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 TC1507

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

Method development and single laboratory validation:
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GeneScan Analytics GmbH

Method testing and confirmation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Contents

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1. Introduction

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 TC1507, 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 gelfiltration 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 30ml 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 1ml of the supernatant to a 2ml microcentrifuge tube containing 5µl RNase A.
6. Incubate for 15 minutes at 60° C.
7. Centrifuge at room temperature for 1 minute at approximately 20000x *g*.
8. Transfer 900µl 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 100µl 0,2x TE buffer. Let stand for 15min 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) (Follow also manufacturer's instructions!)

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 1,5ml 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 - 200µl. 100 -1000µl
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 1l CTAB buffer weigh out 81,8g NaCl. 20g CTAB. 12,1g Tris-Base and 5,84g 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

20mg/ml H_2O

For 10ml proteinase K solution dissolve 200mg proteinase K in 10ml H_2O_{deion},

Store at -20°C for up to 2 years,

RNase A

91mg/ml

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

Store at -20°C for up to 2 years.

Glycogen

20mg/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 200ml combine 150ml 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 100ml 1x TE buffer combine 1ml 1M Tris (pH 8,3) and 200µl 0,5M EDTA (pH 8,0) and adjust the volume to 100ml 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 100ml 0,2x TE buffer dilute 20ml 1x TE buffer with 80ml H_2O_{deion}. Autoclave.

Store at room temperature for up to 2 years.

80% (v/v) Isopropanol

For 500ml combine 400ml 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**
50ml conical tubes	e. g. Sarstedt, 62.547 254
1,5ml microcentrifuge tube	e. g. Roth, 4182.1
2ml 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 sixfold.

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 die extracted DNA was determined by spectrophotometric measurement. 5µl 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 260nm 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):

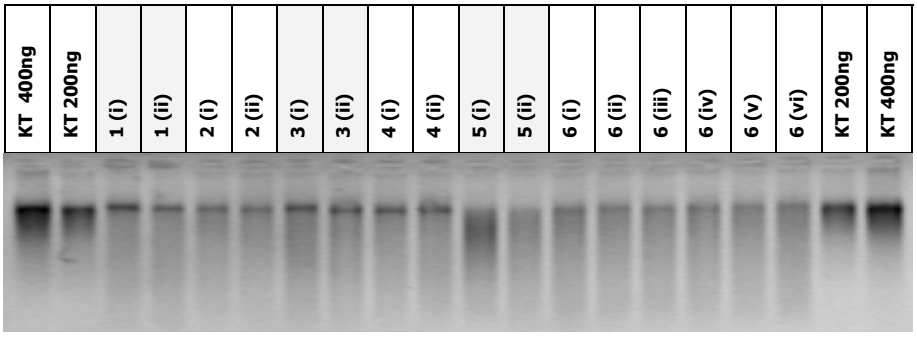
Overall average of all samples:	50 ng/µl
Average (sample no. 6, sixfold extraction):	45.4 ng/µl
Standard deviation (sample no. 6, sixfold extraction):	3.1 ng/µl
Coefficient of variation (sample no. 6, sixfold extraction):	7%

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

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.

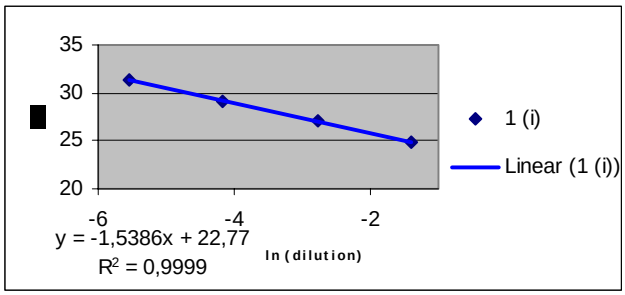
4.3. Purity / absence of PCR-inhibitors

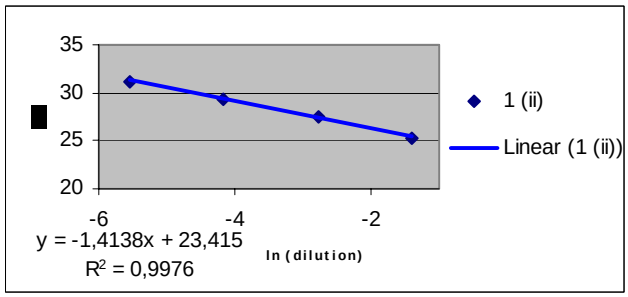
In order to assess the PCR quality of the extracted DNA fourfold serial dilutions of each extract were prepared with 0,2x TE buffer (1:4, 1:16, 1:64, 1:256) and analyzed using a maize specific Realtime PCR-system. To measure inhibition, the Ct values of the four diluted samples were plotted against the 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.

