EU-RL could verify that the taxon-specific primers correctly detect the endogenous gene target in genomic DNA of a conventional carnation line (negative control) and in the genomic DNA of the GM carnation line, while the GM target is detected by the GM specific primers only in genomic DNA of 26407 GM line (positive control).

Quality assurance

The EU-RL 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 EU-RL GMFF quality system.

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

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Content

1. Introduction

Suntory Holdings Ltd has submitted an application for marketing (C/NL/09/02) of a genetically modified carnation line 26407 (Unique identifier: IFD-26407-2). In this context, the European Union Reference Laboratory for GM Food and Feed (EU-RL 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 26407, developed by the applicant.

The EU-RL 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 641/2004 and according to its operational procedures ("Description of the EU-RL 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 ring trial was carried out.

2. Materials and Methods

2.1 Materials

The EU-RL GMFF received the following DNA samples from Suntory Holdings Ltd (Table 1), the DNA being extracted by the applicant from leaf material of GM and non-GM carnation.

Table 1. Positive and negative control samples (genomic DNA)

Sample name	Line information	DNA Concentration*
Cerise Westpearl	Parent carnation line, non-GM	296 ng/µL
26407	GM	85 ng/µL

*concentration was determined by the applicant using a spectrophotometer

In addition, the EU-RL GMFF received the following reagents from the applicant:

- Primer set 1 [non GM-line positive control ANS.F (#1056), ANS.R (#1057)] targeting the *anthocyanidin synthase* carnation gene (*ANS*) with an expected amplicon size of 1279 bp.
- Primer set 2 [RB forward (#1334), IFD-26407-2 reverse (#1608)] targeting the GM line 26407 with an expected amplicon size of 491 bp.

2.2 Estimation of DNA concentration

The concentration of the DNA samples provided by the applicant was verified by the EU-RL GMFF prior to use by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, cat. No P7589). Each DNA extract was measured three times, and the three 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 26407

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 491 bp fragment of the inserted sequence. For detection of the 26407 target a reverse primer (IFD-26407-2 reverse) based on the carnation endogenous genomic DNA flanking sequence, and a forward primer complementary to the insertion sequence (RB Forward) 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)**
IFD-26407-2		
IFD-26407-2 Reverse	5'- CAC GGG TAC AAA TTG GAT GAG -3'	21
RB Forward	5'- ATT TCC ACC TTC ACC TAC GAT GG -3'	23
ANS		
ANS Forward	5'-CTA GAT CGG AGG TCA CCA TAC C-3'	22
ANS Reverse	5'-GAA ACC GTG ACC ATG GTC TCG-3'	21

The method described above was applied by the EU-RL GMFF to genomic DNA of the conventional carnation line Cerise Westpearl (negative control) and of the GM line 26407 (positive control) according to the conditions described in Table 3 and Table 4. The EU-RL 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 26407 reverse and the RB forward primers targeting the GM line 26407. PCR was performed in duplicate.

Table 3. Reaction mastermix for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (*ANS*) and the GM carnation event 26407

Reagent	**Concentration stock**	**Concentration/ amount final**	**µL/reaction**	**Reference reagent**
PCR Buffer	10 x	1 x	2.5 µL	Qiagen (203205)
dNTPs	10 mM (each)	0.2 mM (each)	0.5 µL	Qiagen (201900)
IFD-26407-2 Reverse	50 ng/ µL	100 ng	2.0 µL	Eurofins (lot# 15809874)
RB Forward	50 ng/ µL	100 ng	2.0 µL	Eurofins (lot#16153267)
ANS Forward	50 ng/ µL	100 ng	2.0 µL	Eurofins (lot#14249102)
ANS Reverse	50 ng/ µL	100 ng	2.0 µL	Eurofins (lot#14249103)
HotStarTaq DNA Polymerase	5 units/ µL	2.5 units	0.5 µL	Qiagen (203205)
Genomic DNA template	33.3 ng/ µL	100 ng	3 µL	-
Nuclease free water			10.5 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 carnation 26407

Step		**Temperature**	**Time**
1	Activation/Initial Denaturation	95°C	15 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	5 min
6	Hold	4°C	

2.4 Purification of PCR products and restriction analysis

PCR amplification products were generated by the EU-RL GMFF using as template the 26407 positive control sample in accordance with the primer sequences, reaction set up and amplification conditions described in Tables 2, 3 and 4. After gel excision, the PCR products were purified with the GeneJET Gel Extraction Kit (cat # K0691), according to the manufacturer's instructions.

