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INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Sampling and DNA Extraction of Potato

Report from the Validation of a "CTAB / Microspin" Method for DNA extraction from Freeze-dried Potato Tubers

Method development and single laboratory validation:
BASF Plant Science Holding GmbH

Method testing and confirmation:
Joint Research Centre – European Commission
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Contents

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1. Introduction

Purpose of the DNA extraction procedure described is to serve as a method to provide DNA for subsequent PCR-based detection methods. The method should yield DNA of sufficient quality and quantity and suitable for routine use in terms of ease of operations, sample throughput and costs. This Report describes the method and the validation experiments including results.

These protocols are recommended to be executed by skilled laboratory personal, the procedures comprise working with hazardous chemicals and materials. It is advised to take notice of the safety recommendations and guidelines.

2. Description of the methods

Sampling:

The method for sampling will follow the Commission's Recommendation on technical guidance for sampling and detection of genetically modified organisms (2004/787/EC).

For details concerning requirements of particle size/particle number, official ISTA guidelines (International Rules for Seed Testing, 1999, Volume 27, Supplement, Rules, 1999.) and/or sampling according to prCEN/TS 21568 (2005 - Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Sampling strategies) are proposed.

Scope and applicability:

The “CTAB/Microspin” 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 freeze-dried potato tubers. 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/Microspin” 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. Subsequently remaining inhibitors are removed by a gel filtration step using the commercially available product S-300 HR Microspin Columns (Amersham Pharmacia).

Note:

Milling/grinding of tubers 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. prCEN/TS 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 15 ml CTAB buffer and 60 µl Proteinase K into 50 ml conical tube.
2. Weigh out 3 g of freeze-dried homogenised potato tubers into the tube containing CTAB buffer and Proteinase K and mix thoroughly.
3. Incubate for 2-4 hours at 65 °C with agitation.
4. Spin down at room temperature for 10 min at 2700-3000 x 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 20000 x g.
8. Transfer 900 µl of the supernatant to a 2 ml microcentrifuge tube containing 600 µl chloroform.
9. Vortex, centrifuge at room temperature for 10 minutes at approximately 20000 x g.
10. Transfer 625 µl of the upper phase to a 1.5 ml microcentrifuge tube containing 500 µl isopropanol and 2µl glycogen (glycogen is pipetted into the lid, do not combine the glycogen and the isopropanol[1]).

[1] In order to ensure efficient working of the glycogen as a carrier for nucleic acid precipitation

11. Mix completely (invert tubes several times) and let stand at room temperature for 30 minutes to allow precipitation to form.
12. Centrifuge at room temperature for 10 minutes at approximately 20000 x *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 20000 x *g*.
15. Carefully remove and discard the supernatant. Centrifuge again and remove remaining ethanol. If any fluid remains, allow the pellet to dry at room temperature.
16. Resuspend the pellet in 100 µl 0.2 TE buffer. Let stand for 15 minutes at room temperature with occasional mixing. Make sure that the pellet is dissolved, then centrifuge for 2 minutes at approximately 20000 x g and transfer the supernatant into fresh 1.5 ml microcentrifuge tube.

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

1. Label fresh 1.5 ml microcentrifuge tube using permanent marker.
2. Vortex MicroSpin microcolums to homogenize the gel filtration material.
3. 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.
4. Place the MicroSpin microcolumn in a 2 ml microcentrifuge tube and centrifuge for 2 minutes at 735 x g.
5. Place the MicroSpin microcolumn in a 1.5 ml microcentrifuge tube and pipette DNA solution onto the centre of the MicroSpin microcolumn.
6. Centrifuge at room temperature for 2 minutes at 735 x g.
7. Discard the MicroSpin Microcolumn. The purified DNA solution is collected in the bottom of the 1.5 ml 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 – 20 µl. 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 50 ml 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

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
S-300 HR MicroSpin Columns (50)	Amersham Pharmacia

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

CTAB buffer

1,4 M NaCl

2% (w/v) CTAB

0,1 M Tris-Base

0,015 M EDTA

For 1 litre 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 800 ml H_2O_{deion}. Adjust pH with HCl to pH 8,0, stir until all reagents are dissolved. Adjust volume to 1 litre 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 0,01 M 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 of H_2O.