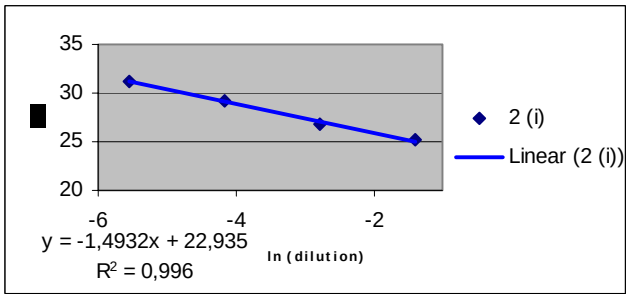
Ct values of undiluted and fourfold serial diluted DNA extracts:

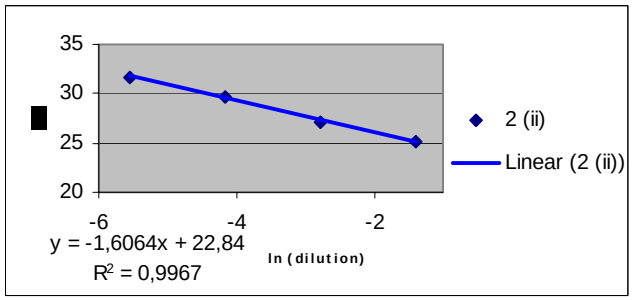
	undiluted	diluted			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1 (i)	22,73	24,89	27,04	29,20	31,28
1 (ii)	23,19	25,26	27,51	29,29	31,20
2 (i)	23,15	25,14	26,83	29,23	31,24
2 (ii)	22,97	25,08	27,16	29,75	31,64
3 (i)	22,91	25,03	27,02	29,00	30,96
3 (ii)	22,30	24,92	27,04	29,12	31,30
4 (i)	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

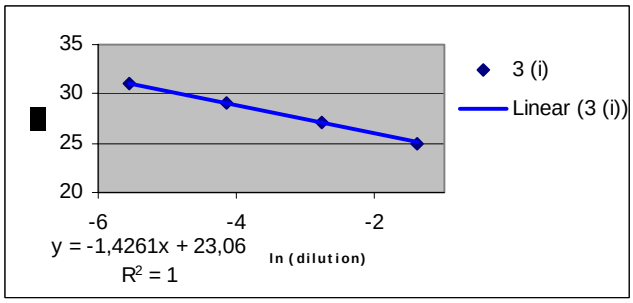
Plot of the Ct values of the four diluted samples against the natural logarithm of the dilution:

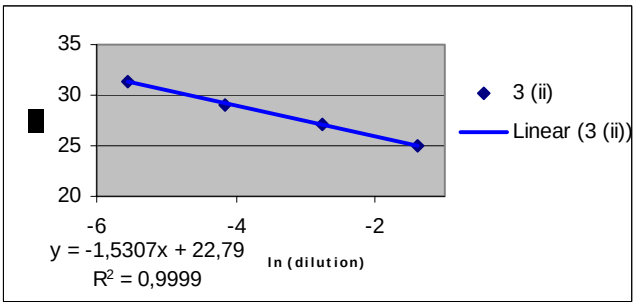


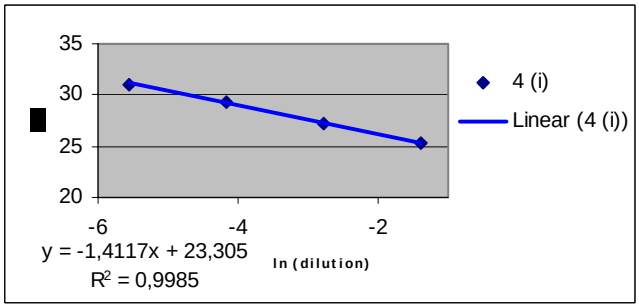


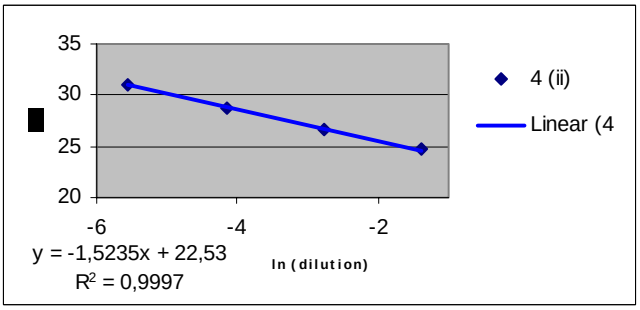


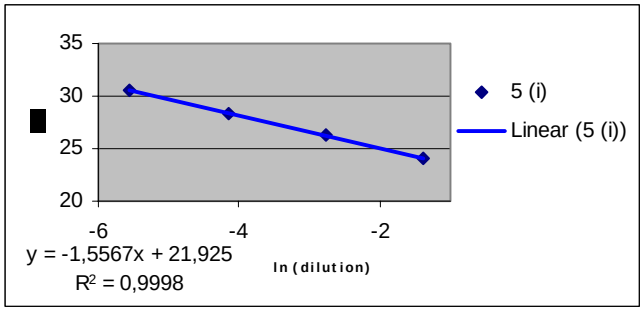








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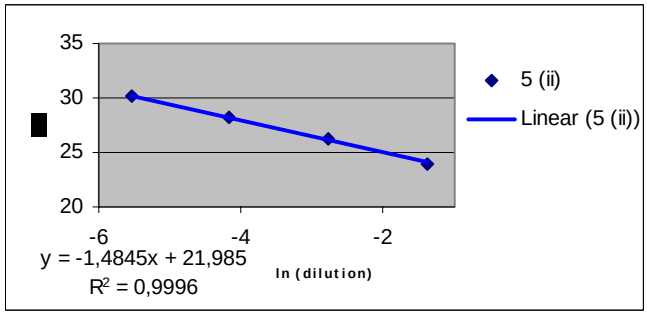
30
25
20
-6
-4
-2
5 (i)
Linear (5 (i))
y = -1,5567x + 21,925
R² = 0,9998
ln (dilution)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Linear (5 (ii))
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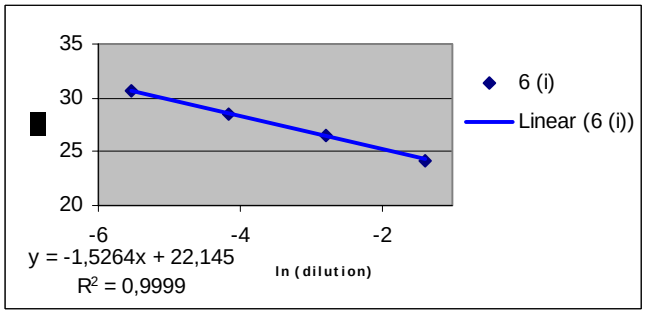
R² = 0,9996
ln (dilution)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Linear (6 (i))
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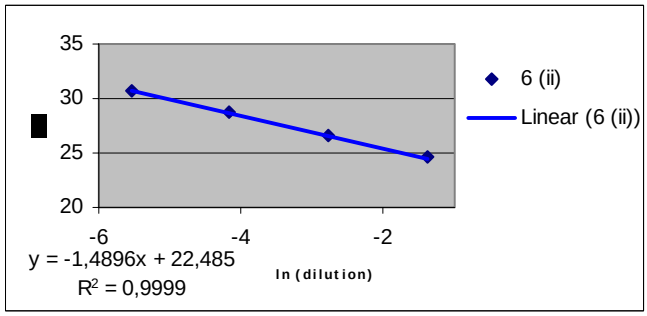
R² = 0,9999
