EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

EURL
European Union Reference Laboratory
for GM Food & Feed

Report on the Single-Laboratory Validation of a DNA Extraction Method from Carnation Leaves

Validation Report and Validated Method

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 DNA extraction protocol to extract genomic DNA from carnation plant leaves for subsequent polymerase chain reaction (PCR) based detection methods and its applicability on the samples provided by the applicant.

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

The data reported confirm that the extraction method, applied to samples of carnation leaves, produces DNA of suitable quantity and quality for subsequent PCR-based methods.

JRC publication 77732

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.

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit – European Union Reference Laboratory for Genetically Modified Food and Feed
Via Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

Content

1. INTRODUCTION 4

2. MATERIALS (EQUIPMENT/CHEMICALS/PLASTICWARE) 4

2.1. EQUIPMENT 4
2.2. ABBREVIATIONS 5
2.3. CHEMICALS 5
2.4. SOLUTIONS 5
2.5. PLASTICWARE 7
2.6. PRECAUTIONS 8

3. DESCRIPTION OF THE METHOD 8

3.1. SCOPE AND APPLICABILITY 8
3.3. PRINCIPLE 8
3.4. GENOMIC DNA ISOLATION FROM CARNATION PLANT MATERIAL 8

4. TESTING OF THE DNA EXTRACTION METHOD BY THE METHOD DEVELOPER 10

4.1. DNA CONCENTRATION 10
4.2. PURITY/ABSENCE OF PCR INHIBITORS 10

5. TESTING OF THE DNA EXTRACTION METHOD BY THE EUROPEAN UNION REFERENCE LABORATORY FOR GENETICALLY MODIFIED FOOD AND FEED 13

5.2. PREPARATION OF SAMPLES AND DNA EXTRACTION 14
5.3. DNA CONCENTRATION, YIELD AND REPEATABILITY 14
5.4. FRAGMENTATION OF DNA 15
5.5. PURITY/ABSENCE OF PCR INHIBITORS 16

6. CONCLUSION 20

7. QUALITY ASSURANCE 20

8. REFERENCES 21

1. Introduction

This report describes the validation of a small-scale DNA extraction protocol to extract genomic DNA from carnation plant leaves for subsequent qualitative polymerase chain reaction (PCR) based detection methods and its applicability on the samples provided by the applicant. The protocol is a modified "Dellaporta-derived" [1] DNA extraction method.

The purpose of the DNA extraction method described is to provide DNA with purity suitable for real-time PCR based detection methods. The method should have a high yield and should be tailored for routine analysis in terms of ease of operations, sample throughput and costs.

The protocol should be executed by skilled laboratory personnel since hazardous chemicals and materials are used at some steps. It is strongly advised to take particular notice of all product safety recommendations and guidelines.

2. Materials (Equipment/Chemicals/Plasticware)

2.1. Equipment

The following equipment is used in the DNA extraction procedure described (equivalents may be used):

1. Mortar and pestle (130 x 62 mm), porcelain
2. Water bath at 65ºC
3. Benchtop centrifuge
4. F34-6-38 Rotor (e.g. Eppendorf)
5. Miracloth filter (Calbiochem-Novabiochem Corporation)
6. Fluorometer
7. Micropipettes (e.g. 0.5-10 µL, 10-100 µL, 100-1000 µL)
8. Refrigerator at 4 ºC
9. Freezer at 20 ºC
10. Ultrafreezer at -75 to -80 ºC
11. 250 mL Beaker
12. Paper towels or tissues
13. Centrifuge adaptors for 14 mL Falcon tubes

2.2. Abbreviations

EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
KAc	potassium acetate
NaAc	sodium acetate
NH_4Ac	ammonium acetate
PCR	polymerase chain reaction
PVP-40	polyvinylpyrrolidone-40
RNase A	ribonuclease A
SDS	sodium dodecyl sulphate
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane

2.3. Chemicals

The following chemicals are used in the DNA extraction procedure described (equivalents may be used):

1. Trizma base (p. a. quality or Molecular biology grade)
2. Concentrated HCl (p. a. quality or Molecular biology grade)
3. NaOH (p. a. quality or Molecular biology grade)
4. Na_2EDTA (p. a. quality or Molecular biology grade)
5. NaCl (p. a. quality or molecular biology grade)
6. β-mercaptoethanol (p. a. quality or Molecular biology grade)
7. PVP-40 (p. a. quality or Molecular biology grade)
8. SDS (p. a. quality or Molecular biology grade)
9. KAc (p. a. quality or Molecular biology grade)
10. Isopropanol (p. a. quality or Molecular biology grade)
11. NH_4Ac (p. a. quality or Molecular biology grade)
12. 95% ethanol (EtOH) (p. a. quality or Molecular biology grade)
13. NaAc (p. a. quality or Molecular biology grade)
14. Glacial acetic acid (p. a. quality or Molecular biology grade)
15. RNase A (p. a. quality or Molecular biology grade)
16. H_2O_{deion}
17. Liquid nitrogen

2.4. Solutions

The following buffers and solutions are used in the DNA extraction procedure described:

1. **2M Tris-HCl pH 8.0**

For 1 L 2M Tris-HCl pH 8.0 dissolve 242.2 g Trizma base in about 500 ml H_2O_{deion}; adjust pH to 8.0 with about 65 mL concentrated HCl under fumehood and bring the volume to 1 Liter. Autoclave.
Store at 4ºC for up to 5 years.

