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Report on the single-laboratory validation of a PCR-based Detection Method for Identification of Florigene™ IFD-25958-3 GM Carnation

Validation Report and Validated Method

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Report on the single-laboratory validation of a PCR-based Detection Method for Identification of Florigene™ IFD-25958-3 GM Carnation

Validation Report

13 December 2012

European Union Reference Laboratory for Genetically Modified Food and Feed

Executive Summary

In the context of the application for marketing submitted by Florigene Pty Ltd for a genetically modified carnation line (C/NL/09/01) IFD-25958-3, the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) has carried out a single-laboratory validation to assess the performance of a polymerase chain reaction (PCR)-based detection method for detecting and identifying the carnation GM line IFD-25958-3.

This report describes the results of tests carried out by the EU-RL GMFF on control samples provided by the method developer and according to the detection method described by the applicant.

The taxon-specific method correctly detects the endogenous gene target in genomic DNA of a conventional carnation line (negative control) and in the genomic DNA of the GM carnation line; the same method can also detect the GM target DNA in IFD-25958-3 GM line (positive control) in the experimental conditions described in this report.

The Limit of Detection (LOD) of the method has been estimated to be at least 50 copies for the taxon-specific gene and at least 100 copies for the GM insert, based on haploid genome copy number.

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and 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.

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Content

1. Introduction

In the context of the application for marketing submitted by Florigene Pty Ltd for a genetically modified carnation line (C/NL/09/01) IFD-25958-3, under Directive 2001/18/EC the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) has carried a single-laboratory validation study to assess the performance of a PCR-based detection method developed to detect and identify the IFD-25958-3 GM carnation.

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 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EU-RL GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The EU-RL GMFF received the method description and positive and negative samples for carnation event IFD-25958-3 (C/NL/09/01) in March 2010.

During step 2, four subsequent scientific assessments were performed, due to the need to clarify various elements of the application; the scientific assessment (step 2) was positively concluded in September 2011.

Between February and May 2012, the EU-RL GMFF verified the purity of the control samples provided and conducted the in-house experimental testing of samples and methods (step 3).

2. Materials and Methods

2.1 Materials

The EU-RL GMFF received the following DNA samples from Florigene Pty Ltd (Table 1). DNA was extracted by the applicant from leaf material of carnation.

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

Line name	Line information	Concentration*
Cerise Westpearl	Parent Carnation line, non-transformed	531 ng/µL
IFD-25958-3	GM	87 ng/µL

*concentration was determined using a spectrophotometer by the applicant

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

- 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 [Florigene Pty Ltd IFD-25958-3 RB forward (#1333), IFD-25958-3 reverse (#1599)] targeting the GM line IFD-25958-3 with an expected amplicon size of 482 bp.

2.2 PCR-based detection method for identification of IFD-25958-3

The method developed and optimised by Florigene Pty Ltd. is a duplex end-point PCR in which two targets are amplified in the same reaction, specifically according to the applicant a 1279 bp fragment of the *anthocyanidin synthase* carnation gene (*ANS*) and a 482 bp fragment of the inserted sequence. For detection of the IFD-25958-3 target a reverse primer (IFD-25958-3 reverse) based on the carnation endogenous genomic DNA flanking sequence, and a forward primer complementary to the insertion sequence (RB Forward) were designed (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-25958-3		
IFD-25958-3 reverse	5'-CTT GTA CAC CGC GTT ATG CTC-3'	21
RB forward	5'-TGG ACC CTT GAG GAA ACT GGT AGC-3'	24
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

Genomic DNA of the conventional carnation line Cerise Westpearl (negative control) and of the GM line IFD-25958-3 (positive control) were amplified by PCR according to the conditions described in Table 3 and Table 4. The test was conducted 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 IFD-25958-3 reverse and the RB forward primers targeting the GM line IFD-25958-3. PCR was performed in duplicate.

