EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Sampling and DNA extraction of maize DAS 59122

Report from the Validation of the "CTAB/Wizard" method for DNA extraction from ground maize grain/seed

Method development and single laboratory validation:
Pioneer Hi-Bred International
GeneScan Analytics GmbH

Method testing and confirmation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM

Contents

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1. Introduction

The purpose of the DNA extraction method described is to serve as a method to provide DNA for subsequent PCR based detection methods. The method does not only have to yield DNA of sufficient quality and quantity but is also required to be suitable for routine use in terms of ease of operations, sample throughput and costs. This Report describes the method and validation experiments including results.

2. Description of the methods

Sampling:
For sampling of seeds and grains of maize DAS 59122, the Applicant refers to the technical guidance documents and protocols described in Commission Recommendation 2004/787/EC on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003.

Scope and applicability:
The "CTAB/Wizard" method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data presented here are restricted to ground maize grain/seed. Application of the method to other matrices may require adaptation and needs specific validation.

Principle:
The basic principle of DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors. The "CTAB/Wizard" method starts with a lysis step (thermal lysis in the presence of CTAB, EDTA and proteinase K) followed by removal of RNA by digestion with RNase A and removal of contaminants such as lipophilic molecules and proteins by extraction with chloroform. Afterwards a crude DNA-extract is generated by precipitation with isopropanol. This first crude extract is subsequently purified using two different commercially available products: At first the Wizard® DNA Clean-Up System (Promega) is applied which makes use of silica resin. This purification step is based on the selective binding of nucleic acids to silica in the presence of chaotropic reagents. Afterwards remaining inhibitors are removed by a gel filtration step using S-300 HR MicroSpin Columns (Amersham Pharmacia).

Note:
When starting from intact maize grain/seed samples instead of finely ground maize grain/seed samples, a grinding step with an appropriate milling/grinding device such as a laboratory mill or blender has to be performed. Milling/grinding of grain/seed not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable

for the generation of representative test portions by reducing the particle size. For details concerning requirements of particle size/particle number, generation of the test portions etc. please refer to the literature (e. g. prEN ISO 21568 sampling).
DNA extractions are recommended to be carried out at least on two test portions. Extraction blanks in duplicate (negative controls; handled identically but without sample material) are mandatory throughout extraction and subsequent PCR.

Lysis / isopropanol precipitation of DNA

1. Transfer 30 ml CTAB buffer and 60 µl Proteinase K to 50 ml conical tube.
2. Weigh out 10 g of ground grain/seed into tube containing CTAB buffer and Proteinase K, mix thoroughly.
3. Incubate for 2 - 4 hours at 60°C with agitation.
4. Spin down at room temperature for 5 minutes at 2700 – 3000x *g*.
5. Transfer 1 ml of the supernatant to a 2 ml microcentrifuge tube containing 5 µl RNase A.
6. Incubate for 15 minutes at 60° C.
7. Centrifuge at room temperature for 1 minute at approximately 20000x *g*.
8. Transfer 900 µl of the supernatant to a 2 ml microcentrifuge tube containing 600µl chloroform.
9. Vortex, centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
10. Transfer 625 µl of the upper phase to a 1,5 ml microcentrifuge tube containing 500 µl isopropanol and 2 µl glycogen (glycogen is pipetted into the lid, do not combine the glycogen and the isopropanol).
11. Mix completely (invert tubes several times) and let stand at room temperature for 30 minutes to allow precipitate to form.
12. Centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
13. Discard supernatant. Add 500 µl 75% ethanol and pipette carefully up and down until pellet is detached from the wall of the microcentrifuge tube.
14. Centrifuge at room temperature for 5 minutes at approximately 20000x *g*.
15. Carefully remove and discard the supernatant. Centrifuge again and remove all remaining ethanol. If any fluid remains, allow pellet to dry at room temperature.
16. Resuspend the pellet in 100 µl 0,2x TE buffer. Let stand for 15 min at room temperature with occasional mixing. Make sure that the pellet is dissolved, then centrifuge for 2 minutes at approximately 20000x g and transfer the supernatant into a fresh 1,5 ml microcentrifuge tube.

Purification using Wizard® DNA Clean-Up System (Promega)

