Report on the Testing of a PCR-based Detection Method for Identification of *Florigene™ Moonlite* GM Carnation

Protocol Version 2

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Joint Research Centre – European Commission
Institute for Health and Consumer Protection
Biotechnology and GMOs Unit

Method development:
Florigene Pty. Ltd.

Method Testing and Confirmation:
Community Reference Laboratory for GM Food and Feed (CRL-GMFF)

EXECUTIVE SUMMARY

In the context of the application for marketing submitted by Florigene Pty. Ltd. for a genetically modified carnation line (C/NL/04/02) "Moonlite™" (123.2.38), the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) reported on 14 September 2006 the results of in-house tests carried out to verify the performance of a PCR-based detection method developed to identify the GM carnation line. In the conclusion of the report the CRL-GMFF recommended that further optimisation of the detection method was to be carried out.

In January 2007, the CRL-GMFF received additional control samples and a revised method of detection for the genetically modified carnation line (C/NL/04/02) "Moonlite™" (123.2.38).

The present report describes the results of tests carried out by the CRL-GMFF on the control samples according to the revised detection method.

The assay correctly detects the control target in genomic DNA of conventional carnation lines and in the genomic DNA of the tested GM Carnation lines; the 123.2.38 assay (Moonlite™-specific) can detect the control target in Moonlite™ GM line (positive control) in the experimental conditions described in this report.

A robustness test confirmed the Florigene Pty. Ltd. findings according to which the detection method is sensitive to changes in Taq polymerase brand.

The Limit of Detection (LOD) of the method has been estimated at 2500 copies, based on haploid genome copy number.

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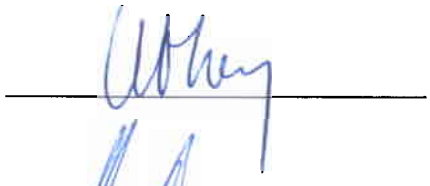

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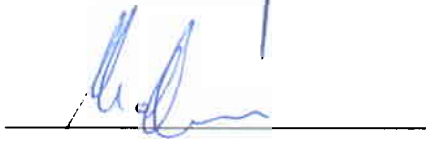

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CONTENT

1. Introduction

In the context of the application for marketing submitted by Florigene Pty. Ltd. for a genetically modified carnation line (C/NL/04/02) "Moonlite™" (123.2.38), the Community Reference Laboratory (CRL) for GM Food and Feed has carried out tests to verify the performance of a revised PCR based detection method developed to identify the GM carnation line.
Upon reception of the protocol and control samples, the JRC performed the tests in February 2007.

2. Documentation, Materials and Methods

The CRL received the revised version of the detection method from Florigene Pty. Ltd. The document can be found in Annex 1 of this report.

On 16/01/2007 the CRL received the following DNA samples from Florigene Pty. Ltd.:

Line name	Line information	Concentration
Moonvista™	GM	620 ng/μl
Moonaqua™	GM	1200 ng/μ
Moonlite™	GM	1200 ng/μl
Moonshadow™	GM	1762 ng/μl
Moondust™	GM	1273 ng/μl
123 (CC)	Parent carnation line	1494 ng/μl
Piccola DNA	Carnation line, non-transformed	306 ng/μl
Rendezvous	Carnation line, non-transformed	80 ng/μl

In addition, the CRL-GMFF received the following reagents:

- Qiagen HotStart Taq and Qiagen PCR buffer
- 10mM dNTPs

- Primer set 1 (all-lines positive control ANS.F (#1056), ANS.R (#1057) amplicon: 1300 bp)
- Primer set 2 (Florigene Pty. Ltd. Moonlite™ LB inside.R – LB123.2.38-R – amplicon: 380 bp)

The concentration of the DNA solutions received was verified prior to the use in PCR by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).
DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/ml to 500 ng/ml using a Bio-Rad VersaFluor™ Fluorometer as fluorescence detector.
The following values were observed:

Carnation line	**Concentration (ng/μl)***
Moonvista™	127
Moonaqua™	1078
Moonlite™	1443
Moonshadow™	891
Moondust™	5049
Cream Cinderella	1234
Piccola	107
Rendezvous	25.4

* average of three readings, except Rendezvous 1 reading due to low sample volume

Amplification conditions were as described in Annex 1. The PCR analysis was performed on an ABI 9700 apparatus.