2. **10M NaOH**

For 1 L solution, slowly add 800 g NaOH to about 150 ml H_2O_{deion} in a beaker placed on ice under a fumehood. Adjust volume to 1 Liter with H_2O_{deion}.
Store in plastic bottles at room temperature for up to 5 years.

3. **500mM EDTA pH 8.0**

For 1 L 500mM EDTA pH 8.0 add 186.12 g Na_2EDTA to about 700 ml H_2O_{deion}; adjust pH to 8.0 with about 45 mL 10M NaOH; adjust volume to 1 Liter with H_2O_{deion}. Autoclave.
Store at 4ºC for up to 5 years.

4. **5M NaCl**

For 1 L 5M NaCl dissolve 292.2 g NaCl in about 900 mL H_2O_{deion}. Adjust volume to 1 Liter with H_2O_{deion}. Autoclave.
Store at 4ºC for up to 5 years.

5. **Dellaporta DNA extraction buffer**
    - 100 mM Tris HCl pH 8.0
    - 50 mM EDTA pH 8.0
    - 500 mM NaCl
    - 14.3 M β-mercaptoethanol
    - 2% (w/v) PVP-40

For 1 Liter of DNA extraction buffer mix 50 mL 2M Tris-HCl pH 8.0, 100 mL 500mM EDTA and 100 mL 5M NaCl; and adjust volume to 1 L with sterile H_2O_{deion}. Dispense 500 mL of the solution in 2 x 500 mL Schott bottles and add 350 µL of β-mercaptoethanol into each bottle under fumehood. Shake well and store at 4 °C.
Prior to use, dispense required amount of Dellaporta DNA Extraction Buffer and add PVP-40 at a concentration of 2% (w/v), mix well and use solution within 1 week.

6. **100 x TE buffer**
    - 1 M Tris-HCl pH 8.0
    - 100 mM EDTA

For 1 L 100 x TE buffer mix 500 mL 2M Tris-HCl pH 8.0, 500 mL 200 mM EDTA pH 8.0 and about 100 mL H_2O_{deion}. Adjust pH with HCl to pH 8.0. Adjust volume to 1 Liter with H_2O_{deion}. Autoclave.
Store at 4ºC for up to 5 years.

7. 1 x TE buffer

- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA

For 500 mL 1 x TE buffer mix 5 mL 100 x TE and about 400 mL H_2O_{deion}. Adjust the volume to 500 mL with H_2O_{deion}. Autoclave.
Store at 4ºC for up to 5 years.

8. 10% (w/v) SDS

For 1 L 10% (w/v) SDS dissolve 100 g Sodium Dodecyl Sulphate in 800 mL H_2O_{deion}, using a microwave to heat the solution to 68°C. Adjust the volume to 1 L with H_2O_{deion}. Do not autoclave or refrigerate.
Store at room temperature for up to 5 years.

9. 5M KAc

For 1 L 5M KAc dissolve 490.7 g KAC in about 350 mL H_2O_{deion}. Adjust volume to 1 Liter with H_2O_{deion}. Autoclave.
Store at 4ºC for up to 5 years.

10. 7.5 M NH_4Ac

For 1 L 7.5 M NH_4Ac dissolve 578.1 g NH_4Ac in about 300 mL H_2O_{deion}. Adjust volume to 1 Liter with H_2O_{deion} in a cylinder and divide the solution in 2 x 500 mL Schott bottles. Autoclave.
Store at 4ºC for up to 5 years.

11. 70% (v/v) ethanol

For 500 mL 70% (v/v) ethanol mix 368 mL 95% ethanol with about 130 mL H_2O_{deion}; bring volume to 500 mL.
Store at room temperature for up to 6 months.

12. 3 M sodium acetate (NaAc) pH 5.2

For 1 L 3 M NaAc pH 5.2 slowly add 246.09 g NaAc to about 400 mL H_2O_{deion} and adjust pH to 5.2 adding about 200 mL Glacial Acetic Acid under fumehood. Adjust volume to 1 Liter. Autoclave.
Store at 4ºC for up to 5 years.