17. Agitate well the Wizard resin. If crystals or aggregates are present, dissolve by warming the resin to 37°C for 10 minutes. Cool to 25 - 30°C before use. Add 900µl to sample DNA. Mix carefully by repeatedly pipetting up and down. Incubate for 5 minutes at 60°C.
18. Use one Wizard® minicolumn for each sample. Attach syringe barrels (included in the kit) to Wizard® minicolumns (label minicolumns!) and then attach the minicolumn/syringe barrel assembly to the valves of the vacuum manifold (Promega Vac-Man® Laboratory Vacuum Manifold).
19. Allow samples to cool to room temperature, then spin down briefly (pulse spin).
20. Mix sample carefully by repeatedly pipetting up and down, transfer to the Wizard minicolumn. Apply a vacuum to draw the solution through the minicolumn. Break the vacuum to the minicolumn.
21. Wash the DNA-resin mixture once by applying 2ml 80% isopropanol to the syringe barrel and reapply a vacuum to draw the solution through the minicolumn.
22. Close the valve when all 80% isopropanol has run through the Wizard minicolumn. Do not let the Wizard minicolumn run dry.
23. Remove the syringe barrels from the vacuum manifold and place Wizard® minicolumns in 1,5ml microcentrifuge tubes. Centrifuge at room temperature for 2 to 4 minutes at 10000x g to remove any residual isopropanol.
24. Place Wizard® minicolumns into clean 1,5 ml microcentrifuge tubes and add 100µl 0,2x TE buffer (prewarmed to 65°C). Let stand at room temperature for 1 minute.
25. Centrifuge at room temperature for 1 minute at approximately 10000x g to elute DNA. Remove the Wizard® minicolumn. Centrifuge the eluted DNA for 2 minutes at approximately 20000x *g*. transfer the supernatant into a fresh 1,5ml microcentrifuge tube.

Purification using S-300 HR MicroSpin Columns (Amersham Pharmacia)

26. Label fresh 1,5 ml microcentrifuge tube using permanent marker.
27. Vortex MicroSpin microcolumns to homogenize the gel filtration material.
28. Slightly unscrew the MicroSpin microcolumn caps (1/4 turn but do not remove caps). Break off the bottom end of the MicroSpin microcolumn using clean gloves, clean forceps or the tool supplied by Amersham Pharmacia.
29. Place the MicroSpin microcolumn in a 2 ml microcentrifuge tube and centrifuge for 2 minutes at 735x *g*.
30. Place the MicroSpin microcolumn into a 1,5 ml microcentrifuge tube and pipette DNA solution onto the center of the MicroSpin microcolumn.
31. Centrifuge at room temperature for 2 minutes at 735x *g*.

32. Discard the MicroSpin microcolumn. The purified DNA solution is collected in the bottom of the 1,5ml microcentrifuge tube.

3. Equipment / Reagents / Plasticware

3.1. Equipment

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

Equipment	**Example of appropriate apparatus**
Pipettes with adjustable volume	e. g. Eppendorf Research. 2 - 20µl. 20 – 200 µl. 100 -1000 µl
Incubator with shaker or shaking water bath	e. g. Heraeus Function Line B12 in combination with shaker GFL 3005 (Gesellschaft für Labortechnik mbH)
Balances for the preparation of buffers and solutions and for sample weigh in	e. g. Ohaus Scout II. Ohaus Adventurer
Centrifuge with rotors for 50ml centrifuge tubes and microcentrifuge tubes	e. g. Sigma 4 K 15C with suitable rotors
Thermoblock for 1,5 / 2,0ml microcentrifuge tubes	e. g. Bioblock Scientific 92333
Vortex	e. g. NeoLab Vortex VM-300
Vacuum manifold	Promega Vac-Man® Laboratory Vacuum Manifold. 20-sample capacity
Diaphragm vacuum pump	e. g. KNF Neuberger Diaphragm vacuum pump N840 FT,18

3.2. Reagents

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

Reagent	Specification
NaCl	p. a. quality or Molecular biology grade
CTAB	p. a. quality or Molecular biology grade
Tris	p. a. quality or Molecular biology grade
EDTA · Na_2-salt	p. a. quality or Molecular biology grade
HCl	p. a. quality
Proteinase K	from Tritirachium album, DNAses, Rnases, Exonucleases not detectable, Molecular biology grade
RNase A	from bovine pancreas, salt free, protease free and chromatographically homogeneous, ca. 90 Kunitz units/mg
Sodium acetate	p. a. quality or Molecular biology grade
Chloroform	p. a. quality
Isopropanol	p. a. quality
Glycogen	from oyster, research grade
Ethanol	p. a. quality
Wizard 'Clean up' Kit (100 Preps)	Promega, A7280
S-300 HR MicroSpin Columns (50)	Amersham Pharmacia, 27-5130-01

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

CTAB buffer

1,4 M NaCl
2% (w/v) CTAB
0,1M Tris-Base
0,015M EDTA

For 1 liter CTAB buffer weigh out 81,8 g NaCl. 20 g CTAB. 12,1 g Tris-Base and 5,84 g EDTA in an appropriate beaker and add about 800ml H_2O_{deion}. Adjust pH with HCl to pH 8,0, stir until all reagents are dissolved. Adjust volume to 1l with H_2O_{deion}. Do not autoclave,
Store at room temperature for up to 2 years.

Proteinase K
20 mg/ml H_2O
For 10 ml proteinase K solution dissolve 200 mg proteinase K in 10 ml H_2O_{deion},
Store at -20°C for up to 2 years.

RNase A
91 mg/ml
Dissolve 0,5 g RNase A in 5 ml 0,01M sodium acetate (pH 5,2), aliquot in 1 ml portions, boil for 15 minutes to inactivate DNases, cool slowly to room temperature and add 100 µl 1M Tris-HCl (pH 7,4) to each aliquot.
Store at -20°C for up to 2 years.

