



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



Sampling and DNA Extraction of Rice

Report on the Validation of a "Dellaporta-Derived" Method for DNA Extraction from Ground Rice Grains/Seeds

Method development and single laboratory validation:

Bayer CropScience

Method testing and confirmation:

Joint Research Centre – European Commission

Biotechnology & GMOs Unit

Community Reference Laboratory for GM Food and Feed

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1. Introduction

The 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 should result 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 rice LLRICE62, the Applicant refers to the technical guidance documents and protocols described in:

- International Organization for Standardizations, Switzerland: ISO standard 6644, ISO standard 13690, ISO standard 5725;
- International Rules for Seed Testing (2004) International Seed Testing Association (ISTA), Switzerland. ISBN 3-906549-38-0;
- USDA-GIPSA (2001) Sampling grains for the detection of Biotech grains <http://www.usda.gov/gipsa/>.

Scope and applicability

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

Principle

The basic principle of the 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 method starts with a lysis step (thermal lysis in the presence of 2-mercaptoethanol and EDTA) followed by two precipitations with potassium acetate and isopropanol. Afterwards removal of RNA by digestion with RNase A and contaminants, such as lipophilic molecules and proteins, by extraction with phenol:chloroform:isoamylalcohol are performed. This first crude extract is subsequently re-suspended and precipitated with isopropanol and washed with ethanol. The DNA is purified using the "DNA Clean & Concentrator"™ - 25 (ZYMO RESEARCH CORP.).

Seed crushing procedure

When starting from intact rice grain/seed samples instead of finely ground rice 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.

The seeds are crushed using a Waring blender; grind in intervals of 10 seconds for 4 times at maximum speed. Shake between intervals until all power is loose to improve crushing procedure; all seeds should be crushed until a fine powder is obtained. Thorough grinding will also produce a homogenous powder; prevent cross-contamination between the samples by dust particles.

DNA extraction procedure

Note:

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.

1. Transfer 10 g powder into a 250 ml centrifuge bucket
2. Add 100 ml Extraction Buffer
3. Add 7 ml 20% SDS, mix well by inversion
4. Incubate at 65°C for 40 minutes
Note: Mix samples every 10 minutes by inversion
5. Centrifuge 10 minutes at 10,000 x g
6. Filter the supernatant over a Miracloth filtration membrane
7. Transfer 30 ml filtered supernatant to a new 50 ml Falcon tube
8. Add 9 ml 5M KAc, shake vigorously for 1 minute
9. Incubate on ice for 30 minutes
Note: Mix samples every 10 minutes by inversion
10. Centrifuge for 20 minutes at 3,000 x g
11. Transfer 25 ml supernatant to a new 50 ml Falcon tube using a 25 ml pipette
12. Add 20 ml isopropanol, mix gently for 1 minute
13. Incubate on ice for 5 minutes
14. Centrifuge for 20 minutes at 3,000 x g
15. Remove supernatant and air-dry the pellet at 37°C until all isopropanol residue is evaporated
16. Dissolve the pellet in 1 ml TE
17. Transfer the DNA solution to a new 2.0 ml Eppendorf tube
18. Add 10 µl RNase A (10 mg/ml), mix gently and incubate for 20 minutes at 37°C
19. Add 800 µl phenol:chloroform:isoamylalcohol (25:24:1)
20. Mix well for 1 minute
21. Centrifuge for 10 minutes in a micro centrifuge at maximum speed
22. Transfer the upper aqueous phase (900 µl) to a new 2 ml Eppendorf tube. Do not disturb the interphase
23. Add 800 µl chloroform
24. Mix well for 1 minute
25. Centrifuge for 10 minutes in a micro centrifuge at maximum speed

26. Transfer the upper aqueous phase (800 µl) to a new 2 ml Eppendorf tube containing 90 µl 3M NaAc. Do not disturb the interphase
27. Add 600 µl isopropanol
28. Mix gently by inversion for 1 minute
29. Centrifuge for 1 minute in a micro centrifuge at maximum speed to pellet the DNA
30. Remove all supernatant
31. Add 1 ml 70% ethanol to wash the DNA pellet. Make sure the DNA pellet is not stuck on the bottom. Shake the samples for 1 hour
32. Centrifuge for 5 minutes in a micro centrifuge at maximum speed
33. Remove supernatant and air-dry the pellet at 37°C until all ethanol residue is evaporated
34. Add 100 µl TE0.1 to the DNA pellet
35. Store samples over night at 4°C
36. Shake samples for 1 hour
37. Centrifuge for 1 minute in a micro centrifuge at maximum speed
38. Purify the DNA samples using the DNA Clean & Concentrator™-25 kit according to the manufacturer s instructions
39. Elute the DNA from the column twice using two times 50 µl TE0.1