2.5. Plasticware

1. 10-1000 µL tips (e.g. Molecular Bioproducts)
2. 50 mL Falcon tubes (e.g. Greiner)
3. 14 mL Falcon tubes (e.g. Greiner)
4. SnapLock re-sealable bags 18 x 17 cm (e.g. GLAD, USA)
5. 5-10 mL serological pipettes (e.g. Greiner Bio One)
6. Rack to hold 14 mL and 50 mL Falcon tubes (e.g. Starlab)

Note: All plasticware has to be sterile and free of DNases, RNases and nucleic acids.

2.6. Precautions

- When handling β-mercaptoethanol work under fumehood, wear gloves and protective clothing.
- Wear safety glasses, mask and gloves when weighing SDS, in powder form SDS must be confined to fumehood.
- Wear protective clothing and work in fumehood when handling Glacial Acetic Acid.
- Hydrochloric acid is highly toxic and corrosive; safety glasses, gloves and lab coat must be worn. Work in fumehood when handling concentrated HCl.
- Sodium hydroxide is both toxic and corrosive and will dissolve glass over time. Do not store solutions in glass bottles. Wear gloves, safety glasses and lab coat and work under the fumehood when handling sodium hydroxide.
- All tubes and pipette tips have to be discarded as biological hazardous material.

3. Description of the method

3.1. Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from carnation leaves. Application of the method to other matrices may require adaptation.

3.3. Principle

The basic principle of the DNA extraction consists of first releasing the DNA present in the tissues into aqueous solution and then purifying the DNA from PCR inhibitors. The method starts with a lysis step (thermal lysis in the presence of β-mercaptoethanol and EDTA) followed by two precipitations with potassium acetate and isopropanol. Afterwards two other precipitations, with ammonium acetate and ethanol, are performed. RNA is then removed by digestion with RNase A followed by precipitation with sodium acetate and a wash with ethanol.

3.4. Genomic DNA isolation from carnation plant material

1. Collect leaves of carnation and separate them in 6 g amounts. Place these immediately at -80°C in 50mL Falcon tubes or SnapLock resealable bags.
2. Grind six grams of frozen leaf material to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle (mortar and pestle were pre-cooled in a -75 to -80°C ultrafreezer for at least 10 minutes prior to use). It is critical that the powder is not allowed to thaw.
3. Add immediately 30 mL (at least 5 mL/g) Dellaporta DNA extraction buffer (premeasured into a 50 mL Falcon tube) to the mortar and continue grinding until the mixture freezes.
4. Allow the mixture to thaw at room temperature, grind further with the pestle and then transfer to two 50 mL Falcon tubes.
5. Add 2 mL of 10% (w/v) SDS to each tube.

6. Vigorously mix the samples and incubate in a waterbath set at 65ºC for 10 min.
7. Add 5 mL of 5 M KAc to each sample; mix vigorously the mixtures and incubate on ice for 20 min.
8. Centrifuge the samples at 5,000 x *g* for 15 min using the benchtop centrifuge.
9. Filter each supernatant through 1 layer of Miracloth into a clean 50 mL Falcon tube.
10. Add 10 mL of isopropanol to each tube before incubation at -20ºC (freezer) for at least 30 min.
11. Following centrifugation at 5,000 x *g* for 15 min using the benchtop centrifuge, gently pour off the supernatant into a beaker and lightly dry the resulting pellets by inverting the tubes on paper towels (or tissues).
12. Add 3 mL of TE buffer to each tube and allow the pellet to resuspend by an overnight incubation at 4 ºC (refrigerator).
13. Gently mix the suspension by shaking the tubes by hand.
14. Add 1.5 mL of 7.5 M NH_4Ac to each tube; mix the samples by inverting the tubes five times before incubation at -20°C (freezer) for 20 min.
15. Centrifuge the samples at 5,000 x *g* for 10 min using the benchtop centrifuge.
16. Transfer each supernatant into a clean 50 mL Falcon tube.
17. Add 3 x volumes of 95% ethanol to each tube; mix the samples by inverting the tubes five times.
18. Place the tubes at -20ºC (freezer) for at least 30 min.
19. Centrifuge the samples at 5,000 x *g* for 15 min using the benchtop centrifuge.
20. Carefully pour off and discarded the supernatant and resuspend each pellet in 3 mL TE buffer (essentially as described above in points 10 and 11).
21. Transfer each gDNA solution to a 14 mL Falcon tube.
22. Add RNAse A at a final concentration of 200 µg/mL to the genomic DNA; incubate the mixture for 30 min at 37ºC.
23. Add 0.1 x volume of 3 M NaAc (pH 5.2) to each sample and gently mix the solution.
24. Add 2.5 x volumes of 95% ethanol and gently mix the samples by inverting the tubes five times.
25. Incubate the samples at -20ºC for at least 30 min.
26. Remove the lids of the Falcon tubes and centrifuge the samples at 5,000 x *g* for 15 min using tube adaptors and the benchtop centrifuge.
27. Carefully pour off and discard the supernatant.
28. Add 1 mL of 70% ethanol to the pellets and gently rotate the samples so as to wash the pellet without dislodging it. Pour off the ethanol (if the pellets are dislodged then centrifuge the tubes as described in point 25 for 5 minutes).
29. After removal of the 70% ethanol, allow the tubes to sit at room temperature in a fume hood for between 5 and 10 min to allow vaporization of most of the ethanol (do not allow the DNA pellet to become too dry as this will interfere with proper resuspension).
30. Resuspend the DNA pellets in 300 µL of TE buffer overnight at 4ºC (refrigerator).