Glycogen
20 mg/ml H_2O
For 5ml glycogen solution dissolve 100mg glycogen in 5ml H_2O_{deion}.
Store at -20°C for up to 2 years.

75% (v/v) Ethanol
For 200 ml combine 150 ml 100% ethanol with 50ml H_2O_{deion}.
Store at room temperature for up to 5 years.

1x TE buffer
10mM Tris. pH 8,3
1mM EDTA
For 100 ml 1x TE buffer combine 1ml 1M Tris (pH 8,3) and 200µl 0,5M EDTA (pH 8,0) and adjust the volume to 100 ml with H_2O_{deion}. Autoclave.
Store at room temperature for up to 2 years.

0,2x TE buffer
2mM Tris, pH 8,3
0,2 mM EDTA
For 100 ml 0,2x TE buffer dilute 20 ml 1x TE buffer with 80 ml H_2O_{deion}. Autoclave.
Store at room temperature for up to 2 years.

80% (v/v) Isopropanol
For 500 ml combine 400 ml 100% isopropanol with 100ml H_2O_{deion}.
Store at room temperature for up to 5 years.

3.3. Plasticware

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

Item	**Specification**
50 ml conical tubes	e. g. Sarstedt, 62.547 254
1,5 ml microcentrifuge tube	e. g. Roth, 4182.1
2 ml microcentrifuge tube	e. g. Eppendorf, 30.120.094
filter tips	fitting the pipette models used

4. Experimental Validation and Results

For experimental validation six different samples of ground maize kernels were extracted using the above described method. Five samples were extracted in duplicate, one sample was extracted six fold.
The following performance characteristics were determined:
- DNA concentration / yield, Repeatability
- Fragmentation state of DNA
- Purity / absence of PCR-inhibitors

4.1. DNA concentration / Yield, Repeatability

The concentration of the extracted DNA was determined by spectrophotometric measurement. 5 µl of the extracted DNA was diluted 1:50 in 0,2x TE buffer. Absorption was measured for both blank (only 0,2x TE) and diluted DNA solutions at 260 nm using the GeneQuant II (Amersham Pharmacia). DNA concentration was calculated based on the assumption that an OD of 1 corresponds to 50µg/ml DNA.

Sample	Replicate	Concentration [ng/µl]
1	i	49,2
	ii	37,9
2	i	56,9
	ii	35,2
3	i	49,7
	ii	49,9
4	i	65,9
	ii	58,2
5	i	62,1
	ii	59,1
6	i	47,0
	ii	46,7
	iii	40,0
	iv	49,2
	v	44,7
	vi	44,5

DNA concentration (ng/µl):

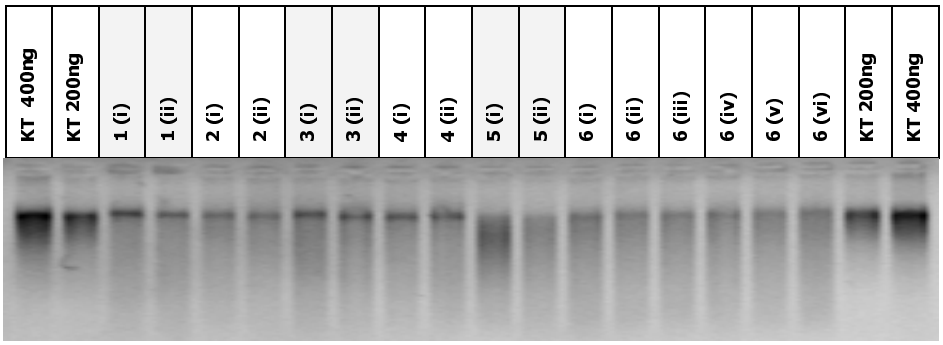
Overall average of all samples:	50 ng/µl
Average (sample no. 6, sixfold extraction):	45.4 ng/µl
Standard deviation (sample no. 6, sixfold extraction):	3.1 ng/µl
Coefficient of variation (sample no. 6, sixfold extraction):	7%

Yield (total volume of DNA solution: 100 µl):

Overall average of all samples:	5 µg
Average (sample no. 6, sixfold extraction):	4.5 µg
Standard deviation (sample no. 6, sixfold extraction):	0.3 µg
Coefficient of variation (sample no. 6, sixfold extraction):	7%

4.2. Fragmentation state of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis performed essentially according to Sambrook et al.. 5,0 µl of each DNA solution was analyzed on a 1,5% agarose gel (TAE buffer system). Defined amounts of calf thymus DNA (KT) were loaded as DNA quantity standards. After electrophoretic separation the gel was stained in ethidium bromide solution for 30 min and the DNA visualized using an UV transilluminator.



Medium to high molecular weight DNA was observed.

