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

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

Method development:
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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 for a genetically modified carnation line (C/NL/06/01) Moonaqua™ (123.8.12), the JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), has carried out tests to verify the performance of a PCR-based detection method developed to identify the said GM carnation line.

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

The assay correctly detects the control target in genomic DNA of conventional carnation lines (negative control) and in the genomic DNA of the tested GM Carnation lines; the 123.8.12 assay (Moonaqua™-specific) can detect the control target in Moonaqua™ GM line (positive control) in the experimental conditions described in this report.

The Limit of Detection (LOD) of the method has been estimated as at least 100 copies, based on haploid genome copy number.

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CONTENT

1. Introduction

In the context of the application for marketing submitted by Florigene for a genetically modified carnation line (C/NL/06/01) Moonaqua™ (123.8.12), the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) has carried out tests to verify the performance of a PCR-based detection method developed to identify the said GM carnation line.

Upon reception of the protocol and control samples, the JRC performed the tests from July to November 2007.

2. Documentation, Materials and Methods

The CRL-GMFF received the description of the detection method from Florigene (e-mail of 14/06/2007). The document can be found in Annex 1 of this report.

On 25/06/2007 the CRL-GMFF received the following DNA samples from Florigene:

Line name	Line information	Concentration
Cream Cinderella	Parent carnation line	894 ng/µL
Cerise Westpearl	Carnation line, non-transformed	893 ng/µL
Rendezvous	Carnation line, non-transformed	2.14 µg/µL
Moonaqua™	GM	1.15 µg/µL
Moonshade™	GM	151 ng/µL
Moonvista™	GM	1.13 µg/µL
Moonlite™	GM	1.2 µg/µL
Moonshadow™	GM	383 ng/µL

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

- 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 Moonaqua™ rRB (7-26) (#819B)– 123.8.12-2.1-R (#853H) – amplicon: 750 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/ µL to 500 ng/ µL using a Bio-Rad VersaFluor™ Fluorometer as fluorescence detector.

The following values were observed:

Carnation line	Concentration (ng/μL)
Cream Cinderella *	934
Cerise Westpearl *	113
Rendezvous *	859
Moonaqua™ **	1075
Moonshade™ *	140
Moonvista™ **	57.6
Moonlite™ **	1085
Moonshadow™ *	91.2

* average of two readings

** one reading due to low sample volume

Amplification conditions were as described in Annex 1. The PCR analysis was performed on a Bio-Rad PCR apparatus (iCycler).

3. Results

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

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

The test was conducted 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 GM line Moonaqua™. PCR analysis was performed in duplicate.

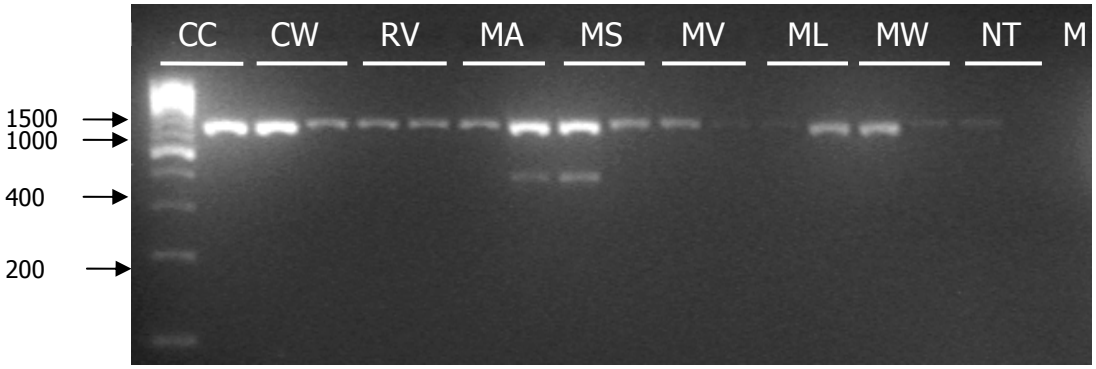
The amplification products were separated by agarose gel electrophoresis on a 2.5% gel (Figure 1).

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 target line Moonaqua™ reacted with the event-specific primers yielding a band (~750 bp) in accordance with the expected size for Moonaqua™ specific amplicon.

Results 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:

CC = Cream Cinderella; CW = Cerise Westpearl; RW = Rendezvous; MA = Moonaqua™; MS = Moonshade™;

MV = Moonvista™; ML = Moonlite™; MW = Moonshadow™; NT= No template Control

M= Molecular Weight Marker (bp): 3000, 2800, 2600, 2400, 2200, 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200

The PCR analysis demonstrates that the application of 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 in the DNA of Moonaqua™ carnation line.