4. Testing of the DNA extraction method by the method developer

4.1. DNA concentration

DNA was extracted in parallel from six analytical samples of ground carnation leaves (6 grams each) during three consecutive days (total of 18 extracts). An additional step of homogenization al low speed for 5-10 sec with a Polytron Ultra-Turrax was performed by the method developer after step 4, and steps 8, 11, 15, 19 and 26 were performed with the JA20 rotor in a high speed centrifuge, at the same speed as indicated in the protocol above. The DNA concentration was evaluated by measuring the absorbance at 260 nm using a Nanodrop ND-1000 while the DNA purity was assessed by the $A_{260/280}$ ratio using a CARY 50 Bio spectrophotometer. The concentration of DNA measured in each sample is shown in Table 1.

Table 1. DNA concentration (ng/µL) of 18 samples extracted in three days.

Day	Sample	Concentration ng/µl
1	#1	294
1	#2	716
1	#3	724
1	#4	288
1	#5	208
1	#6	887
2	#7	217
2	#8	181
2	#9	241
2	#10	251
2	#11	429
2	#12	239
3	#13	501
3	#14	310
3	#15	547
3	#16	362
3	#17	490
3	#18	442

DNA concentration (ng/µL)

Overall average of all samples	404.6 ng/µL
Standard deviation of all samples	205.9 ng/µL
Coefficient of variation	50.9 %

4.2. Purity/Absence of PCR inhibitors

Presence of inhibitors in genomic DNA extracted from leaves of carnation was evaluated by real-time PCR using as a template a dilution series of arabidopsis genomic DNA spiked with the

extracted carnation genomic DNA. The whole procedure was applied to 18 carnation DNA extracts (6 samples x 3 days). A fragment of the arabidopsis *actin 2* (*ACT2*) gene (GeneBank accession number U41998) was chosen as amplification target for the arabidopsis genome. Suitable primers and probe were designed and used for real-time PCR analysis.

The arabidopsis genomic DNA sample was reduced to a concentration of 50 ng/µL with TE buffer. This solution was then diluted with a fourfold serial dilution in TE buffer (1:4, 1:16, 1:64 and 1:256). The 18 carnation genomic DNA samples were diluted to a concentration of 100 ng/µL. 2 µL of the series of diluted arabidopsis genomic DNA solution and 1 µL of the diluted carnation genomic DNA solution (100 ng/µL) were combined and mixed (spiking of arabidopsis DNA with carnation DNA samples).

Table 2 summarises the components combined for each PCR reaction, to a total volume of 20 µL. The sequences of the primers and the probe for real-time analysis are shown in Table 3.

Table 2. Reaction mixture for amplification of the *actin 2* target

Component	**Starting concentration**	**Final concentration**	**µL/ reaction**
Template DNA: Arabidopsis	See text above	See text above	2 µL
Template DNA: carnation (when applicable)	100 ng/µL	100 ng	1 µL
2 x PCR mastermix	2 x	1 x	10 µL
Actin 2 FW primer	10 µM	900 nM	1.8 µL
Actin 2 RV primer	10 µM	900 nM	1.8 µL
Actin 2 probe	13.3 µM	250 nM	0.76 µL
Pure water	-	-	3.64 or 2.64 µL

Table 3. Sequence of primers and probe for amplifying the arabidopsis *actin 2* gene

Name	**DNA sequence (5'-3')**
Actin 2 FW primer	GTGTGTCTCACACTGTGCCAATC
Actin 2 RV primer	AAGACGGAGGATGGCATGA
Actin 2 probe	FAM-CGAGGGTTTCTCTCTT-MBG

For each sample extract, PCR reactions were performed in triplicate. Standard real-time PCR cycling and temperature conditions were used as described in Table 4.

Table 4. Thermal profile for amplification of *actin 2* gene

Step			**Temperature**	**Time**	**Cycles**
1	Uracil-N-Glycosylase treatment		50°C	120 sec	1 x
2	Initial Denaturation		95°C	15 sec	1 x
3	Amplification	Denaturation	95°C	15 sec	45 x
		Annealing & Extension	60°C	60 sec	

To assess first the presence of inhibitors in the arabidopsis DNA samples, the Ct values of the diluted samples were plotted against the logarithm of the dilution factor. The Ct value for the "undiluted" sample was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the "undiluted" sample was compared with the measured Ct.