Bioinformatics analyses, carried out by the EU-RL GMFF identified a unique restriction site for the restriction enzyme EcoRV in the 491 bp PCR product. Two fragments of respectively 105 and 386 bp are expected to confirm the identity of the 491 bp amplicon with the 26407 target fragment.

The concentrations of the purified DNA amplicons were measured by the EU-RL GMFF via UV reading at 260 nm; 350 nanograms were digested with 15 Units of EcoR V (Invitrogen 15425010) at 37°C overnight. Lambda DNA (Molecular Probes, cat. No P7589) was used as a restriction control. Results of digestion were visualised in 1.5% (w/v) agarose gel electrophoresis.

2.5 Limit of Detection (LOD)

Suntory Holdings Ltd determined the limit of detection (LOD) of the method by applying it to the following concentrations, measured in genome copies per reaction (ten replicates per reaction): 7500, 5000, 2500, 1000, 500, 160, 80, 0. One copy of the carnation haploid genome is assumed to correspond to 0.63 pg[a].

After amplification, the fragments were resolved by 1% (w/v) agarose gel electrophoresis and visualised by ethidium bromide staining, imaged under UV light.

3. Results of tests conducted by the applicant

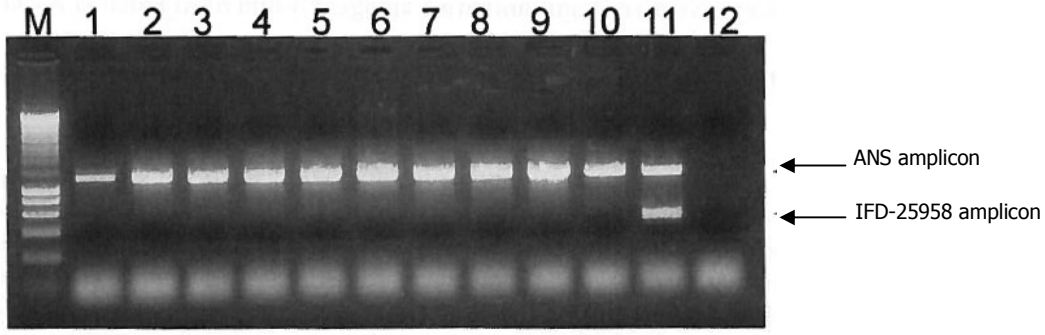
The data reported below were produced by the applicant as a part of the notification dossier.

3.1. Identification and specificity

Suntory Holdings Ltd provided information on specificity and stability of the IFD-26407-2 method. The duplex assay was tested on genomic DNA extracted from non-genetically modified carnation lines (Cream Cinderella, Piccola, Cerise Westpearl) and on genomic DNA from genetically modified carnation lines (Florigene Moonvista™, Moonaqua™, Moondust™, Moonlite™, Moonshadow™, Moonshade, IFD-25958-3 and IFD-26407-2). 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 IFD-26407-2 line (Figure 1). The resulting amplicons were compared by agarose gel electrophoresis to a ladder marker of molecular weights. The size of the *ANS* amplicon was indicated by the applicant as 1,300 bp while it should have been correctly marked as 1,279 bp.

[a] Royal Botanic Gardens, Kew Plant DNA C-values Database (release 4.0, October 2005). http://data.kew.org/cvalues/.

Figure 1. Agarose gel electrophoresis of the PCR products obtained with the duplex method



Lanes M = Hyperladder I (Bioline, Australia) (10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, 1.0, 0.8, 0.6, 0.4 and 0.2 kb fragments); Lane 1 = Cream Cinderella; Lane 2 = Piccola; Lane 3 = Cerise Westpearl; Lane 4 = Moonvista; Lane 5 = Moonaqua; Lane 6 = Moonshadow; Lane 7 = Moondust; Lane 8= Moonlite; Lane 9 = Moonshade; Lane 10 = IFD-25958-3; Lane 11 IFD-26407-2; Lane 12 = 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 26407 is 1000 haploid carnation copies.

4. Results of tests conducted by the EU-RL GMFF

The data reported below were produced by the EU-RL 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 following values were observed (Table 5):

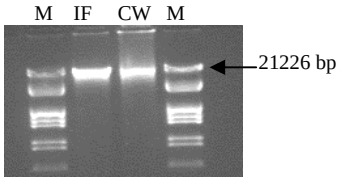
Table 5. Measured concentrations for the negative and positive control samples

Carnation line	Concentration (ng/μL)
Cerise Westpearl	142
26407	65

4.1.2. DNA integrity

The DNA integrity of the control samples was evaluated by agarose gel electrophoresis; 260 ng and 280 ng respectively of the positive (26407) and negative (Cerise Westpearl) control samples were analysed on a 1.0% (w/v) agarose gel at 70 Volts for 1 hour (Figure 2).