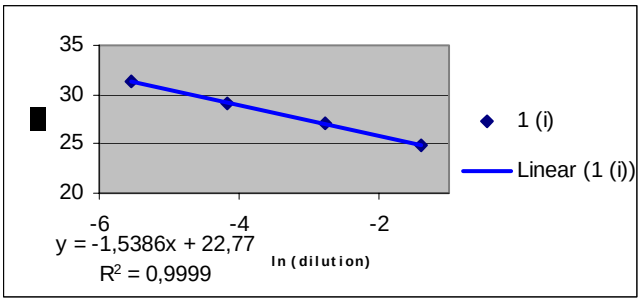
4.3. Purity / absence of PCR-inhibitors

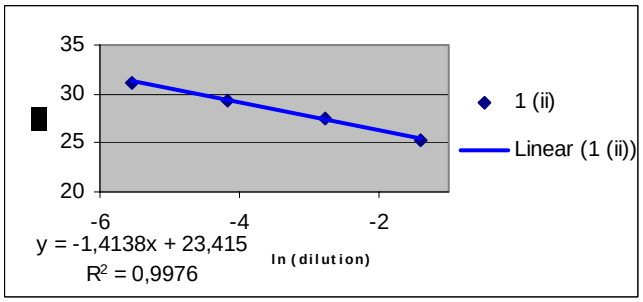
In order to assess the PCR quality of the extracted DNA fourfold serial dilutions of each extract were prepared with 0,2x TE buffer (1:4, 1:16, 1:64, 1:256) and analyzed using a maize specific Real time PCR-system. To measure inhibition, the Ct values of the four diluted samples were plotted against the natural logarithm of the dilution and 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. Evaluation: PCR inhibitor is present if the measured Ct value for the undiluted sample is suppressed by >0,5 cycles from the calculated Ct value.

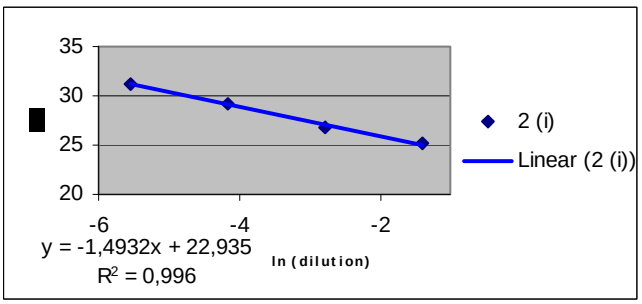
Ct values of undiluted and fourfold serial diluted DNA extracts:

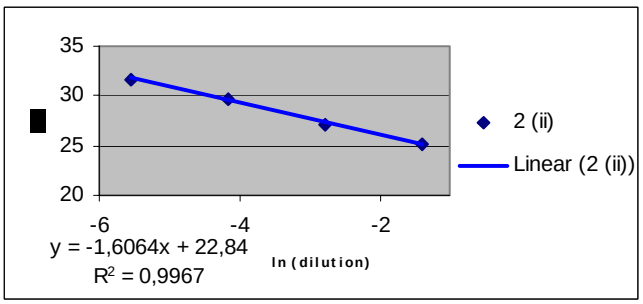
	undiluted	diluted			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1 (i)	22,73	24,89	27,04	29,20	31,28
1 (ii)	23,19	25,26	27,51	29,29	31,20
2 (i)	23,15	25,14	26,83	29,23	31,24
2 (ii)	22,97	25,08	27,16	29,75	31,64
3 (i)	22,91	25,03	27,02	29,00	30,96
3 (ii)	22,30	24,92	27,04	29,12	31,30
4 (i)	23,01	25,24	27,18	29,32	31,05
4 (ii)	22,49	24,68	26,72	28,82	31,02
5 (i)	22,06	24,05	26,28	28,42	30,53
5 (ii)	21,85	23,99	26,17	28,18	30,18
6 (i)	22,04	24,24	26,42	28,47	30,61
6 (ii)	22,20	24,57	26,58	28,69	30,75
6 (iii)	22,26	24,30	26,40	28,40	30,60
6 (iv)	22,10	24,44	26,57	28,94	30,64
6 (v)	22,24	24,47	26,84	28,81	30,69
6 (vi)	22,04	24,29	26,36	28,67	30,36

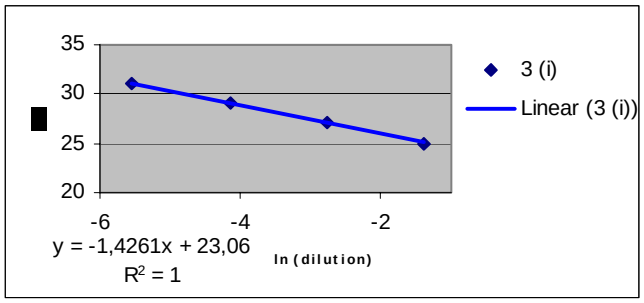
Plot of the Ct values of the four diluted samples against the natural logarithm of the dilution:

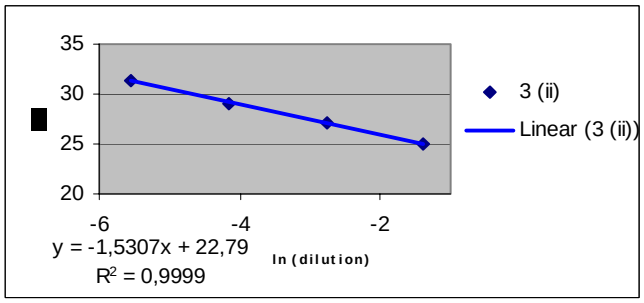


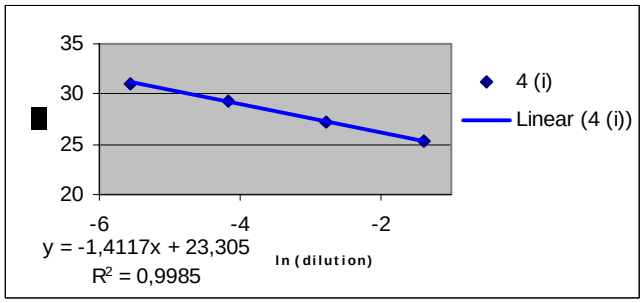


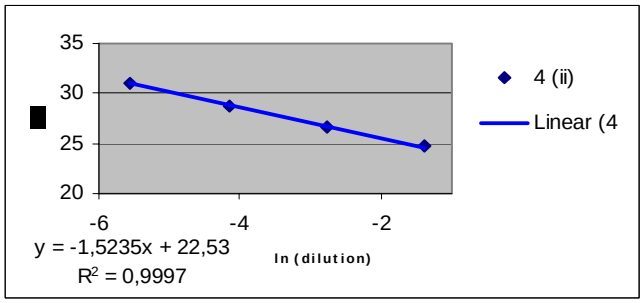


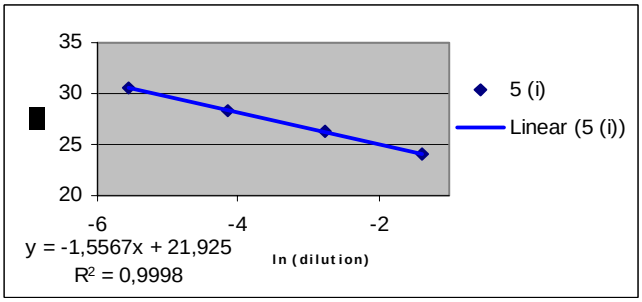


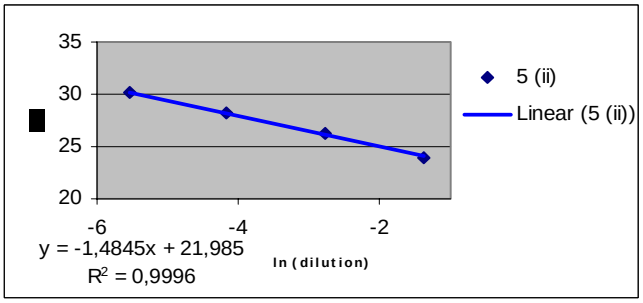

35
30
25
20
-6
-4
-2
y = -1,4261x + 23,06
R² = 1
ln (dilution)
3 (i)
Linear (3 (i))


35
30
25
20
-6
-4
-2
y = -1,5307x + 22,79
R² = 0,9999
ln (dilution)
3 (ii)
Linear (3 (ii))


35
30
25
20
-6
-4
-2
y = -1,4117x + 23,305
R² = 0,9985
ln (dilution)
4 (i)
Linear (4 (i))


35
30
25
20
-6
-4
-2
y = -1,5235x + 22,53
R² = 0,9997
ln (dilution)
4 (ii)
Linear (4


35
30
25
20
-6
-4
-2
y = -1,5567x + 21,925
R² = 0,9998
ln (dilution)
5 (i)
Linear (5 (i))


35
30
25
20
-6
-4
-2
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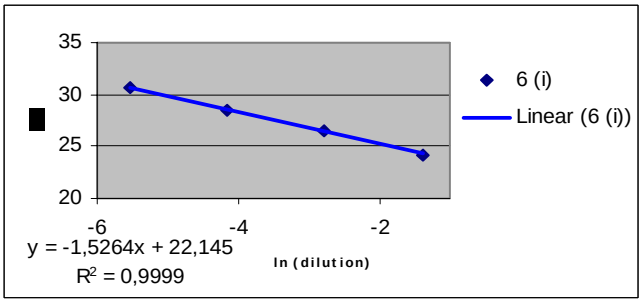
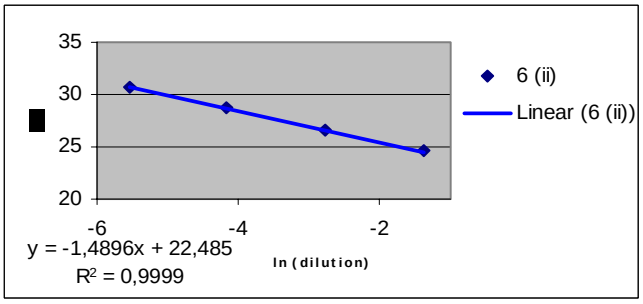
R² = 0,9996
ln (dilution)
5 (ii)
Linear (5 (ii))


35
30
25
20
-6
-4
-2
y = -1,5264x + 22,145
R² = 0,9999
ln (dilution)
6 (i)
Linear (6 (i))