It is assumed that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is 0.5 cycles higher than the calculated Ct value ($\Delta Ct > 0.5$). In addition, the slope of the curve should be between -3.1 and -3.6, and the R_2 value of linear regression should be > 0.98.

Table 5 reports the extrapolated Ct values versus the measured Ct values for the arabidopsis DNA extracts after amplification for the a*ctin 2* gene. No inhibitor effect was observed in the undiluted DNA samples versus the diluted ones. It was concluded therefore, that the DNA extracts were free of inhibiting compounds.

Table 5. Comparison of extrapolated Ct values versus measured Ct values for the arabidopsis DNA extracts after amplification of the a*ctin 2* gene.

Repetition	R^2	Slope	Ct extrapolated	Ct measured	ΔCt
1	0.999	-3.436	24.862	24.853	0.01
2	0.999	-3.436	24.862	24.853	0.01
3	0.999	-3.472	24.89	24.900	0.01
4	0.998	-3.423	24.93	24.942	0.01
5	0.999	-3.517	24.859	24.853	0.01
6	0.999	-3.517	24.859	24.881	0.02
7	0.999	-3.447	24.917	24.960	0.04
8	0.999	-3.447	24.917	24.960	0.04
9	0.999	-3.447	24.917	24.960	0.04
10	0.999	-3.447	24.917	24.960	0.04
11	0.999	-3.447	24.917	24.960	0.04
12	0.999	-3.461	24.912	25.028	0.12
13	0.999	-3.483	24.950	24.884	0.07
14	0.999	-3.483	24.950	24.884	0.07
15	0.999	-3.483	24.950	24.884	0.07
16	0.999	-3.483	24.950	24.884	0.07
17	0.999	-3.483	24.950	24.884	0.07
18	0.999	-3.461	24.912	25.028	0.12

Slope and R^2 values are calculated from the Ct/log-concentration standard curves. ΔCt values are derived from the equation DCt=(Ct extrapolated - Ct measured)

To assess the presence of inhibitors in the carnation DNA extracts, the arabidopsis "undiluted" and diluted DNA samples were spiked with the carnation DNA extracts, and as for non-spiked arabidopsis DNA, the Ct values of the diluted samples were plotted against the logarithm of the dilution factor. The Ct value for the "undiluted" sample was extrapolated from the equation

calculated by linear regression. Subsequently the extrapolated Ct for the "undiluted" sample was compared with the measured Ct.

Table 6 reports on the comparison of extrapolated Ct values versus measured Ct values for the arabidopsis DNA samples spiked with the carnation DNA extracts. The data presented demonstrate that the addition of DNA extracted from carnation into a background of arabidopsis DNA does not induce PCR inhibition.

Table 6. Comparison of extrapolated Ct values versus measured Ct values for the arabidopsis DNA samples spiked with carnation DNA after amplification of the arabidopsis a*ctin 2* gene.

Sample	R^2	Slope	Ct extrapolated	Ct measured	ΔCt
#1	0.998	-3.498	24.862	24.853	0.01
#2	0.999	-3.498	24.719	24.784	0.07
#3	1	-3.478	24.733	24.750	0.02
#4	0.998	-3.425	24.774	24.763	0.01
#5	0.999	-3.455	24.74	24.744	0.00
#6	0.999	-3.507	24.728	24.767	0.04
#7	1	-3.565	24.793	24.812	0.04
#8	1	-3.482	24.910	24.910	0.04
#9	0.999	-3.444	24.913	24.942	0.04
#10	0.999	-3.495	24.915	24.881	0.04
#11	0.999	-3.586	24.991	24.832	0.04
#12	0.999	-3.412	24.966	24.979	0.12
#13	0.998	-3.412	24.813	24.749	0.06
#14	0.999	-3.464	24.761	24.801	0.04
#15	0.999	-3.478	24.837	24.832	0.01
#16	0.998	-3.468	24.787	24.727	0.06
#17	0.999	-3.470	24.772	24.803	0.03
#18	0.999	-3.411	24.880	24.878	0.00

Slope and R^2 values are from the Ct/log-concentration standard curves. ΔCt values are (Ct extrapolated - Ct measured)

5. Testing of the DNA extraction method by the European Union Reference Laboratory for Genetically Modified Food and Feed

The aim of the tests conducted was to verify that the DNA extraction method provides DNA of suitable quantity and quality for the intended purpose.
The DNA extraction method should allow preparation of the analyte in quality and quantity appropriate for the analytical method used to identify the event-specific analyte and the reference analyte.

The EU-RL GMFF tested the method submitted by the applicant on samples of carnation leaves provided by the applicant.