Figure 2. Agarose gel electrophoresis of (DNA solutions from) positive (26407) and negative (Cerise Westpearl) controls



CW= Cerise Westpearl, 1 µL; IF = IFD-26407-2 2 µL; M= Molecular Weight Marker (bp): 21226, 5148-3530, ca. 2000, 1584, 1375, 947, 831, 564.

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

4.1.3. Bioinformatics analysis

Bioinformatics analysis carried out at the EU-RL GMFF, based on similarity searches, could not draw conclusions about the event-specificity of the method proposed. This was due to lack of information on the genomic sequences of *Dianthus caryophyllus* (carnation) in public databases. Therefore, the event-specificity of the method for detection of 26407 could only be based on the documentation and data provided by the applicant. It needs to be noted that the primers for the *ANS* endogenous gene also amplify the transgenic sequences of event 26407. Indeed, the amplicon of the taxon target falls in the promoter of the *ANS* gene, which has also been used for the construction of the carnation GM event. Therefore, while the *ANS* endogenous gene assay is fit for the purpose of qualitative detection of carnation, it might not be the most suitable target in case of quantitative analysis.

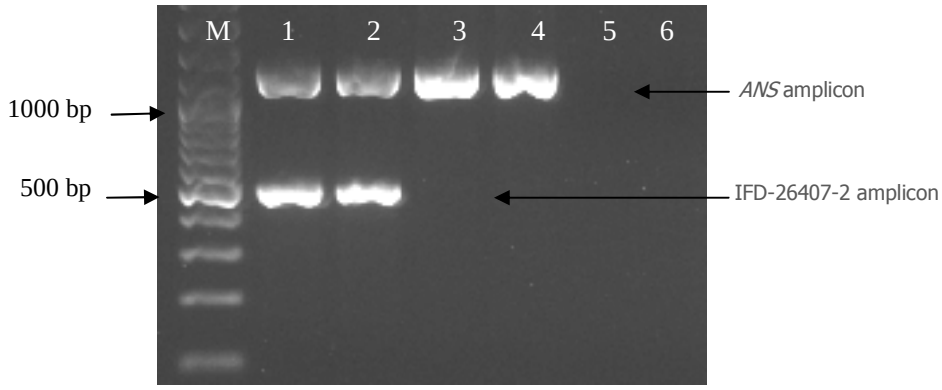
4.2. Identification of 26407 carnation

The protocol provided by the method developer for specific identification of 26407 was applied by the EU-RL 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 assay was run in duplicate on the positive and negative control samples. Two DNA amounts (100 and 150 nanograms) were loaded for the positive and negative control samples.

The amplification products were separated by agarose gel electrophoresis on a 1.5% (w/v) agarose gel (Figure 3).

Figure 3. Agarose gel electrophoresis of PCR products obtained by the EU-RL GMFF from PCR amplification of genomic DNA of the carnation conventional line Cerise Westpearl and of the GM line 26407



1-2 = IFD-26407-2 (100 ng genomic DNA in reaction); 3-4 = Cerise Westpearl (100 ng genomic DNA in reaction); 5-6 = No template control; M= Molecular Weight Marker (Fermentas SM0324) bp: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100

Both the 26407 and the conventional carnation genomic DNA reacted with the *ANS* amplification system, resulting in the amplification of a fragment of 1279 bp corresponding to the expected length for the target sequence of the carnation *anthocyanidin synthase* gene.

Only the GM target line 26407 reacted with the event-specific amplification system yielding a fragment of 491 bp in accordance with the expected size for the 26407 amplicon.