3.3. Limit of detection

The CRL-GMFF carried out experiments to estimate the limit of detection (LOD) of the method. The LOD was calculated by amplifying in the described conditions (Annex 1) Moonaqua™ 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
20000	15	15	0
15000	15	15	0
10000	15	15	0
7500	15	15	0
5000	14	14	0
2500	15	15	0
2000	15	15	0
500	15	15	0
200	15	15	0
100	15	15	0
50	15	11	4

Therefore, the absolute LOD of the method is at least 100 copies under the experimental conditions described.

4. Conclusions

The present study demonstrates that the application of the method proposed by Florigene Pty. Ltd. for detection of carnation Moonaqua™ 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 Moonaqua™ specific amplicon only in this line.

The fragment size of both the endogenous marker (~1300 bp) and the specific fragment (~750 bp) corresponds to the expected values.

The estimated absolute LOD is at least 100 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 Moonaqua™ (123.8.12)

C/NL/06/01

'Unique Identifier' PCR Reactions for FLORIGENE® carnation product, FLORIGENE Moonaqua™

This document is comprised of:
(1) PCR-based detection method and (2) Amplicon sequence including primer pair used.

(1) PCR based detection method

Introduction

This report provides a method enabling simple PCR-mediated positive and specific identification for the trade marked product FLORIGENE Moonaqua being always selected from line 123.8.12. 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 Moonaqua (of line 123.8.12) 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 or non-transgenic lines are used as template in the PCR. Primers amplifying a carnation anthocyanidin synthase (ANS) gene fragment (1300 bp) are included as an internal positive control.

Methodology for identifying and detecting the GMO product.

(i) PCR primers

- ***Unique identifier PCR primers*** - Sequences adjacent to transformation vector-derived RB sequences integrated into the carnation genome of FLORIGENE Moonaqua (of line 123.8.12) were generated using procedures as described in Liu *et al*. (1995) or Zhou *et al*. (1997). PCR primers, rRB(7-26) and 123.8.12-2.1R designed to this sequence are shown in Table 1.
  These primers were also tested on several transgenic carnation lines to show that the specific primers amplified a 750 bp product only when the appropriate, specific genomic DNA from FLORIGENE Moonaqua (of line 123.8.12) was used as template.

- ***ANS primers*** - 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 1300 bp fragment was therefore expected in each reaction where genomic DNA isolated from carnation was used as template.

Table 1. PCR primers designed to amplify carnation ANS and FLORIGENE Moonaqua unique identifier products.

Primer Set	Line	Primer name	Primer sequence (5' to 3')
1	all lines-positive control	ANS.F	CTAGATCGGAGGTCACCATACC
		ANS.R	GAAACCGTGACCATGGTCTCG
2	FLORIGENE Moonaqua (of line 123.8.12)	rRB(7-26)	GAATAGAGTGGACTACTTAC
		123.8.12-2.1R	GGAGGTGTACTATGGGAATCC

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

(ii) Carnation lines used:
A summary of the carnation lines used in this method is described in Table 2. The negative controls include:
a) no DNA
b) genomic DNA isolated from non-transgenic carnation lines including the parent carnation line used as the transformation target
c) genomic DNA isolated from transgenic carnation products other than FLORIGENE Moonaqua.

Table 2. Summary of carnation lines described in this report.

Line	Trade Name	Transformation Vector
123	-	Parent carnation line, non-transformed
145	-	Carnation line, non-transformed
18	-	Carnation line, non-transformed
123.8.12	FLORIGENE Moonaqua	pCGP1991
123.8.8	FLORIGENE Moonvista™	pCGP1991
123.2.2	FLORIGENE Moonshade™	pCGP1470
123.2.38	FLORIGENE Moonlite™	pCGP1470
11363	FLORIGENE Moonshadow™	pCGP1991

(iii) PCR conditions - Crude genomic DNA used in PCRs as template was isolated from leaf tissue essentially as described by Edwards *et al*. (1991). The cycle sequencing reactions were performed using a Bio-Rad PCR machine (iCycler). PCR reaction solution included 2.5 µL 10 x Qiagen DNA Polymerase buffer (Qiagen), 1 µL 10 mM dNTPs, 2 µL each primer (50 ng/µL), 2 µL genomic DNA template (50 ng/ul), 0.5 µL Qiagen Hotstar Taq DNA Polymerase (Qiagen) and pure water to a total volume of 25 µL. The

PCR was incubated at 95ºC for 15 minutes, followed by 35 cycles of 94ºC for 1 minute, 52ºC for 1 minute and 72ºC for 1 minute and then a final incubation at 72ºC for 10 minutes with subsequent storage at 4ºC. 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 145 (non-transgenic negative control), carnation variety line 18 (non-transgenic negative control), FLORIGENE Moonaqua (of line 123.8.12), and the transgenic carnation lines 123.2.2, 123.2.38, 123.8.12 and 11363 (transgenic negative controls).