To assess the suitability of the DNA extraction method for PCR analysis, the extracted DNA was tested on a real-time PCR equipment.

5.2. Preparation of samples and DNA extraction

DNA was extracted following the method described above (see paragraph 3. "Description of the methods"); the DNA extraction was carried out on 6 test portions (replicates) and repeated over three days, giving a total of 18 DNA extractions.

5.3. DNA concentration, yield and repeatability

Concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).

Each DNA extract was measured three times, and the three values were averaged. DNA concentration was determined on the basis of a five point standard curve ranging from 1 to 500 ng/µL using a Biorad VersaFluor fluorometer.

The DNA concentration for all samples is reported in Table 7 below.

Table 7. DNA concentration (ng/µL) of eighteen samples extracted in three days.

Day	**Sample**	**Concentration (ng/µL)**
1	**1**	317.0
1	**2**	275.1
1	**3**	485.7
1	**4**	467.8
1	**5**	472.2
1	**6**	329.7
2	**7**	284.3
2	**8**	316.2
2	**9**	300.2
2	**10**	310.2
2	**11**	136.2
2	**12**	190.7
3	**13**	108.7
3	**14**	90.4
3	**15**	126.8
3	**16**	92.2
3	**17**	166.7
3	**18**	70.4

✓ **DNA concentration (ng/µL)**

Overall average of all samples 252.3 ng/µL
Standard deviation of all samples 136.5 ng/µL
Coefficient of variation 54.1%

✓ **Yield (total volume of DNA solution: approx. 300 µL)**

Overall average of all samples 75.7 µg
Standard deviation of all samples 40.9 µg
Coefficient of variation 54.1%

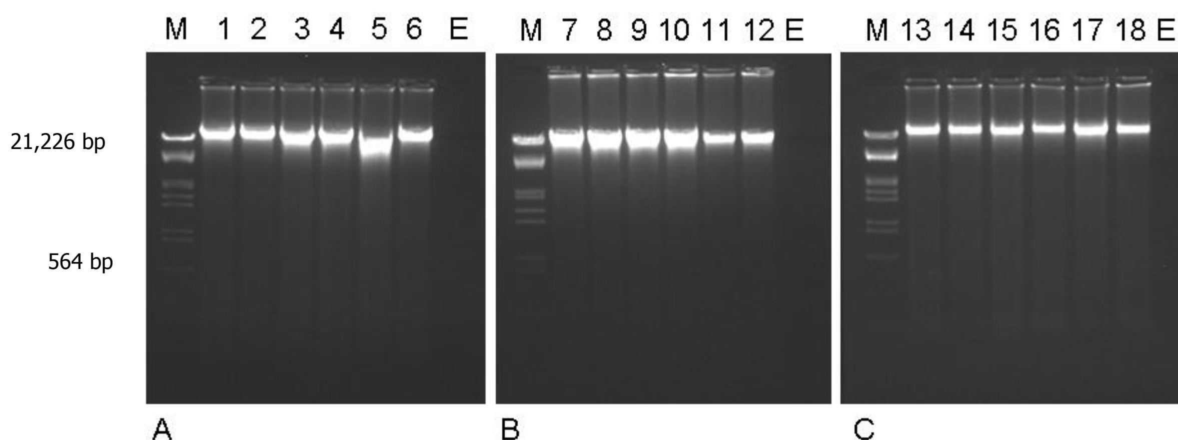
The yield of the extraction method obtained is appropriate; in fact the amount of DNA and its concentration is always sufficient for the purpose of subsequent analyses by PCR. However, the coefficient of variation for DNA concentration yield is high, thus confirming the data provided by the applicant. This is likely due to the fact that the starting samples were not homogenous in terms of DNA content per mass of material (flowers, leaves).

5.4. Fragmentation of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 4 µL of the DNA solution were analysed on a 1.0% agarose gel (Figure 1 A-C).

The eighteen genomic DNA samples extracted as described above appeared as high molecular weight DNA. None of the DNA samples showed indications of significant degradation.

Figure 1. Agarose gel electrophoresis of genomic DNA samples. A, Lanes 1-6: samples extracted on day 1; B, lanes 7-12 samples extracted on day 2; C, lanes 13-18 samples extracted on day 3; M: 1 kb DNA molecular weight marker; E: extraction control negative.



5.5. Purity/Absence of PCR inhibitors

In the absence of a real-time PCR method developed for carnation DNA, to verify the absence of PCR inhibiting compounds in the extracted carnation DNA samples, maize genomic DNA is diluted to perform an inhibition run and spiked with the extracted carnation DNA samples.

The use of a real-time method developed for the maize endogenous gene *high mobility group* (*hmg*) allows evaluating the presence of inhibitors in the extracted carnation samples even in the absence of a developed carnation-specific real-time PCR method.

For *hmg* method details refer to the report 'Event-specific Method for the Quantification of Maize MON 87460 Using Real-time PCR' (http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_ Method.pdf).