35
30
25
20
-6
-4
-2
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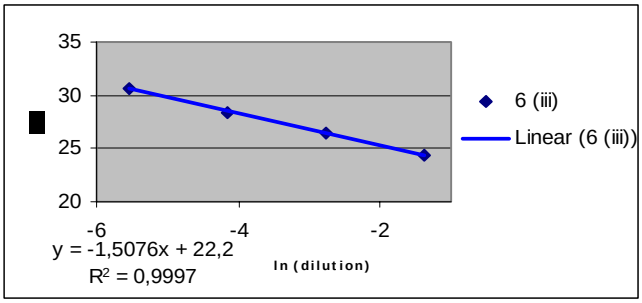
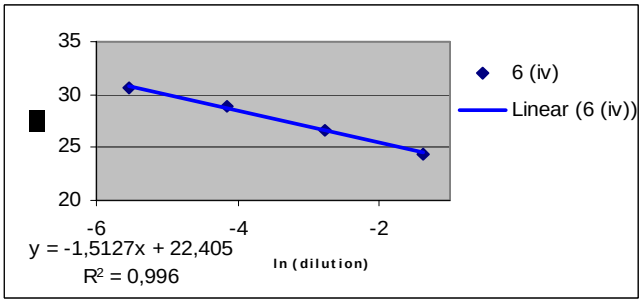
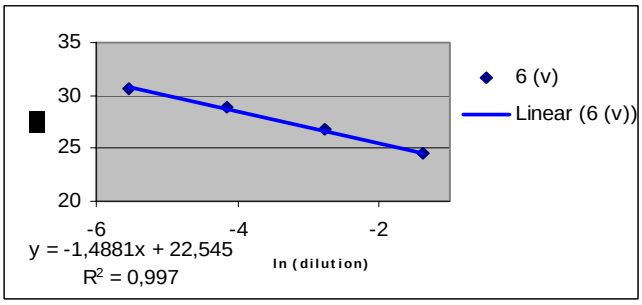
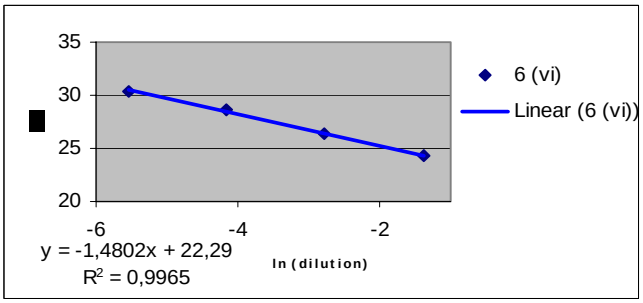
R² = 0,9999
ln (dilution)
6 (ii)
Linear (6 (ii))









Comparison of extrapolated Ct values versus measured Ct values:

delta Ct = abs(Ct extrapolated - Ct measured)

DNA extract	R^2	Ct extrapolated	C_T measured	delta Ct
1 (i)	0,9999	22,77	22,73	0,04
1 (ii)	0,9976	23,42	23,19	0,22
2 (i)	0,9960	22,94	23,15	0,22
2 (ii)	0,9967	22,84	22,97	0,13
3 (i)	1,0000	23,06	22,91	0,15
3 (ii)	0,9999	22,79	22,30	0,49
4 (i)	0,9985	23,31	23,01	0,29
4 (ii)	0,9997	22,53	22,49	0,04
5 (i)	0,9998	21,93	22,06	0,13
5 (ii)	0,9996	21,99	21,85	0,13
6 (i)	0,9999	22,15	22,04	0,11
6 (ii)	0,9999	22,49	22,20	0,29
6 (iii)	0,9997	22,20	22,26	0,06
6 (iv)	0,9960	22,41	22,10	0,31
6 (v)	0,9970	22,55	22,24	0,31
6 (vi)	0,9965	22,29	22,04	0,25

All delta Ct values < 0,5.
All R^2 > 0,99.
The data observed indicate absence of PCR inhibitors.

Abbreviations:

CTAB	cetyltrimethylammoniumbromide
PCR	polymerase chain reaction
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
TAE	tris-acetate
KT	calf thymus
TE	tris EDTA

5. Experimental testing of the DNA extraction method by the Community Reference Laboratory

The aim of the experimental testing is to verify that the method of DNA extraction provides DNA of suitable quantity and quality for the intended purpose.
The DNA extraction method should allow preparation of the analyte in amounts and quality appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.
The CRL tested the CTAB/Wizard DNA extraction method proposed by the applicant on the DAS 59122 (100% GM) maize line.
To assess the suitability of the extraction method for Real Time PCR analysis, the DNA extracted was tested using a qualitative PCR run on the real time PCR equipment.
The 100% DAS 59122 maize seeds were provided by the applicant.
Tests were carried out by two analysts.

5.1 Preparation of samples

About 300 g of seeds of the transgenic maize line 59122 (Lot n. PIV1PNE11040-00) were grinded using the GRINDOMIX mixer.

5.2 DNA extraction

DNA was extracted by means of the CTAB/Wizard-based method described above and in-house validated by the applicant.