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

Reaction #	Primer set	DNA template
1	1 and 2	No DNA - negative control
2	1 and 2	Parent carnation line (line 123)
3	1 and 2	Carnation line - Cerise Westpearl (line 145)
4	1 and 2	Carnation line – Rendezvous (line 18)
5	1 and 2	Transgenic line 123.8.12
6	1 and 2	Transgenic line 123.8.8
7	1 and 2	Transgenic line 123.2.2
8	1 and 2	Transgenic line 123.2.38
9	1 and 2	Transgenic line 11363

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.

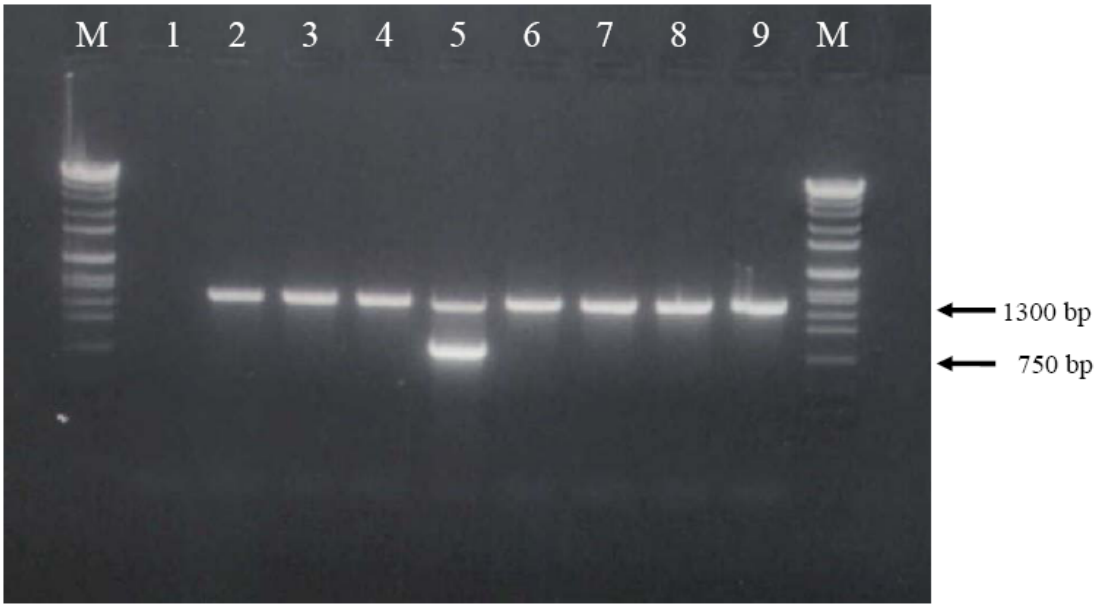
Table 4. Summary of the sizes (bp) of products detected in PCR reactions shown in Figure 1.

Reaction No.	Template DNA	PCR Product Sizes (bp)
1	No DNA	-ve
2	Parent variety line 123	1300
3	Carnation variety line 145	1300
4	Carnation variety line 18	1300
5	123.8.12	1300+750
6	123.8.8	1300
7	123.2.2	1300
8	123.2.38	1300
9	11363	1300

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

The data presented demonstrates that we have provided a unique set of primers that are able to detect and differentiate the carnation product FLORIGENE Moonaqua of line 123.8.12 from other transgenic carnation lines (123.2.2, 123.2.38, 123.8.8 and 11363), and non-transgenic carnation varieties 123, 145 and 18. A product of the expected size (750 bp) was only detected in the carnation product FLORIGENE Moonaqua (of line 123.8.12) when the unique primer set was included in the PCR. No PCR products were detected in reactions that did not contain genomic DNA. All reactions contained primer set 1 (primers to an endogenous carnation *ANS* gene) which resulted in amplification of the expected 1300 bp product showing that the PCR conditions were optimal for product amplification.

Figure 1. Scanned photograph of an agarose gel containing the PCR products of reactions set up according to Table 3. M = standard marker (i.e. EcoRI digested SPP1 DNA).



Literature cited

1. 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
2. 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
3. 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
RB: right border