3. Results

3.1. Testing of the method and of test samples (genomic DNA and primers)

Genomic DNA from conventional carnation lines or GM- lines Moonvista™, Moonaqua™, Moonshadow™, Moondust™ (negative control) and Moonlite™ (positive control) were amplified by PCR according to the conditions described in Annex 1.

Three tests were conducted to assess the method specificity using the duplex configuration as specified by the method developer (see Annex 1) with primer set 1 targeting the anthocyanin synthase carnation gene (ANS) and primer set 2 targeting the the GM line Moonlite™ in the same reaction. PCR analysis was performed in duplicate.

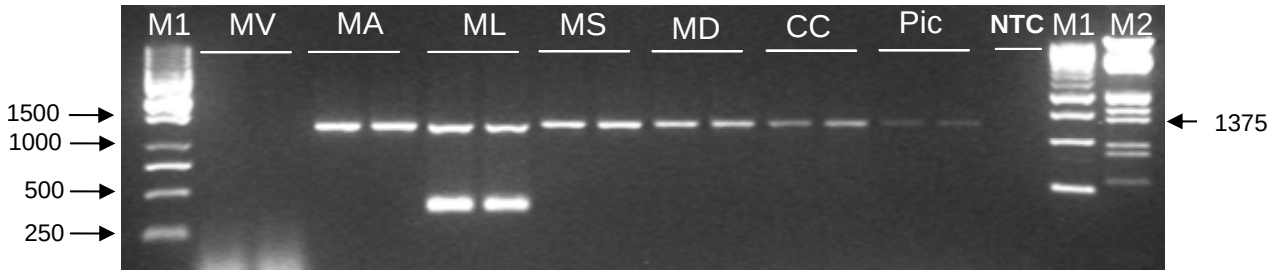
The conventional line Rendezvous was amplified in one experiment only due to low amount of sample available.

All the samples reacted with the ANS primers, thus resulting in the amplification of a fragment (amplicon size ~1300 bp) corresponding to the expected length for the target sequence of the carnation anthocyanin synthase gene.
GM line Moonvista™, however, resulted in inconstant amplification of the 1300 bp DNA sequence.

GM target line Moonlite™ reacted with the event-specific primers yielding a band (~380 bp) in accordance with the expected size for Moonlite™ specific amplicon.
The amplification products were separated by agarose gel electrophoresis on a 1% gel.

Results from one experiment are shown in Figure 1.

Figure 1: Agarose gel electrophoresis of PCR products obtained from PCR amplification of genomic DNA of the carnation conventional lines and of the GM-lines.



Legend:
MV= Moonvista; MA= Moonaqua; ML= Moonlite; MS= Moonshadow, M=, Moondust; CC+Cinderella cream; Pic= Piccola; NTC= No template Control
M1= Molecular Weight Marker (bp): 6000, 5500, 5000, 4500, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250
M2= Molecular Weight Marker (bp); 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 654

The PCR analysis demonstrates that applying the proposed duplex PCR setup allows amplifying *i*) a fragment corresponding to the endogenous marker, the carnation anthocyanidin synthase (ANS) gene fragment in all carnation lines and *ii*) a fragment corresponding to the GM specific amplification product only with the DNA of Moonlite™ carnation line.

3.2. Robustness

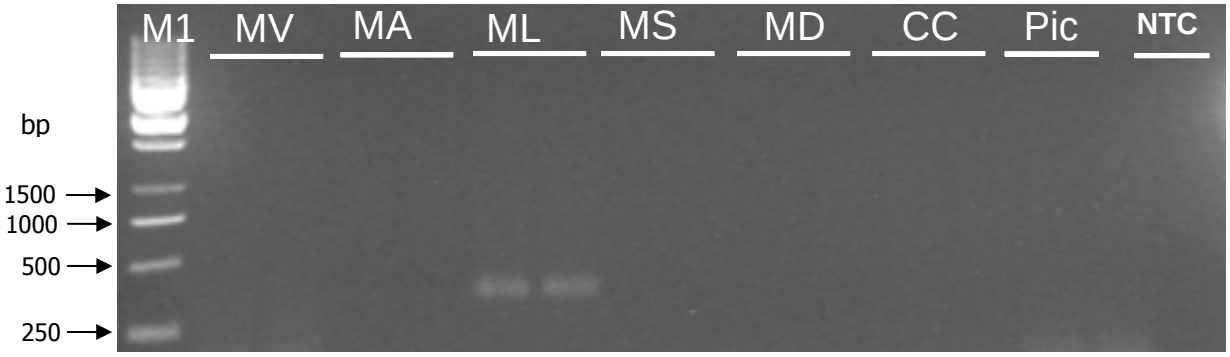
Documentation received by Florigene Pty. Ltd. referred that when AmpliTaq Gold was used in substitution of the HotStar Taq (Qiagen), the amplification reactions did not generate the desired products when both primer sets were used in the same reaction (duplex test configuration).
With the aim of verifying the behavior of the method when such reagent was substituted, the CRL-GMFF replicated the experiments described, introducing the following modifications:

- The PCR program was as described in Annex 1 with 10 minutes activation at 95 °C instead of 15 minutes.
- The AmpliTaq Gold polymerase and the 10X PCR Gold Buffer (Applied Biosystems part number 43.11.814) were used instead of the Qiagen Hotstart

Taq DNA Polymerase and 10x Qiagen DNA Polymerase buffer. The PCR buffer was complemented with 1.5 mM $MgCl_2$ final concentration.

The amplification products were separated by agarose gel electrophoresis on a 1% gel (Figure 2).

Figure 2: Agarose gel electrophoresis of PCR products obtained from PCR amplification of genomic DNA of conventional and GM-carnation lines with the AMpliTaq Gold in the conditions described above



Legend:
MV= Moonvista; MA= Moonaqua; ML= Moonlite; MS= Moonshadow, M=, Moondust; CC+Cinderella cream; Pic= Piccola; NTC= No template Control
M1= Molecular Weight Marker (bp): 6000, 5500, 5000, 4500, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250

As observed in Figure 2, only the band corresponding to the 380 bp amplicon specific to the Moonlite™ carnation line was visible. No reference amplicon could be detected.

3.3. Limit of detection

The CRL-GMFF carried out experiments to estimate the practical limit of detection (LOD) of the method. The LOD was calculated by amplifying in the described conditions (Annex 1) Moonlite™ gDNA at defined copy numbers.

Acceptance criterion was defined as the lowest copy number at which the presence of the amplicon could be detected at least 95% of the times, ensuring a ≤5% false negative rate (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, 2005 at http://gmo-crl.jrc.it/guidancedocs.htm). In the model, one copy of carnation haploid genome is considered to correspond to 0.63 pg [(1)].

Results are shown in Table 1.

Table 1. Results of experiments for the determination of the Limit of Detection (LOD)

Copy numbers/reaction	Number of replicates	Positive results	Negative results
10000	10	10	0
7500	10	10	0
5000	10	10	0
2500	10	10	0
1000	10	0	10
500	10	0	10

Hence, the absolute LOD of the method is at least 2500 copies under the experimental conditions described.

4. Conclusions

The present study demonstrates that applying the revised method proposed by Florigene Pty. Ltd. for detection of carnation Moonlite™ event allows amplifying a fragment corresponding to the endogenous marker, the carnation anthocyanidin synthase (ANS) gene fragment, in both the parental and the GM lines and a fragment corresponding to the Moonlite™ specific amplicon only in this line.
The fragment size of both the endogenous marker (~1300 bp) and of the specific fragment (~380 bp) corresponds to the expected values.

A robustness test confirmed the Florigene Pty. Ltd. findings according to which the detection method is sensitive to changes in Taq DNA polymerase brand (Figure 2).

The estimated absolute LOD is at least 2500 copies.

5. References

1. Royal botanic Gardens, Kew. Plant c-DNA value database (release 4.0, October 2005). http://www.rbgkew.org.uk/cval/homepage.html

Annex 1. PCR method for specific identification of *Florigene Pty. Ltd. Moonlite™* (123.2.38)

C/NL/04/02

(2) PCR based detection method

Introduction

This report provides a method enabling simple PCR-mediated positive and specific identification for FLORIGENE Moonlite™ (123.2.38). Data is presented here showing that the test is able to distinguish this line from different transgenic and non-transgenic carnation lines, including the parent carnation line used as the transformation target.

This report also provides the sequence of a unique PCR primer pair that can be used to amplify a product of a designated size when genomic DNA isolated from FLORIGENE Moonlite™ (123.2.38) is used as template. The product has been identified by visualization under UV illumination in an agarose gel stained with ethidium bromide. No products are detected when genomic DNA from non-specific transgenic lines is used as template in the PCR (transgenic or non-transgenic). Primers amplifying a carnation anthocyanidin synthase (ANS) gene fragment (~1300bp) are included as an internal positive control.