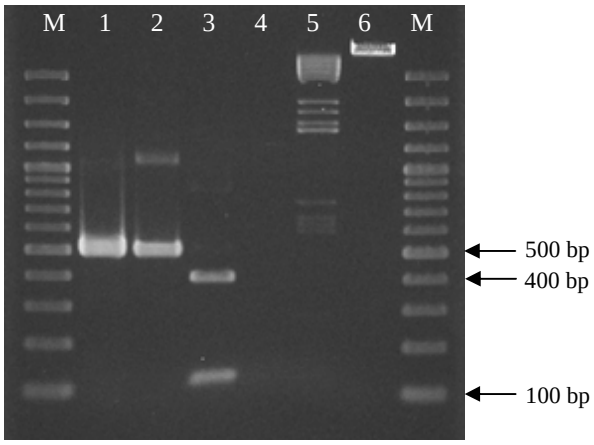
The analysis demonstrates that the application of the proposed duplex PCR assay allowed amplifying: i) a fragment corresponding to the endogenous reference gene, the carnation *anthocyanidin synthase* (*ANS*) gene fragment in the carnation samples (Cerise Westpearl and IFD-26407-2) and ii) a fragment corresponding to the GM specific amplification product only in the positive control IFD-26407-2 carnation line.

4.3. Confirmation of PCR products

The amplicon sizes generated by the duplex assay for simultaneous detection of 26407 and *ANS* carnation gene were in agreement with the expected sizes determined via bioinformatics analysis. However, a confirmation step of the identity of the GM amplification product was carried out via purification of the 491 bp amplicon and analysis of restriction digestion with the EcoR V enzyme.

Figure 4 shows the results of the EcoR V restriction analysis.

Figure 4. Restriction analysis of the 491 bp amplicon



1 = PCR product, not purified; 2 = PCR purified product, no digest; 3 = PCR product after restriction (105 + 386 bp); 4 = no template control of digestion; 5 = positive control of EcoR V digestion: lambda DNA restricted with EcoR V (expected: ~23 fragments); 6 = lambda DNA; M = Molecular Weight Marker (Fermentas SM0324) bp: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100

The restriction digestion with EcoR V of the 491 bp PCR product produced two fragments consistent with the predicted size of 105 bp and 386 bp, thus confirming that the event-specific duplex PCR system allows detection and identification of the IFD-26407-2 carnation GM-line.

4.4. Robustness

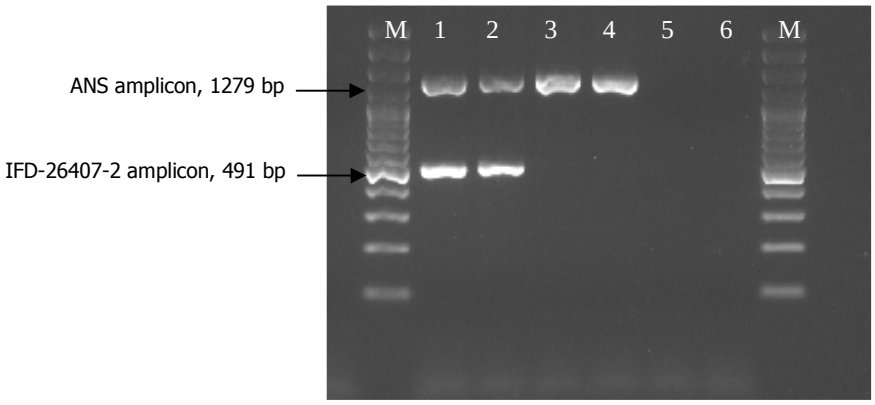
The robustness of the method was tested by changing Taq DNA polymerase and the reaction buffer. The AmpliTaq Gold (Roche) and its respective reaction buffer were used in place of the HotStarTaq DNA polymerase, (Qiagen), in combination with its reaction buffer. The reactions were carried out according to the amplification conditions described in Table 4. The reaction mixture was prepared as described in Table 6.

Table 6. Reaction mastermix with AmpliTaq Gold for the duplex PCR targeting *anthocyanidin synthase* carnation gene (*ANS*) and the carnation GM event 26407

Reagent	**Concentration stock**	**Concentration/ amount final**	**µL/reaction**	**Reference reagent**
10 x PCR Buffer II	10 x	1 x	2.5 µL	Applied Biosystems (N808-0243)
dNTPs mix	10 mM each	0.2 mM each	0.5 µL	Promega (U1240), in house dilution to 10 mM each)
$MgCl_2$	25 mM	1.5 mM	1.5 µL	Applied Biosystems (N808-0243)
IFD-26407-2 Reverse	50 ng/µL	100 ng	2.0 µL	Eurofins (lot# 15809874)
RB Forward	50 ng/µL	100 ng	2.0 µL	Eurofins (lot#16153267)
ANS Forward	50 ng/µL	100 ng	2.0 µL	Eurofins (lot#14249102)
ANS Reverse	50 ng/µL	100 ng	2.0 µL	Eurofins (lot#14249103)
AmpliTaq Gold	5 units/µL	2.5 units	0.5 µL	Applied Biosystems (N808-0243)
Genomic DNA template	33.3 ng/µL	100 ng	3 µL	
Nuclease free water			9 µL	Promega, P119C
Total volume µL	25 µL			

The amplification products were separated by agarose gel electrophoresis on a 1.5% (w/v) gel (Figure 5).