Note: the following modification to the method was introduced: step n.16, due to the presence of a large pellet, the DNA was kept overnight at 4 ºC, and before the purification with the Wizard columns was not centrifuged and transferred to a new tube; the purification was carried out on the whole precipitated DNA.

5.3 DNA concentration / Yield, Repeatability

DNA concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).
Each DNA extract was measured twice, and the two 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 (ng/µl) for all samples (yellow boxes samples from 1 to 6 extracted on 26.07.2005, green boxes samples from 1-6 extracted on 02.08.2005 and blue boxes samples extracted on 09.08.2005) is reported in the table below.

Sample	Concentration (ng/µl)
1	100
2	94.0
3	80.5
4	92.6
5	60.9
6	66.4
1	58.8
2	63.0
3	65.7
4	66.3
5	68.2
6	52.1
1	29.5
2	25.3
3	26.2
4	28.0
5	24.5
6	25.7

DNA concentration (ng/µl)

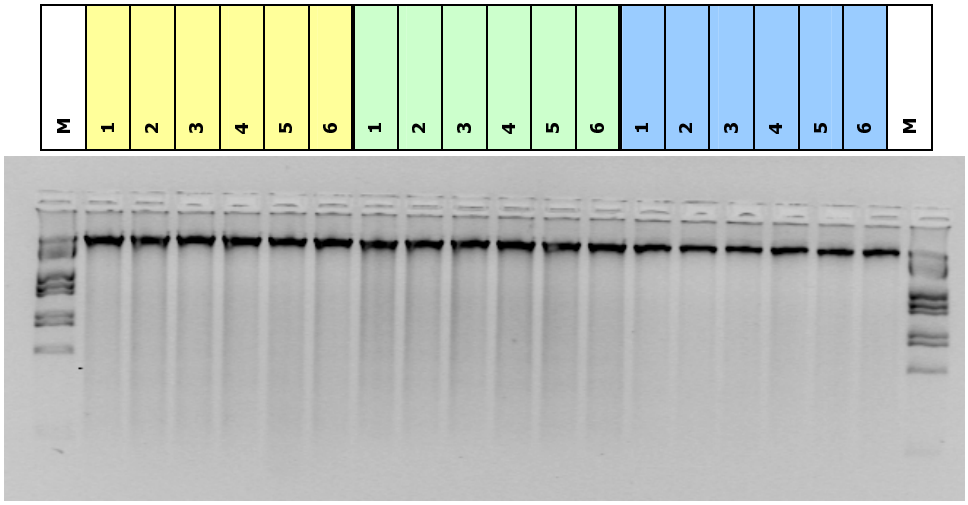
Overall Average of all samples	57.1 ng/µl
Standard deviation	25.5 ng/µl
Coefficient of variation	44.7%

Yield (total volume of DNA solution: 100 µl)

Overall Average of all samples	5.71 µg
Standard deviation	2.55 µg
Coefficient of variation	44.6 %

5.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 5 µl of the DNA solution were analyzed on a 1.5% agarose gel. In the yellow boxes samples from 1 to 6 extracted on 26.07.2005; in the green boxes samples from 1-6 extracted on 02.08.2005; in the blue boxes samples extracted on 09.08.2005. A DNA ladder 1kb (M) was used.



High and medium molecular weight DNA distribution was observed for all samples.

5.5 Purity / Absence of PCR inhibitors

To assess the PCR quality of the DNA extracted, the experimental approach previously described (paragraph 4.3) was followed.
The study was conducted on all DNA preparations by applying real time PCR targeting both a maize specific reference sequence (Hmg - High mobility group) and a DAS-59122 event-specific sequence (for a validation report on both systems see http://gmo-crl.jrc.it/statusofdoss.htm)
Yellow boxes represent samples from 1 to 6 extracted on 26.07.2005, green boxes samples from 1-6 extracted on 02.08.2005 and blue boxes samples extracted on 09.08.2005).

Ct values of undiluted and fourfold serial diluted DNA extracts after amplification of maize reference gene Hmg

	Undiluted (40 ng/µl)	diluted			
DNA extract	1:1	1:4	1:16	1:64	1:256
1	21,82	23,78	25,64	27,76	29,69
2	21,47	23,93	25,67	27,79	29,86
3	21,45	23,51	26,00	27,86	29,80
4	21,74	23,55	25,59	28,32	30,06
5	21,64	23,59	25,58	27,42	29,75
6	21,80	23,74	26,03	27,91	29,77
1	21,63	23,61	25,78	27,67	29,70
2	21,51	23,74	25,79	27,83	29,84
3	21,51	23,58	25,88	27,61	29,55
4	22,17	23,98	25,98	27,88	29,93
5	21,70	24,20	25,96	27,74	29,96
6	21,63	23,80	26,48	28,34	30,44
1	22,20	24,15	25,86	28,51	30,25
2	22,29	24,47	26,50	28,60	30,82
3	22,35	24,33	26,46	28,36	30,25
4	22,13	24,24	26,29	28,31	30,24
5	22,30	24,57	26,61	28,58	30,67
6	22,22	24,38	26,38	28,49	30,49