Methodology for identifying and detecting the GMO product.

(i) Unique PCR primers - Sequences adjacent to transformation vector-derived LB or RB sequences integrated into the carnation genome of FLORIGENE Moonlite™ (123.2.38) were generated using procedures as described in Liu *et al* (1995) or Zhou *et al*. (1997). These are shown in Table 1.

Table 1. PCR primers designed to specific sequence generated at the LB flanking region of* FLORIGENE Moonlite™ *(123.2.38) carnation line

1	all lines-positive control	ANS.F	CTAGATCGGAGGTCACCATACC
		ANS.R	GAAACCGTGACCATGGTCTCG
2	FLORIGENE Moonlite™ (123.2.38)	LB inside.R	ACGTGAATGTAGACACGTCG
		LB123.2.38-R	CAATGCCTCGCCTTTTGTGG

ANS = primers designed to the anthocyanidin synthase gene from Carnation; LB = Left Border, F = Forward, R = Reverse.

In order to show that the PCR reaction conditions were optimal and that the DNA was amplifiable, primers designed to the promoter fragment of the carnation ANS gene were included in each reaction (Table 1). A 1300bp fragment was therefore expected in each reaction where genomic DNA isolated from carnation was used as template.

PCR primers were subsequently designed to the specific LB flanking sequence generated for FLORIGENE Moonlite™ (123.2.38) and then tested on several transgenic carnation lines to show that the specific primers amplified a product only when the appropriate, specific genomic DNA from FLORIGENE Moonlite™ (123.2.38) was used as template. Negative controls were
a) no DNA,
b) genomic DNA isolated from non-transgenic carnation lines including the parent carnation line used as the transformation target (Table 2),
(c) genomic DNA isolated from transgenic carnation lines other than FLORIGENE Moonlite (Table 2).

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Table 2. Summary of carnation lines described in this report

123	-	Parent carnation line, non-transformed
5	*Piccola*	Carnation line, non-transformed
18	*Rendezvous*	Carnation line, non-transformed
7442	FLORIGENE Moondust™	pCGP1470
123.2.38	FLORIGENE Moonlite™	pCGP1470
123.8.8	FLORIGENE Moonvista™	pCGP1991
123.8.12	FLORIGENE Moonaqua™	pCGP1991
11363	FLORIGENE Moonshadow™	pCGP1991

(ii) PCR conditions - Genomic DNA used in PCRs as template was isolated from either petal or leaf tissue essentially as described by Dellaporta, *et al.* (1983). The cycle sequencing reactions were performed using a BioRad iCycler PCR machine. PCR reaction solution included 2.5 µL 10 x Qiagen DNA Polymerase buffer (Qiagen), 1 µL 10 mM dNTPs, 2 µL each primer (50ng/µL), 2 µL genomic DNA template (50ng/µL), 0.5 µL Qiagen Hotstar Taq DNA Polymerase (Qiagen) and pure water to a total volume of 25 µL. The PCR was incubated at 95°C for 15 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes and then a final incubation at 72°C for 10 minutes with subsequent storage at 4°C. The reactions were set up according to Table 3 using the following genomic DNA samples as templates: no DNA (negative control), parent variety line 123 (non-transgenic negative control), carnation variety line 5 (non-transgenic negative control), carnation variety line 18 (non-transgenic negative control), FLORIGENE Moonlite™ (123.2.38), and the transgenic carnation lines developed by Florigene, 7442, 123.8.8, 123.8.12 and 11363.

Table 3. Primer sets and template DNA from carnation lines included in each reaction.

1	1 and 2	No DNA - negative control
2	1 and 2	Parent carnation line (line 123)
3	1 and 2	Carnation line - Piccola (line 5)
4	1 and 2	Carnation line - Rendezvous (line 18)
5	1 and 2	Transgenic line 123.2.38 - FLORIGENE Moonlite™
6	1 and 2	Transgenic line 7442 - FLORIGENE Moondust™
7	1 and 2	Transgenic line 123.8.8 - FLORIGENE Moonvista™
8	1 and 2	Transgenic line 123.8.12 - FLORIGENE Moonaqua™
9	1 and 2	Transgenic line 11363 - FLORIGENE Moonshadow™

The reaction products were electrophoresed through a 1% (w/v) agarose gel alongside 10 µL 100 µg/µL standard DNA markers *Eco*RI digested SPPI (Geneworks) and visualised under UV light.