The DNA samples were adjusted to a concentration of 100 ng/µL (hereafter referred as "undiluted" samples), or in case of samples 14, 16 and 18 to a concentration of 90.4 ng/µL, 92.2 ng/µL and 70.4 ng/µL, respectively.

In parallel, genomic DNA extracted from maize was adjusted to a concentration of 50 ng/µL ("undiluted" DNA) and then diluted with buffer TE (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) to fourfold serial dilutions (1:4, 1:16, 1:64, 1:256).

Real-time PCR experiments were performed by mixing 2 µL of maize undiluted and serially diluted DNA with 1 µL of undiluted carnation DNA samples.

A sample containing only 2 µL of maize undiluted and serially diluted DNA was also included in each real-time experiment to provide data on maize DNA Ct values in absence of carnation DNA extracts (negative control).

Table 8 reports the DNA amount of maize and carnation genomic DNA used for each sample in real-time PCR experiments to evaluate the presence of inhibition in carnation DNA extracts.

Table 8. Amount in ng of maize and carnation genomic DNA used in real time PCR experiments for inhibition evaluation.

			DNA amount in (ng)				
Day	**DNA extraction**	**DNA source**	**Undiluted**	**1:4 diluted**	**1:16 diluted**	**1:64 diluted**	**1:256 diluted**
1	**1**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
1	**2**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
1	**3**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
1	**4**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
1	**5**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
1	**6**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**7**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**8**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**9**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**10**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**11**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
2	**12**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
3	**13**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
3	**14**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	90.40	-	-	-	-
3	**15**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
3	**16**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	92.20	-	-	-	-
3	**17**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	100.00	-	-	-	-
3	**18**	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	70.40	-	-	-	-
	Negative control	**maize**	100.00	25.00	6.25	1.56	0.39
		carnation	-	-	-	-	-

The Ct values obtained for "undiluted" and diluted maize DNA samples are reported in Table 9.

Table 9. Ct values of 'undiluted' and fourfold serially diluted maize DNA samples after amplification of the maize endogenous gene, *hmg*.

Repetition	Ct Undiluted (50 ng/µL)	Ct Diluted			
		1:4	1:16	1:64	1:256
1	22.24	24.18	26.20	28.32	30.39
2	22.83	24.84	26.84	28.93	30.81
3	22.20	24.17	26.26	28.30	30.43
Average	22.42	24.40	26.43	28.52	30.54

Table 10 below reports the comparison of extrapolated Ct values versus measured Ct values and the values of linearity (R^2) and slope for all measurements of the maize DNA samples.

Table 10. Comparison of extrapolated Ct values versus measured Ct values (amplification of maize *hmg* gene) for maize DNA samples.

Repetition	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.999	-3.45	22.09	22.24	0.16
2	0.999	-3.33	22.85	22.83	0.03
3	0.999	-3.46	22.08	22.20	0.12
Average	0.999	-3.41	22.34	22.42	0.10

*The expected slope for a PCR with 100% efficiency is -3.32; **delta Ct = abs (Ct extrapolated - Ct measured)

The Ct values obtained for "undiluted" and diluted maize DNA spiked with 100 ng carnation DNA extracts are reported in Table 11.

Table 11. Ct values of undiluted and fourfold serially diluted maize DNA samples spiked with carnation DNA extracts after amplification of the maize endogenous gene, *hmg*.

Day	DNA extraction	Ct Undiluted	Ct Diluted			
			1:4	1:16	1:64	1:256
1	**1**	22.35	24.30	26.37	28.28	30.41
1	**2**	22.36	24.43	26.4	28.32	30.48
1	**3**	22.36	24.28	26.37	28.49	30.50
1	**4**	22.46	24.48	26.43	28.36	30.55
1	**5**	22.32	24.23	26.20	28.27	30.49
1	**6**	22.27	24.23	26.22	28.34	30.42
2	**7**	22.90	24.92	26.93	29.02	30.96
2	**8**	23.04	25.06	27.03	29.00	31.10
2	**9**	23.22	24.96	26.98	28.93	31.10
2	**10**	22.91	24.81	26.78	28.69	30.92
2	**11**	22.91	24.88	26.94	28.92	30.99
2	**12**	23.01	24.82	26.95	28.96	31.01
3	**13**	22.37	24.31	26.35	28.42	30.55
3	**14**	22.30	24.40	26.44	28.42	30.46
3	**15**	22.61	24.38	26.36	28.42	30.53
3	**16**	22.54	24.36	26.30	28.32	30.52
3	**17**	22.36	24.44	26.40	28.36	30.57
3	**18**	22.27	24.28	26.24	28.47	30.33

Table 12 reports the comparison of extrapolated Ct values versus measured Ct values for maize DNA samples spiked with 100 ng of carnation DNA extracts, the values of linearity (R^2) and slope of all measurements and the comparison of measured Ct values for maize DNA samples from Table 10 and measured Ct values for maize DNA samples spiked with carnation DNA extracts

Table 12. Comparison of extrapolated Ct values versus measured Ct values (amplification of maize *hmg* gene) for maize DNA samples spiked with 100 ng of carnation DNA extracts.