Table 3. Reaction mastermix for the duplex PCR targeting the *anthocyanidin synthase* carnation gene (*ANS*) and the GM carnation IFD-25958-3

Reagent	Concentration stock	Concentration final	µL / reaction	Reference reagent
PCR Buffer	10 x	1 x	2.5 µL	Qiagen (203205)
dATPs	10 mM	0.2 mM	0.5 µL	Promega (U120A)
dCTPs	10 mM	0.2 mM	0.5 µL	Promega (U122A)
dGTPs	10 mM	0.2 mM	0.5 µL	Promega (U121A)
dUTPs	10 mM	0.2 mM	0.5 µL	Promega (U123A)
IFD-25958-3 Reverse	50 ng/ µL	100 ng	2.0 µL	M Medical (lot# 14249100)
RB Forward	50 ng/ µL	100 ng	2.0 µL	M Medical (lot#14249101)
ANS Forward	50 ng/ µL	100 ng	2.0 µL	M Medical (lot#14249102)
ANS Reverse	50 ng/ µL	100 ng	2.0 µL	M Medical (lot#14249103)
HotStar Taq DNA Polymerase	5 units/ µL	2.5 units	0.5 µL	Qiagen (203205)
Genomic DNA template	50 ng/ µL	100 ng	2 µL	-
Nuclease free water			10 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 IFD-25958-3

Step		Temperature	Time
1	Initiation denaturation and enzyme activation	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	

2.3 PCR-based detection method for determination of the Limit of Detection

Florigene Pty Ltd determined the limit of detection (LOD) of the method for detecting IFD-25958-3 carnation based on a simplified approach. A simplex format, including only the primers for amplification of the IFD-25958-3 target in carnation, was used according to the conditions referred to in Table 5. The LOD of the assay was tested at the following genome copies per reaction (ten replicates per reaction): 7500, 5000, 2500, 1000, 500, 160, 80, 0. One copy of the carnation haploid genome was assumed to correspond to 0.63 pg [(1)].

Table 5. Reaction components and cycling conditions for the LOD test

Reaction components			
1x PCR Buffer (Qiagen, Australia)		2.5 µL	-
dNTPs (Takara, Japan)		0.2 mM each	
Primer IFD-25958-3 Reverse		50 ng/µL	100 ng
Primer RB Forward		50 ng/µL	100 ng
HotStar Taq DNA Polymerase (Qiagen, Australia)		2.5 units	
Genomic DNA template		7500, 5000, 2500, 1000, 500, 160, 80, 0 copies	
Deionised water		Up to 25 µL	
Total volume µL		25 µL	
Cycling conditions			
Step 1	Denaturation and enzyme activation	95°C	15 min
Step 2	Denaturation	95°C	30 sec
Step 3	Annealing	55°C	30 sec
Step 4	Extension, 30 cycles	72°C	1 min
Step 5	Extension	72°C	5 min

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

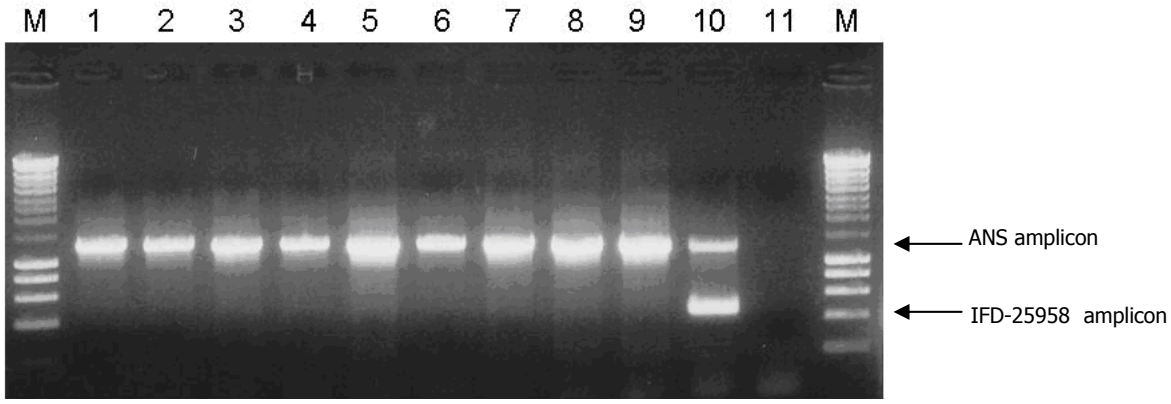
3. Results of tests conducted by the applicant

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

a. Identification and specificity

Florigene Pty Ltd provided information on identification, specificity and stability of the IFD-25958-3 method. According to the data provided by the applicant 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 and IFD-25958-3). The end-point PCR duplex 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-25958-3 line (Figure 1).