Figure 5: Agarose gel electrophoresis of PCR products obtained (from PCR amplification of genomic DNA of the GM-line 26407 and of the carnation conventional line Cerise Westpearl) with AmpliTaq Gold (Roche)



1-2 = IFD-26407-2 (100 ng genomic DNA in reaction); 3-4 = Cerise Westpearl (100 ng genomic DNA in reaction) M= Molecular Weight Marker (Fermentas SM0324) bp: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100

The results of the amplification are in line with the results obtained using the Qiagen components. In particular, the PCR amplification generated *i)* a fragment of a size corresponding to the endogenous carnation *anthocyanidin synthase* (*ANS*) gene fragment in the carnation samples (Cerise Westpearl and IFD-26407-2) and *ii*) a fragment corresponding to the GM specific amplification product only in the positive control IFD-26407-2 carnation GM-line.
Therefore, the robustness testing showed that the proposed detection method tolerates changes in the reaction components described.

4.5. Limit of detection (LOD)

The EU-RL GMFF carried out tests to estimate the limit of detection (LOD) of the method, a duplex end-point PCR for identification of 26407 and the carnation taxon-target *ANS*.

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 time and hence ensuring ≤5% false negative results, with a 0.95 confidence level.

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 statistical approach (for a more complete description of it see Annex 1). Based on this approach, 60 replicates were found to be needed to be tested with the GM duplex system per each DNA copy number at different concentration-levels set up as serial dilutions. Genomic DNA from carnation line 26407 was amplified at defined copy numbers in the described conditions (Tables 3 and 4).

One copy of carnation haploid genome is considered to correspond to 0.63 pg [(1)].

Amplification results were evaluated by gel electrophoresis. A PCR volume of 20 µL was loaded on a 1% (w/v) agarose gel and run at 100 volts for 1 hour. Results are shown in Table 7.

Table 7. Results of the determination of the limit of detection (LOD)

Copy number/ reaction	**Number of replicates**	**Positive results GM (26407)**	**Negative results GM (26407)**	**Positive results reference (*ANS*)**	**Negative results reference (*ANS*)**
50	60	60	0	60	0
10	60	42	18	60	0
0.1	60	0	60	0	60

Therefore, the absolute LOD of the method is between 50 and 10 copies for the GM target and below 10 copies for the taxon target.

5. Conclusions

The presented study demonstrated that the duplex end-point PCR method proposed by Suntory Holdings Ltd for the identification of 26407 and of the carnation target *ANS,* that amplifies a fragment of 1279 bp corresponding to the carnation *anthocyanidin synthase* (*ANS*) reference gene, in both the parental and the GM line, and a fragment of 491 bp corresponding to the event 26407 only in the respective GM line, is applicable for the detection (LOD between 10 and 50 copies for the GM target) and identification and of the GM event.

The two DNA fragments generated by the restriction analysis of the 491 bp amplicon correspond to the expected pattern. The method is robust, i.e. functions with different master mix and should therefore be widely applicable.

Annex 1. Determination of the limit of detection

For an accurate estimate of the 0.95 (1-α) confidence interval (depending on the degrees of freedom used to compute p), the F-distribution is based on the relationship between such distribution and the binomial distribution[b]. The method is derived from Bliss[c] and recently re-proposed by Zar[d] According to this method, 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 and α= 0.05, L_2 = 0.05 results from n = 100.

As suggested by various statisticians[e] 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):

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

[b] Fisher R.A., Yates F., 1963. Statistical tables for biological, agricultural and medical research, 6th edition. Hafner, New York, USA, 146 pp.

[c] Bliss C.I., 1967. Statistical biology, Vol. 1. McGraw-Hill, New York, USA, 558 pp.

[d] Zar J.H., 1999. Biostatistical analysis, 4th edition. Prentice Hall, New Jersey, 663 pp.

[e] Cochran, 1977. Sampling techniques, 3rd edition. John Wiley, New York, 428 pp.

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

Based on this simplified approach, with X = 1 and α = 0.05, L2 = 0.05 results from n = 60, thus resulting in an experimental absolute LOD set at 59 positive tests (n - X) over 60 replicates.

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Abstract

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