Day	DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**	ΔCt*** maize DNA - spiked DNA
1	**1**	0.999	-3.36	22.27	22.35	0.08	0.11
1	**2**	0.999	-3.33	22.39	22.36	0.03	0.12
1	**3**	1.000	-3.45	22.21	22.36	0.14	0.12
1	**4**	0.999	-3.35	22.42	22.46	0.04	0.22
1	**5**	0.999	-3.46	22.09	22.32	0.23	0.08
1	**6**	0.998	-3.44	22.13	22.27	0.14	0.03
2	**7**	1.000	-3.36	22.91	22.90	0.00	0.07
2	**8**	0.999	-3.34	23.02	23.04	0.02	0.21
2	**9**	0.998	-3.38	22.90	23.22	0.32	0.39
2	**10**	0.998	-3.36	22.74	22.91	0.17	0.08
2	**11**	1.000	-3.37	22.85	22.91	0.06	0.08
2	**12**	0.999	-3.42	22.79	23.01	0.22	0.18
3	**13**	0.999	-3.45	22.22	22.37	0.15	0.17
3	**14**	1.000	-3.35	22.39	22.30	0.10	0.10
3	**15**	0.999	-3.41	22.29	22.61	0.32	0.41
3	**16**	0.999	-3.40	22.25	22.54	0.30	0.34
3	**17**	0.999	-3.38	22.35	22.36	0.01	0.16
3	**18**	0.999	-3.38	22.24	22.27	0.03	0.07

*The expected slope for a PCR with 100% efficiency is -3.32; **delta Ct = |Ct extrapolated - Ct measured|; ***delta Ct = |mean Ct measured maize DNA (from table 10) – mean Ct measured maize DNA spiked with carnation DNA extract|. Values compared are from the same PCR run (repetition 1 for DNA extractions 1-6; repetition 2 for DNA extractions 7-12; repetition 3 for DNA extractions 13-18).

To measure inhibition, the Ct values of the four diluted maize genomic DNA samples were plotted against the logarithm of the dilution and the Ct value for the "undiluted" sample was extrapolated from the equation calculated by linear regression. To exclude the presence of inhibitory compounds in maize genomic DNA, the difference (ΔCt) average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the test should be < 0.5. In addition the slope of the curve should be between -3.6 and -3.1 and the R_2 value of linear regression should be > 0.98 (see EU-RL GMFF guidance document 'Definition of

minimum performance requirements for analytical methods of GMO testing', http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_ Requirements_Analytical_methods.pdf).

The data obtained from the inhibition evaluation of maize DNA samples confirm that there is no inhibition in maize DNA extracts. In fact the ΔCt average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the inhibition test is < 0.5. Moreover the curve slopes is between -3.1 and -3.6 and the R_2 of linear regression equals 0.999 (Table 9 and Table 10).

As for maize genomic DNA samples, the Ct values of the four diluted maize genomic DNA samples spiked with carnation DNA extracts were plotted against the logarithm of the dilution and the Ct value for the "undiluted" sample was extrapolated from the equation calculated by linear regression. The extrapolated Ct for the "undiluted" sample of maize DNA spiked with carnation DNA was compared with the measured Ct. The evaluation is carried out assuming that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is > 0.5 cycles in comparison with the calculated Ct value (ΔCt > 0.5). In addition, the slope of the curve should be between -3.1 and -3.6. The Ct values obtained with maize DNA were compared with Ct values obtained with maize DNA spiked with carnation DNA extracts, to confirm that the difference was < 0.5 cycles (Table 11 and Table 12).

All ΔCt values of extrapolated versus measured Ct are < 0.5. R^2 of linear regression is > 0.99 for all DNA samples. The slopes of the curves are all between -3.1 and -3.6.

The difference between measured Ct values for maize DNA (from table 10) and measured Ct values for maize DNA spiked with carnation DNA measured in the same real time run are < 0.5 cycles. Therefore, the carnation DNA extracts 1 to 18 are considered not inhibited.

6. Conclusion

The data reported confirm that the extraction method, applied to samples of carnation leaves, produces DNA of suitable quantity and quality for subsequent PCR-based methods.

However, the coefficient of variation for DNA concentration yield is high. This is likely due to the lack of homogenous DNA content per mass in the starting material (e.g. older or younger leaves); it is therefore advisable to use as starting material young leaf tissue.

7. 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].

8. References

1. Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. Plant. Mol Biol. Rep. 1: 19-21.