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 = no template control.

b. Determination of the Limit of Detection (LOD)

According to Florigene the LOD of the assay for detection of IFD-25958-3 was 500 genome 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.

a. Quality checks on the control samples

i. DNA concentration

The concentration of the DNA solutions received was verified prior to use by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). 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.

The following values were observed (Table 6):

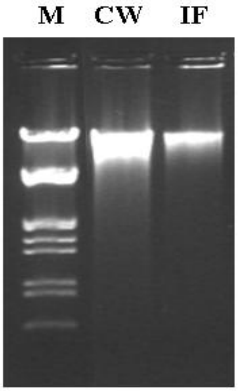
Table 6. Measured concentrations for the negative and positive control samples

Carnation line	Concentration (ng/μL)
Cerise Westpearl	473
IFD-25958-3	82

ii. DNA quality or integrity

The DNA intactness of the control samples was evaluated by agarose gel electrophoresis; 1 μL (Cerise Westpearl) or 2 μL (IFD-25958-3) of the DNA solutions received were analysed on a 1.0% (w/v) agarose gel at 70 Volts for 1 hour (Figure 1).

Figure 1. Agarose gel electrophoresis of DNA solutions from positive (IFD-25958-3 line) and negative (Cerise Westpearl) controls.



CW= Cerise Westpearl, 1 μL ; IF = IFD-25958-3 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. None of the DNA samples showed signs of significant degradation, thus indicating satisfactory DNA quality.

iii. Bioinformatics analysis

Bioinformatics analysis carried out at the EU-RL GMFF, based on search for similarity, 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 IFD-2598-3 could only be based on the declaration and documentation provided by the applicant. It needs to be noted that the primers for the *ANS* endogenous gene also amplify the transgenic sequences of event IFD-25958-3.

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.

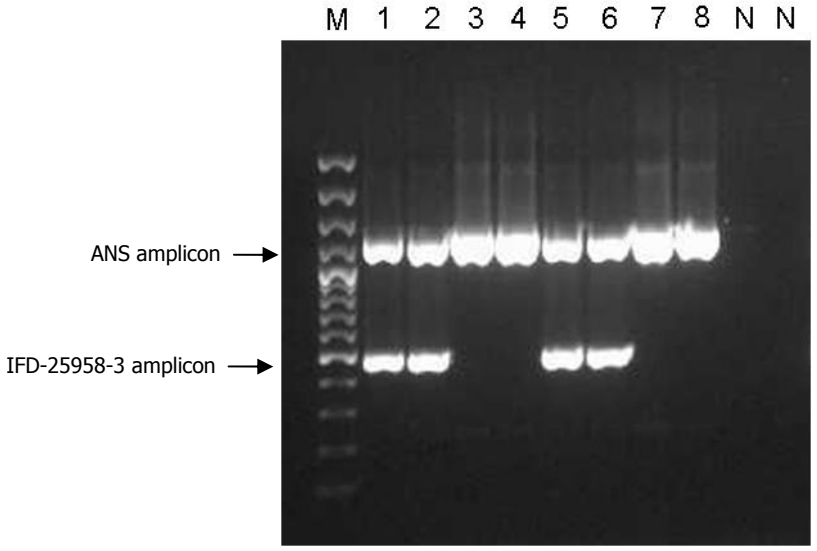
b. Identification of IFD-25958-3

The protocol provided by the method developer for specific identification of IFD-25958-3 was applied by the EU-RL GMFF according to the described amplification conditions (Paragraph 2.2) 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 were loaded for the positive and negative control samples, respectively 100 and 150 nanograms.

The amplification products were separated by agarose gel electrophoresis on a 1% (w/v) agarose gel (Figure 3).