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
Waring blender	e. g. model 7010S/7010G/7010HS/7010HG
70mm Blender Base for Waring blender or equivalent	e. g. Eberbach Corp. Cat.No.
Centrifuge with rotors for 250 ml centrifuge tubes and microcentrifuge tubes	e. g. Sorvall RC-5B Superspeed Centrifuge with SLA-1500 rotor (or equivalent)
Micro centrifuge with 18,000 x g for Eppendorf tubes	
Mira cloth filtration membrane	e. g. Calbiochem Cat.No. 475855
Table centrifuge with 3000 x g for Falcon tubes	With swinging buckets
Water bath adjustable to 65°C 1°C	Promega Vac-Man® Laboratory Vacuum Manifold. 20-sample capacity
UV spectrophotometer for DNA quantitation	

3.2. Reagents

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

Reagent	Specification
NaCl	Duchefa Cat.No. S0520
CTAB	p. a. quality or Molecular biology grade
Tris-HCl	USB Cat.No. 22676
EDTA · Na ₂ -salt	Titriplex III (Merck Cat.No. 1.08418.1000)
RNase A	Roche Cat.No. 0109-142
Sodium acetate	Merck Cat.No. 1.06268.1000
Potassium acetate	Merck Cat.No. 1.04820.1000
Sodium Dodecyl Sulphate (SDS)	BDH Cat.No. 442444H
2-mercaptoethanol	Sigma Cat.No. M6250
Chloroform	p.a. (Merck Cat.No. 1.02445.2500)
Isopropanol	p.a. (Merck Cat.No. 1.09634.2500)
Phenol:chloroform:isoamylalcohol (25:24:1)	Sigma Cat.No. P-3803
Ethanol	p.a. (Merck Cat.No. 1.00983.1000)
DNA Clean & Concentrator™ 25	Zymo Research Cat.No. D4005

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

Extraction buffer

100 mM Tris HCl pH8
 50 mM EDTA pH8
 500 mM NaCl
 10 mM 2-mercaptoethanol

RNase A

10 mg/ml

1x TE buffer

10 mM Tris pH 8.3
 1 mM EDTA pH 8.3

0,1x TE buffer

10 mM Tris pH 8.3

0.1 mM EDTA pH 8.3

SDS 20%

70% (v/v) Ethanol

Potassium acetate (KAc) 5 M

Sodium acetate (NaAc) 3 M

3.3. Plasticware

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

Item
250 ml centrifuge bucket
50 ml conical tubes
1,5 ml microcentrifuge tube
2 ml microcentrifuge tube

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

Experimental data is provided from an in-house validation in which the method module has been successfully applied to the relevant matrix in the context of the application for authorization. Four samples of 100 g rice seed were ground to fine powders, and 20 - 30 mg sub-samples flour were taken from each ground sample for DNA extractions. The procedure was performed three times under repeatability conditions (within short intervals of time (i.e. days) by the same operator, using the same equipment) resulting in 12 DNA samples.

4.1 DNA extraction efficiency

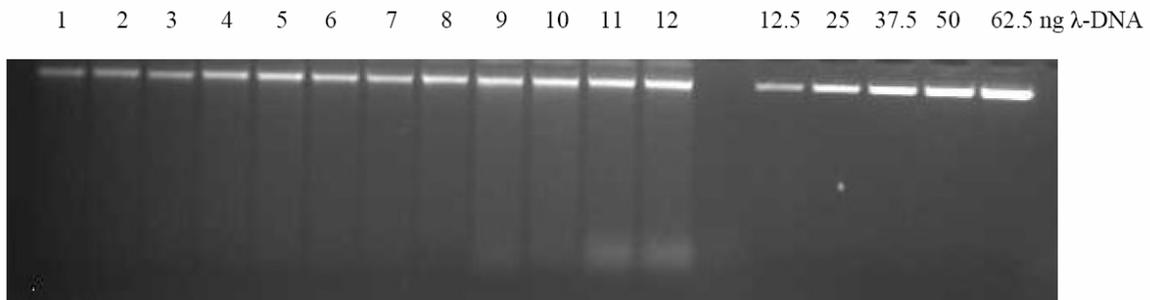
The concentration of the extracted DNA was determined spectrophotometrically (Ultrospec 2000, Pharmacia Biotech). 20 μ l of the extracted DNA was diluted 1:5 in MQ water. Absorption was measured for both blank (TE 0.1 diluted in MQ water) and diluted DNA solutions at 260nm. DNA concentration was calculated based on the assumption that an OD of 1 corresponds to 50 μ g/ml DNA.