For 5 ml glycogen solution dissolve 100 mg glycogen in 5 ml $H_2O_{deion.}$

Store at - 20°C for up to 5 years.

75% (v/v) Ethanol

For 200 ml combine 150 ml 100% ethanol with 50 ml H_2O_{deion}.

Store at room temperature for up to 5 years.

1x TE buffer

10 mM Tris pH 8,3

1 mM EDTA

For 100 ml 1x TE buffer combine 1 ml 1M Tris (pH 8,3) and 200 µl 0,5 M 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

2 mM 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.

3.3. Plasticware

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

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

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

For experimental validation six different samples of freeze-dried potato tubers 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 the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Suitable dilutions of each DNA extract were prepared in triplicates and mixed with PicoGreen reagent. DNA concentration was determined on the basis of a five-point standard curve (triplicates) ranging from 20 ng/µl to 1000 ng/µl using an ABI-PRISM® 7700 as a detector.

Potato line	Replicate	Concentration [ng/µl]	Mean (ng/µl)
EH92-527-1	I	155,4	143,6
	II	131,8	
Prevalent	I	101,2	109,6
	II	142,4	
	III	89,4	
	IV	105,3	
	V	104,4	
	VI	114,5	
Kuras	I	118,7	116,7
	II	114,7	
Dinamo	I	144,2	141,0
	II	137,7	
Sibu	I	131,6	139,2
	II	146,8	
Seresta	I	151,4	145,8
	II	140,2	

DNA concentrations:

Mean of 5 duplicates of 5 different lines:	137.3 ng/µl (total yield: 13.73 µg)
Mean of "Prevalent", sixfold extraction:	112 ng/µl (total yield: 10.96 µg)
Standard deviation of "Prevalent" sixfold extraction:	18.0 ng/µl
Relative standard deviation of sixfold extraction:	16.5%

4.2. Fragmentation state of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis: 5 µl of each DNA solution were 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.

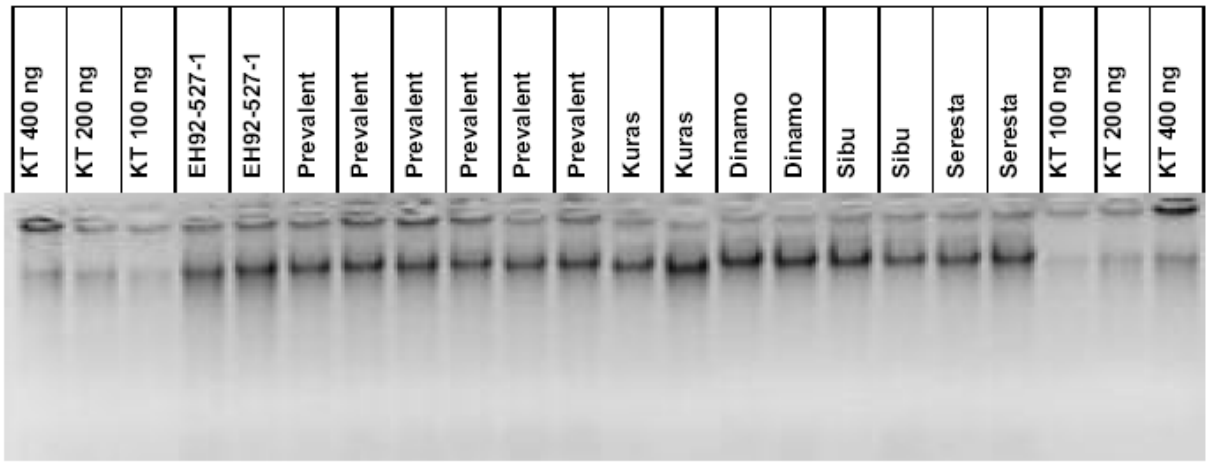


Figure 1. Agarose gel electrophoresis of DNA extract

High rate of 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 potato species 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. PCR inhibitors are 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:

DNA extract	Replicate	undiluted	diluted		
		1:1	1:4	1:16	1:64
EH92-527-1	I	14,42	16,39	18,35	20,04
	II	14,03	15,94	18,07	19,75
Prevalent	I	14,42	16,16	18,22	20,39
	II	14,26	16,31	18,28	20,45
Kuras	I	14,19	16,10	17,78	19,97
	II	14,08	16,19	18,14	20,26
Dinamo	I	14,01	15,96	18,10	19,83
	II	14,31	16,10	17,93	20,18
Sibu	I	14,15	16,41	18,18	20,39
	II	14,71	16,51	18,50	20,24
Seresta	I	14,46	16,31	18,47	20,31
	II	14,47	16,24	18,09	20,48

Comparison of extrapolated Ct values versus measured Ct values:

DNA extract	Replicate	CT extrapol.	CT experim.	delta CT
EH92-527-1	I	14,34	14,42	**0,08**
	II	13,74	14,03	**0,30**
Prevalent	I	14,21	14,42	**0,22**
	II	14,26	14,26	**0,00**
Kuras	I	14,22	14,19	**0,02**
	II	14,12	14,08	**0,04**
Dinamo	I	13,88	14,01	**0,13**
	II	14,03	14,31	**0,29**
Sibu	I	14,37	14,15	**0,22**
	II	14,51	14,71	**0,21**
Seresta	I	14,19	14,46	**0,27**
	II	14,21	14,47	**0,27**

delta Ct = abs (Ct extrapolated - Ct measured)

Delta Ct < 0.5 in 12 of 12 test series were observed; the data observed suggest the absence of PCR inhibitors.

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/Microspin" DNA extraction method proposed by the applicant on the potato materilal. 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 experimental testing was carried out on freeze-dried potato tubers provided by the applicant.

5.1 DNA extraction

DNA was extracted by means of the the CTAB/Microspin" method described above and in-house validated by the applicant.
The DNA extraction was carried out on 6 test portions (replicates) and repeated in three different days, giving a total of 18 DNA extracts.

5.2 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.

Considering that the expected DNA concentration of the samples was close to the upper limit of the range, samples were diluted 1:1 prior to measurement.

The DNA quantification for all samples (yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2, blue boxes for samples extracted on day 3) is reported in the table below.

Sample	Concentration (ng/µl)
1	102,7
2	121,5
3	122,8
4	117,4
5	116,7
6	95,8

1	146,6
2	149,1
3	166,9
4	126,9
5	150,6
6	122,7
1	150,8
2	124,9
3	136,4
4	148,8
5	144,3
6	122,8

DNA concentration (ng/μl)

Overall average of all samples:	131.5 ng/μl
Standard deviation of all samples	18.7 ng/μl
Coefficient of variation	14.2%

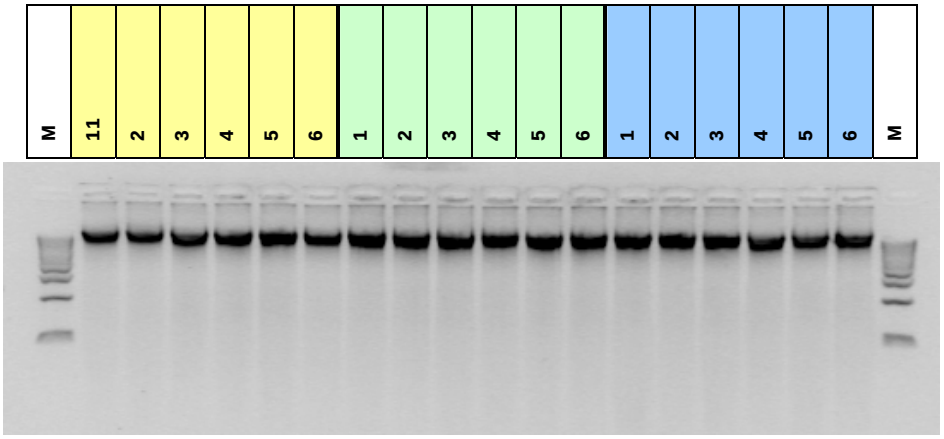
Yield (total volume of DNA solution: 100 μl)