Figure 3: Agarose gel electrophoresis of PCR products obtained from PCR amplification of genomic DNA of the carnation conventional line Cerise Westpearl and of the GM line IFD-25958-3.



Lane 1-2 = IFD-25958-3 (100 ng template DNA in reaction); lane 3-4 = Cerise Westpearl (100 ng template DNA in reaction); lane 5-6 = IFD-25958-3 (150 ng template DNA in reaction); lane 7-8 = Cerise Westpearl (150 ng template DNA in reaction); N= No template Control; M= Molecular Weight Marker (bp): 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100

All the samples reacted with the *ANS* primers, 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.

The GM target line IFD-25958-3 reacted with the event-specific primers yielding a fragment of 482 bp in accordance with the expected size for the IFD-25958-3 amplicon.

The analysis demonstrates that the application of the proposed duplex PCR assay allowed amplifying: i) a fragment corresponding to the endogenous marker, the carnation *anthocyanidin synthase* (*ANS*) gene fragment in the carnation samples (Cerise Westpearl and IFD-25958-3) and ii) a fragment corresponding to the GM specific amplification product only in the positive control IFD-25958-3 carnation line.

c. Robustness

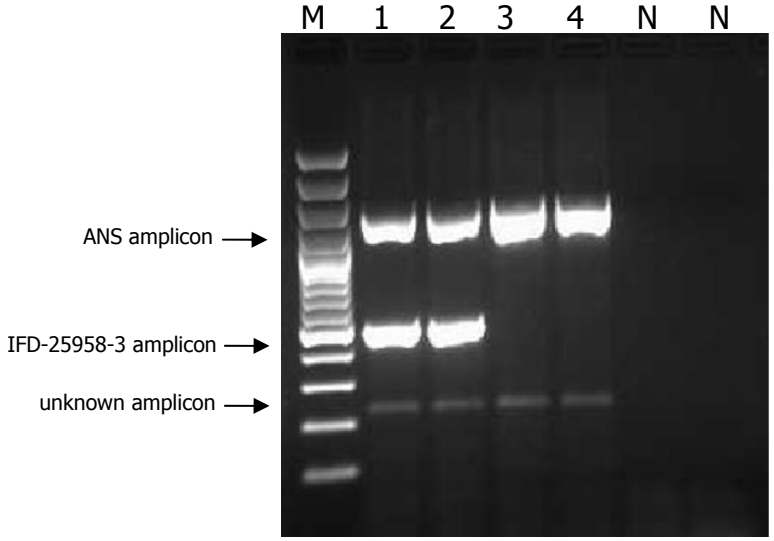
The robustness of the method was tested by changing Taq DNA polymerase and the reaction buffer. The AmpliTaq Gold (Roche) and its respective reaction buffer were used in place of the HotStar Taq DNA polymerase, Qiagen, in combination with its reaction buffer. The reactions were carried out according to the amplification conditions described in Table 4. Reaction mixture was prepared as described in Table 7.

Table 7. Duplex PCR *anthocyanidin synthase* carnation gene (*ANS*) - IFD-25958-3 reaction mastermix with AmpliTaq Gold.

Reagent	**Concentration stock**	**Concentration final**	**µL / reaction**	**Reference reagent**
10 x PCR Buffer II	10 x	1 x	2.5 µL	Roche (N808-0249)
dNTPs mix	10 mM	0.2 mM	0.5 µL	Promega (C114H)
$MgCl_2$	25 mM	1.5 mM	1.5 µL	Roche (N808-0249)
IFD-25958-3 reverse	50 ng/µL	100 ng	2.0 µL	M Medical (lot#14249100)
RB forward	50 ng/µL	100 ng	2.0 µL	M Medical (lot#14249101)
ANS forward	50 ng/µL	100 ng	2.0 µL	M Medical (lot#14249102)
ANS reverse	50 ng/µL	100 ng	2.0 µL	M Medical (lot#14249103)
AmpliTaq Gold	5 units/µL	2.5 units	0.5 µL	Roche (N808-0249)
Genomic DNA template	50 ng/µL	100 ng	2 µL	
Nuclease free water			10 µL	Promega, P119C lot#30810801
Total volume µL	25 µL			

The amplification products were separated by agarose gel electrophoresis on a 1% (w/v) gel (Figure 4).