ln (dilution)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Linear (6 (ii))
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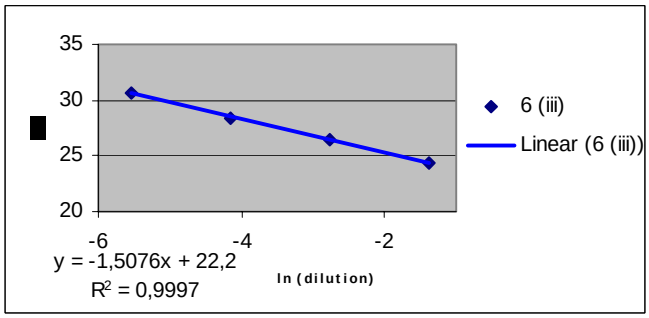
R² = 0,9999
ln (dilution)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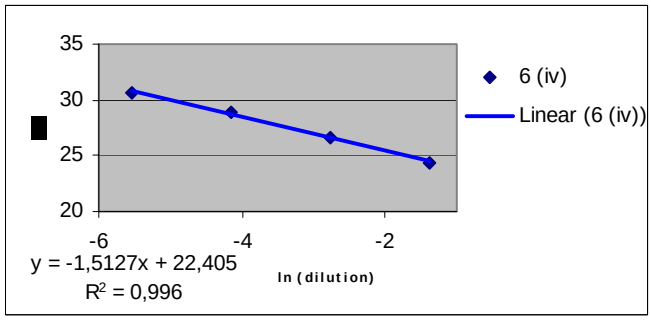
ln (dilution)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6 (iv)
Linear (6 (iv))
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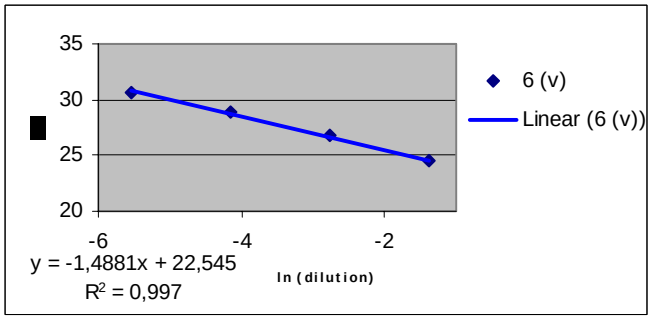
R² = 0,996
ln (dilution)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30
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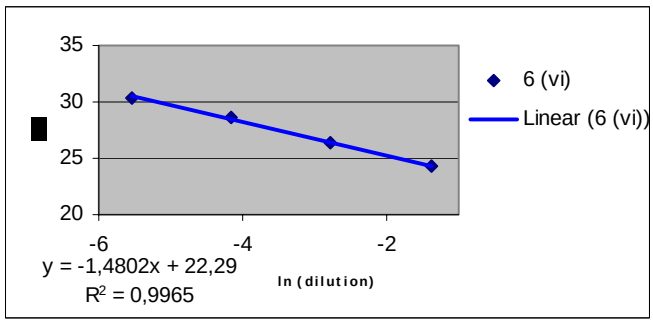
20
-6
-4
-2
6 (v)
Linear (6 (v))
y = -1,4881x + 22,545
R² = 0,997
ln (dilution)