Experimental data demonstrating the specificity of the methodology

A photograph of the agarose gel under UV light conditions was taken by Polaroid camera and is shown in Figure 1. A summary of the results is described in Table 4.

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Table 4. Summary of the sizes (bp) of products detected in PCR reactions shown in Figure 1

Reaction No.	Template DNA	PCR Product Sizes (bp)
1	No DNA	-ve
2	Parent variety line 123	1300
3	Carnation variety line 5	1300
4	Carnation variety line 18	1300
5	**FLORIGENE Moonlite™ 123.2.38**	**1300+ 380**
6	7442	1300
7	123.8.8	1300
8	123.8.12	1300
9	11363	1300

ANS = anthocyanin synthase, bp = base pairs, – ve = negative (i.e. no amplified bands)

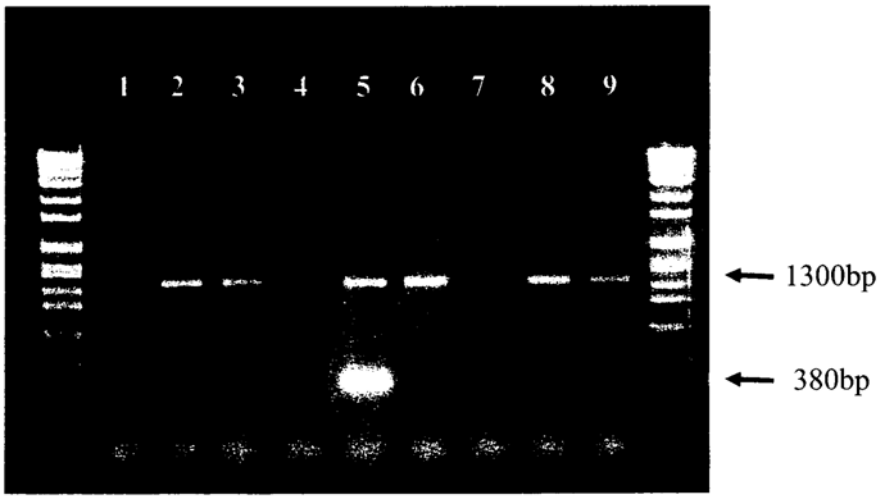

Figure 1. Scanned photograph of an agarose gel containing the PCR products of reactions set up according to Table 4. M = standard marker (i.e. EcoRI digested SPP1 DNA).

The data presented above demonstrates that we have provided a unique set of primers that are able to detect and differentiate the transgenic carnation line FLORIGENE Moonlite™ (123.2.38) from other transgenic carnation lines (7442, 123.8.8, 123.8.12 and 11363), and non-transgenic carnation varieties 123, 5 and 18. A product of the expected size (380bp) was detected only in the transgenic line FLORIGENE Moonlite™ (123.2.38) when the unique primer set was included in the PCR. No products were detected in reactions that did not contain genomic DNA. All reactions contained primer set 1 (primers to an endogenous carnation ANS gene) and

resulted in amplification of the expected 1300bp product showing that the PCR conditions were optimal for product amplification.

Citations

1. Dellaporta, S. J., Wood, J. and Hick, J.B. (1983) A plant DNA mini-preparation, version two. *Plant Mol. Biol. Rep.* 1, 19-21.

2. Edwards K, Johnstone C and Thompson C. A simple and rapid method for the preparation of plant DNA for PCR. *Nucleic. Acids Research* 19:1349, 1991

3. Liu *et al.*, Efficient Isolation and mapping of *Arabidopsis thaliana* T-DNA insert junction by Thermal asymmetric interlaced PCR. *Plant J* **8:** 457-463, 1995

4. Zhou, Y., Newton, R. and J.H. Gould. A simple method for identifying plant/T-DNA junction sequences resulting from *Agrobacterium*-mediated DNA transformation. *Plant Molecular Biology Reporter* 15:246-254, 1997

Abbreviations

PCR: polymerase chain reaction
LB: left border
RB: right border

(3) Amplicon sequences including primer pairs used.

Illustrated below are sequences for FLORIGENE Moonlite locus 1 and the gene for anthocyanin synthase (ANS) from carnation. On each illustration we have indicated primer sets used in the PCR-based assay and the deduced sequence of each amplicon. Forward primers are in green and reverse primers in red and each underlined with directional arrows