Figure 4: Agarose gel electrophoresis of PCR products obtained from PCR amplification of genomic DNA of the GM-line IFD-25958-3 and of the carnation conventional line Cerise Westpearl with AmpliTaq Gold (Roche).



Lane 1-2 = IFD-25958-3; lane 3-4 = Cerise Westpearl; N= No template Control

M= Molecular Weight Marker (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 reaction components indicated in the method submitted (Annex 1). In particular, the PCR amplification generated *i)* a fragment of a size corresponding to the endogenous marker, the carnation *anthocyanidin synthase* (*ANS*) gene fragment in the carnation samples (Cerise Westpearl and IFD-25958-3) and *ii*) a fragment corresponding to the GM specific amplification product only in the positive control IFD-25958-3 carnation line. An unknown and faint amplification product at around 250 bp is detectable in both the negative control and the GM IFD-25958-3 indicating that the duplex assay is sensitive to the modification of the reaction buffer and DNA polymerase (from HotStar Taq DNA polymerase, Qiagen, to AmpliTaq Gold, Roche).

Therefore, the robustness testing showed that the proposed detection method tolerates changes in the reaction components described, though an additional unknown fragment is amplified with low efficiency.

d. Limit of detection

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 IFD-25958-3 and the carnation taxon-target *ANS*. The LOD was calculated by amplifying in the described conditions (Tables 3 and 4) IFD-25958-3 genomic DNA at defined copy numbers.

The optimal sample size (number of replicates n per assayed GM level) was estimated to determine the limit of detection (LOD), defined as the GM level (p) detected at least 95% of the time, thus ensuring ≤5% false negative results. The number n was estimated to generate a 0.95 confidence interval whose upper bound does not exceed 5%.

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 was used based on the relationship between such distribution and the binomial distribution [(2)]. The method is derived from Bliss [(3)] and recently re-proposed by Zar [(4)]. 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 [(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 ɑ= 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 (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, 2005 at http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf).

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, 60 replicates were tested with the GM duplex system in each of four DNA copy number levels set up as serial dilutions.

In the model, one copy of carnation haploid genome is considered to correspond to 0.63 pg [(1)].

The evaluation of positive signals is based on gel electrophoresis. A PCR volume of 20 µL was loaded on a 1% agarose gel and run at 100 volts for 1 hour. Results are shown in Table 8.

Table 8. Results of experiments for the determination of the Limit of Detection (LOD)

Copy number / reaction	**Number of replicates**	**Positive results GM (IFD-25958-3)**	**Negative results GM (IFD-25958-3)**	**Positive results reference (*ANS*)**	**Negative results reference (*ANS*)**
100	60	60	0	60	0
50	60	44	16	60	0
10	60	0	60	0	60
0.1	60	0	60	0	60

Therefore, the absolute LOD of the method is between 100 and 50 copies for the GM target and between 50 and 10 copies for the taxon target under the experimental conditions described.

5. Conclusions

The present study demonstrated that the method, a duplex end-point PCR for the identification of IFD-25958-3 and the carnation target *ANS* proposed by Florigene Pty Ltd amplifies a fragment corresponding to the reference marker, the carnation *anthocyanidin synthase* (*ANS*) gene, in both the parental and the GM line, and a fragment corresponding to the IFD-25958-3 specific amplicon only in the GM line. The fragment size of both the reference marker and the GM specific fragment corresponds to the expected values of r 1279 bp and 482 bp respectively.

Testing of method's robustness by using AmpliTaq Gold (Roche) and its respective reaction buffer in place of the HotStart Taq DNA polymerase (Qiagen) in combination with its reaction buffer, showed that the proposed detection method tolerates changes in the reaction component described, though an additional unknown fragment is amplified with low efficiency.
The estimated absolute LOD is between 100 and 50 copies for the GM target and between 50 and 10 copies for the reference target.

6. References

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Abstract

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The Limit of Detection (LOD) of the method has been estimated to be at least 50 copies for the taxon-specific gene and at least 100 copies for the GM insert, based on haploid genome copy number.

This report is published at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

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