35
30
25
20
-6
-4
-2
6 (vi)
Linear (6 (vi))
y = -1,4802x + 22,29
R² = 0,9965
ln (dilution)

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.

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 TC1507 (0% - 100% GM) maize line. The extraction method was also tested on different TC1507 flour concentration levels prepared in laboratory (0.5% - 2.5% - 5% GM content (w/w).
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 0% and 100% TC1507 maize seeds were provided by the applicant.
Tests were carried out by two analysts.

5.1 Preparation of samples

About 300 g of the transgenic TC1507 maize seeds and about 400 g of the non-transgenic TC1507 maize seeds were grinded using the GRINDOMIX mixer.

Different TC1507 concentration levels were prepared as follows:

- the 5% was prepared by mixing with the Grindomix 400g 0% TC1507 and 100g of 100% TC1507.
- The 0.5%, 2.5% were directly prepared as test portion (10g) by weighting the necessary quantity of 0% and the 5%: 1g of 5% TC1507 + 9 g of 0% TC1507 and 5 g of 5% TC1507 + 5 g 0% TC1507.

5.1 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

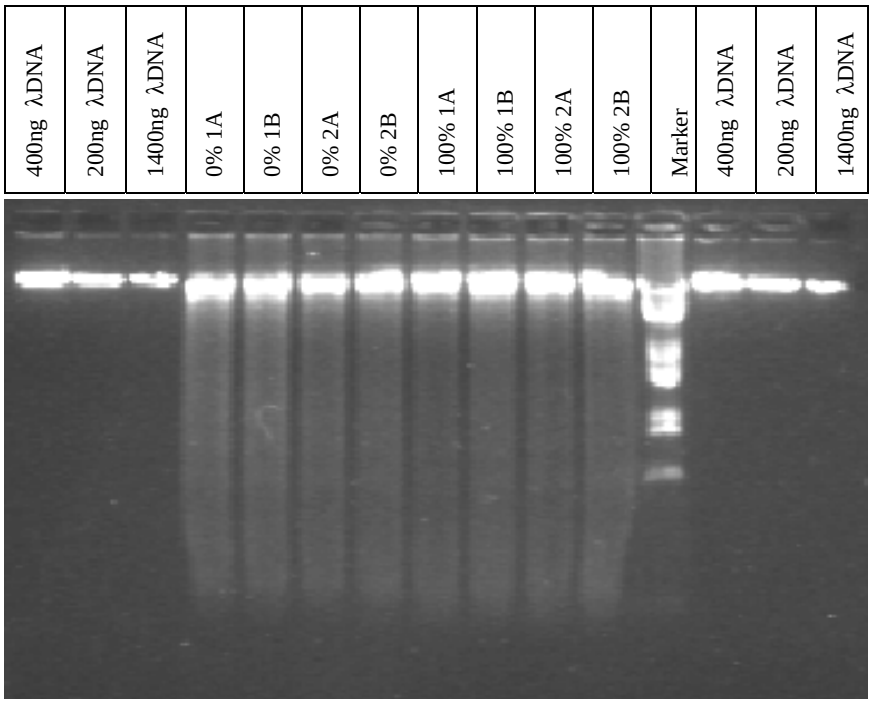
with the Wizard columns was not centrifuged and transferred to a new tube; the purification was carried out on the whole precipitated DNA.

Analyst 1:
Two test portions of four different concentration levels (0%-1; 0%-2; 0.5%-1; 0.5%-2; 5%-1; 5%-2; 100%-1; 100%-2) were extracted.
Each sample was extracted in duplicate (A, B): after cell lysis with the CTAB buffer, two aliquots of 1 ml were transferred to two Eppendorf tubes.

Analyst 2:
Three test portions (1, 2 , 3) of three different concentration levels (0.5%; 2.5%; 5%) were extracted.

5.2 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis.
5µl of the DNA solution were analyzed on a 1.5% agarose gel.
Defined amounts of λDNA were loaded as DNA quantity standards (400-200-100ng total DNA).



High molecular weight was observed

5.3 DNA concentration / Yield, Repeatability

DNA concentration of the DNA extracted was determined by spectrophotometer measurement, following the instructions included in the protocol provided.
The DNA quantification for all samples (analyst 1 and 2, test portion 1, 2 and 3, replicate A and B) are reported in the table below.

Sample	Concentration (ng/µl)	Absorption 260/280
0 1A	105	1.76
0 1B	104	1.75
0 2A	102	1.12
0 2B	89	1.65
0.5 1A	95	1.68
0.5 1B	107	1.62
0.5 2A	118	1.60
0.5 2B	113	1.57
0.5 1	85	1.72
0.5 2	103	1.69
0.5 3	84	1.68
2.5 1	83	1.71
2.5 2	71	1.67
2.5 3	83	1.67
5 1A	84	1.48
5 1B	79	1.70
5 2A	73	1.67
5 2B	118	1.61
5 1	95	1.08
5 2	72	1.59
5 3	79	1.66
100 1A	113	1.67
100 1B	107	1.68
100 2A	100	1.74
100 2B	104	1.79

Note: Samples extracted by the analyst 1 in grey-shaded cells, in white cells samples extracted by the analyst 2.

DNA concentration (ng/µl)

Overall average of all samples:	94.6 ng/µl
Standard deviation	14.6 ng/µl
Average analyst 1	100.6 ng/µl
Average analyst 2	84 ng/µl

Yield (total volume of DNA solution: 100 µl)

Overall average of all samples:	9.5 µg
Standard deviation	1.5 µg
Average analyst 1	10 µg
Average analyst 2	8.4 µg

5.4 Purity / Absence of PCR inhibitors

In order to assess the PCR quality of the DNA extracted, four dilutions of each extraction were prepared with 0.2 TE buffer. The 0% and 100% TC1507 were diluted as follows by the analyst 1: 1:5, 1:25, 1:125, 1:625 dilutions; all the remaining TC1507 concentration levels were diluted 1:4, 1:16, 1:64 and 1:256 and analyzed, in duplicate, in a qualitative PCR run on the real-time PCR equipment.
The HMG maize specific system, used as a reference gene in the Real Time quantification method provided by the applicant, was used.
ΔCt (difference between the measured and expect Ct for 1:5 and 1:4 serial dilutions), slope and the R^2 of the curve derived by plotting the log concentration of each dilution against the corresponding measured Ct, were used as a parameter to evaluate the DNA quality.