Ct values of undiluted and fourfold serial diluted DNA extracts after amplification of DAS-59122 event specific sequence

	Undiluted (40 ng/µl)	diluted			
DNA extract	1:1	1:4	1:16	1:64	1:256
1	21,69	23,83	25,82	27,87	29,90
2	21,54	23,75	25,92	27,99	30,04
3	21,71	23,76	25,80	27,96	29,78
4	21,85	23,71	25,89	28,24	30,16
5	22,04	24,03	26,20	27,90	30,16
6	21,83	23,85	26,41	28,26	30,28
1	21,69	23,83	26,01	28,08	30,19
2	21,72	23,76	25,90	27,78	29,84
3	21,63	23,64	25,54	27,47	29,51
4	23,19	24,43	26,48	28,66	30,61
5	22,80	24,63	26,53	28,39	30,32
6	22,51	24,48	26,69	28,73	30,75

1	22,82	24,85	26,94	28,89	30,94
2	23,30	24,99	26,99	29,17	31,42
3	23,13	24,91	27,07	29,17	30,88
4	23,16	24,79	26,93	28,97	31,06
5	23,24	25,50	27,66	29,62	31,58
6	23,21	25,26	27,11	29,47	31,49

Comparison of extrapolated Ct values versus measured Ct values (amplification of maize reference gene Hmg)

DNA extract	R^2	Slope*	Ct extrapolated	C_T measured	delta Ct**
1	0,9994	-3,297	21,76	21,82	0,06
2	0,9983	-3,308	21,83	21,47	0,37
3	0,9953	-3,446	21,61	21,45	0,16
4	0,9933	-3,700	21,31	21,74	0,43
5	0,9977	-3,372	21,51	21,64	0,13
6	0,9972	-3,318	21,87	21,80	0,07
1	0,9994	-3,346	21,65	21,63	0,02
2	1,0000	-3,378	21,72	21,51	0,21
3	0,9967	-3,260	21,75	21,51	0,24
4	0,9998	-3,281	22,01	22,17	0,16
5	0,9966	-3,165	22,20	21,70	0,50
6	0,9943	-3,616	21,82	21,63	0,20
1	0,9921	-3,481	21,95	22,20	0,25
2	0,9996	-3,511	22,31	22,29	0,03
3	0,9991	-3,268	22,43	22,35	0,09
4	0,9998	-3,327	22,26	22,13	0,14
5	0,9999	-3,365	22,54	22,30	0,24
6	0,9999	-3,393	22,33	22,22	0,10

Note: In the yellow boxes samples from 1 to 6 extracted on 26.07.2005; in the green boxes samples from 1-6 extracted on 02.08.2005; in the blue boxes samples from 1-6 extracted on 09.08.2005

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

All delta Ct values of extrapolated versus measured Ct < 0,5.

Comparison of extrapolated Ct values versus measured Ct values (amplification of DAS-59122 event specific sequence)

DNA extract	R^2	Slope*	Ct extrapolated	C_T measured	delta Ct**
1	0,9999	-3,364	21,79	21,69	0,11
2	0,9998	-3,483	21,68	21,54	0,14
3	0,9989	-3,357	21,77	21,71	0,06
4	0,9984	-3,602	21,58	21,85	0,27
5	0,9972	-3,337	22,05	22,04	0,01
6	0,9949	-3,513	21,91	21,83	0,08
1	0,9999	-3,513	21,74	21,69	0,05
2	0,9995	-3,343	21,79	21,72	0,07
3	0,9997	-3,245	21,65	21,63	0,02
4	0,9996	-3,442	22,36	23,19	0,83
5	0,9999	-3,144	22,73	22,80	0,07
6	0,9996	-3,461	22,45	22,51	0,06
1	0,9998	-3,362	22,84	22,82	0,03
2	0,9993	-3,568	22,77	23,30	0,53
3	0,9971	-3,322	23,01	23,13	0,12
4	0,9999	-3,464	22,73	23,16	0,43
5	0,9994	-3,360	23,53	23,24	0,29
6	0,9980	-3,499	23,07	23,21	0,14

Note: In the yellow boxes samples from 1 to 6 extracted on 26.07.2005; in the green boxes samples from 1-6 extracted on 02.08.2005; in the blue boxes samples from 1-6 extracted on 09.08.2005

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

Sixteen out of eighteen (89%) delta Ct values of extrapolated versus measured Ct < 0,5. One value at 0,53, one value 0,83.

6. Conclusion

The data reported confirm that the method provides DNA of suitable quantity and quality for subsequent PCR based detection applications.
The method is therefore considered fit for the intended purpose.

7. Literature

Sambrook. J., Fritsch. E. F. and Maniatis. T. (1989) Molecular Cloning: a laboratory manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

8. Abbreviations

CTAB	cetyltrimethylammoniumbromide
EDTA	ethylenediaminetetraacetic acid
KT	calf thymus
PCR	polymerase chain reaction
RNase A	ribonuclease A
TAE	tris-acetate
TE	tris EDTA
Tris	tris(hydroxymethyl)aminomethane