Overall average of all samples:	13 μg
Standard deviation	1.87 μg
Coefficient of variation	14.2%

5.3 Fragmentation state of DNA

The size and fragmentation 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 yellow, green and blue boxes, samples from 1 to 6 extracted on days 1, 2 and 3 respectively.

A DNA ladder 1kb (M) was used.



High molecular weight DNA was observed.

5.4 Purity / Absence of PCR inhibitors

To assess the PCR quality of the DNA extracted, the experimental approach previously described (see paragraph 4.3) was followed. In yellow, green and blue boxes, samples from 1 to 6 extracted on days 1, 2 and 3 respectively.

Ct values of undiluted and fourfold serial diluted DNA extracts

	Undiluted (40 ng/µl)	diluted			
DNA extract	1:1	1:4	1:16	1:64	1:256
1	15.28	17.26	19.50	21.82	23.89
2	15.51	17.73	19.78	22.07	24.33
3	15.40	17.31	19.37	21.34	23.63
4	15.55	17.50	19.58	21.72	23.90
5	15.53	17.44	19.55	21.94	23.98
6	15.60	17.45	19.65	21.81	24.03
1	14.30	16.31	18.28	20.11	22.28
2	14.57	16.44	18.35	20.45	22.77
3	14.67	16.36	18.25	20.14	22.32
4	14.52	16.43	18.31	20.21	22.33
5	14.58	16.37	18.39	20.31	22.52
6	14.39	16.19	18.23	20.47	22.33
1	14.47	16.34	18.20	20.40	22.54
2	14.32	16.19	18.08	20.42	22.38
3	14.42	16.09	17.99	20.23	22.13
4	14.54	16.47	18.45	20.70	22.61
5	14.54	16.39	18.52	20.63	23.03
6	14.22	16.19	18.33	20.58	22.58

Note: the PCR quality of the samples number 1 and 4 in the yellow boxes (n.a.), was not assessed due to the low DNA yield obtained after extraction. However it cannot be asserted that the DNA of these samples is not suitable for real-time analysis

Comparison of extrapolated Ct values versus measured Ct values:

DNA extract	R^2	Slope*	Ct extrapolated	C_T measured	delta Ct**
1	0.9993	-3.691	15.06	15.28	-0.22
2	0.9979	-3.670	15.45	15.51	-0.06
3	0.9989	-3.476	15.17	15.40	-0.23
4	0.9986	-3.545	15.33	15.55	-0.21
5	0.9975	-3.657	15.22	15.53	-0.31
6	0.9990	-3.639	15.25	15.60	-0.35

1	0.9982	-3.270	14.32	14.30	0.02
2	0.9966	-3.502	14.23	14.57	-0.35
3	0.9965	-3.284	14.32	14.67	-0.35
4	0.9989	-3.258	14.41	14.52	-0.11
5	0.9978	-3.380	14.30	14.58	-0.28
6	0.9984	-3.434	14.13	14.39	-0.26
1	0.9976	-3.454	14.17	14.47	-0.30
2	0.9976	-3.473	14.04	14.32	-0.28
3	0.9983	-3.385	14.01	14.42	-0.41
4	0.9985	-3.436	14.38	14.54	-0.16
5	0.9986	-3.662	14.13	14.54	-0.41
6	0.9982	-3.557	14.07	14.22	-0.15

***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 are < 0.5

6. Conclusion

The data reported show that the method is fit for the intended purpose.

7. 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