Ct values of undiluted and fivefold serial diluted DNA extracts (analyst 1):

DNA extract	Mean Ct 1:1	ΔCt* Mean Ct 1:5	ΔCt* Mean Ct 1:25	ΔCt* Mean Ct 1:125	ΔCt* Mean Ct 1:625	Slope**	R^2
0%-1A		2.00	2.28	1.94	2.42		
	22.55	24.55	26.84	28.79	31.22	-3.08	0.99
0%-1B		2.94	1.76	2.44	2.46		
	21.87	24.81	26.57	29.01	31.48	-3.35	0.99
0%-2A		2.71	2.53	1.87	2.34		
	22.17	24.89	27.42	29.30	31.64	-3.34	0.99
0%-2B		2.33	2.34	2.64	1.99		
	22.34	24.67	27.01	29.65	31.64	-3.37	0.99
100%-1A		2.33	2.08	2.17	3.15		
	22.42	24.75	26.83	29.01	32.16	-3.40	0.99
100%-2B		2.49	1.89	2.10	3.22		
	22.42	24.91	26.80	28.90	32.12	-3.34	0.98

*The expected ΔCt value for 1:5 dilutions is 2.32.
**The expected Slope for a PCR with 100% efficiency is -3.32

Ct values of undiluted and fourfold serial diluted DNA extracts:

DNA extract	Mean Ct 1:1	ΔCt* Mean Ct 1:4	ΔCt* Mean Ct 1:16	ΔCt* Mean Ct 1:64	ΔCt* Mean Ct 1:256	Slope**	R^2
0.5%-1A		1.96	1.84	2.14	1.89		
	22.48	24.44	26.28	28.42	30.31	-3.26	0.99
0.5%-1B		2.88	1.44	2.17	1.63		
	21.83	24.72	26.17	28.35	29.98	-3.30	0.98
0.5%-2A		2.15	2.39	2.00	1.92		
	21.87	24.02	26.42	28.43	30.35	-3.55	0.99
0.5%-2B		2.00	1.87	2.68	1.8		
	21.96	23.96	25.83	28.51	30.31	-3.53	0.99
0.5%-1		2.05	2.28	1.91	1.95		
	22.41	24.46	26.74	28.65	30.60	-3.41	0.99
0.5%-2		2.08	1.79	1.90	1.65		
	23.18	25.26	27.05	28.95	30.60	-3.60	0.99
0.5%-3		2.54	1.88	2.45	1.77		
	22.15	24.69	26.57	29.02	30.79	-3.53	0.99
2.5%-1		1.90	2.13	2.40	1.94		
	22.51	24.41	26.54	28.94	30.88	-3.53	0.99
2.5%-2		1.77	1.92	2.10	2.14		
	23.03	24.80	26.72	28.82	30.96	-3.30	0.99
2.5%-3		2.41	1.63	1.84	1.77		
	22.66	25.07	26.70	28.54	30.31	-3.11	0.99
5%-1A		2.05	2.11	1.88	2.28		
	22.32	24.38	26.48	28.37	30.65	-3.43	0.99
5%-1B		1.74	2.00	2.42	1.74		
	22.62	24.37	26.37	28.79	30.53	-3.36	0.99
5%-2A		1.99	1.97	1.84	2.14		
	22.95	24.93	26.91	28.75	30.89	-3.27	0.99
5%-2B		2.54	1.85	1.88	2.12		
	21.81	24.36	26.20	28.09	30.21	-3.41	0.99
5%-1		1.67	1.69	1.97	2.18		
	23.09	24.76	26.45	28.42	30.60	-3.10	0.99
5%-2		2.38	1.83	1.93	1.83		
	22.79	25.17	27.00	28.93	30.76	-3.27	0.99
5%- 3		1.70	2.55	2.01	1.94		
	22.70	24.40	26.95	28.96	30.90	-3.48	0.99

*The expected ΔCt value for 1:4 dilutions is 2.
**The expected Slope for a PCR with 100% efficiency is -3.32
Note: Samples extracted by the analyst 1 in grey-shaded cells, in white cells samples extracted by the analyst 2.

6. Conclusion

The data show that the method is 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
PCR	polymerase chain reaction
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
TAE	tris-acetate
KT	calf thymus
TE	tris EDTA