The average DNA efficiency was 10.8 ± 2.7 μ g DNA / 10 g meal.

DNA extraction	Meal mass (gram)	[DNA] (ng/ μ l)	DNA extraction efficiency (μ g DNA / 10 gram meal)
A1	10.0	75	7.5
A2	10.0	80	8.0
A3	10.0	75	7.5
A4	10.0	145	14.5
B1	10.0	125	12.5
B2	10.0	90	9.0
B3	10.0	90	9.0
B4	10.0	160	16.0
C1	10.0	120	12.0
C2	10.0	115	11.5
C3	10.0	105	10.5
C4	10.0	120	12.0

4.2. Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed by ethidium bromide-stained 1% agarose gel electrophoresis compared to a series of standard (non restriction enzyme digested) lambda DNA references of different molecular weight. The 12 genomic DNA samples extracted as described above were apparent as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA.



4.3. Evidence of the absence of PCR inhibitory compounds

PCR inhibitory compounds in the DNA preparations were analyzed by Real-time PCR using the oligonucleotides directed to the endogenous control gene *Phospholipase D* on serial dilutions of the DNA preparations. The threshold cycle (C_T) values of a Real-time PCR analysis between the C_T values corresponding to the dilutions should match the dilution factor applied, e.g. if DNA is diluted 10X then the ΔC_T should be approx. 3.32, if the DNA is diluted 2X, the C_T should be 1, etc. Deviations from this relationship may indicate that the extracted DNA contains PCR inhibitors, or that the DNA solution is not homogenous. This relationship was used to analyze the serial dilutions of the DNA preparations for the presence of PCR inhibitory compounds by plotting the mean C_T values against the logarithm of the DNA mass, and determining the slope (PCR efficiency) and the linearity of the correlation.

A two-fold serial dilution of the extracted DNA was prepared yielding eight different amounts of DNA (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) of which the C_T values were determined in triplicate in a Real-time PCR run with the oligonucleotide primers and probe. In total, the analysis was executed three times with DNA samples extracted on different days. To analyze the data, the mean C_T values (y axis) were plotted against the logarithm of the DNA mass (x axis), and by linear regression a trend line ($y = ax + b$) was calculated, as well as a correlation coefficient, r^2 , as a measure of linearity. The ideal slope value, 'a', (optimal PCR efficiency) then becomes $a = -3.32$ (typically 'a' values between -3.1 and -3.6 indicate excellent PCR efficiencies). Correlation coefficients of $r^2 > 0.98$ indicate an excellent linear relationship, and thus, equally efficient PCR amplification over the measured dynamic range. The results of the three Real-time PCR runs are shown in the table below and show no evidence for PCR inhibitory compounds. The PCR efficiency has been calculated by the following equation: Efficiency (%) = $100 * [10^{(-1/\text{slope})}] - 1$

DNA	Slope, a	Efficiency (%)	Intercept, b	Linearity, r ²
1A	-3.4651	94.35	26.968	0.9997
1B	-3.4592	94.57	27.613	0.9986
1C	-3.4738	94.03	27.398	0.9989

5. 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 DNA extraction method proposed by the applicant. The experimental testing was carried out on samples of food and feed containing 1% of grains harboring the LLRICE62 GM event mixed with grains of the of the non-GM WT 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.

5.1 Preparation of samples

About 300 g of grains were grinded using the GRINDOMIX mixer.

5.2 DNA extraction

DNA was extracted by means of the Dellaporta-derived method described above and in-house validated by the applicant.

The DNA extraction was performed on 6 test portions (replicates) and repeated in three different days, giving a total of 18 DNA extracts.

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/ μ l using a Biorad VersaFluor fluorometer.

The DNA concentration (ng/ μ l) for all samples (yellow boxes samples from 1 to 6 extracted on 28.03.2006, green boxes samples from 1-6 extracted on 04.04.2006 and blue boxes samples extracted on 06.04.2006) is reported in the table below.

Sample	Concentration (ng/ μ l)
1	73.8
2	93.2
3	82.3
4	101.8
5	53.7
6	77.3
1	92.5
2	95.5
3	99.5
4	85.7
5	65.3
6	84.2
1	78.8
2	78.7
3	81.9
4	83.9
5	58.5
6	55.1

DNA concentration (ng/ μ l)

Overall Average of all samples	80.1 ng/ μ l
Standard deviation	14.4 ng/ μ l
Coefficient of variation	18.0%

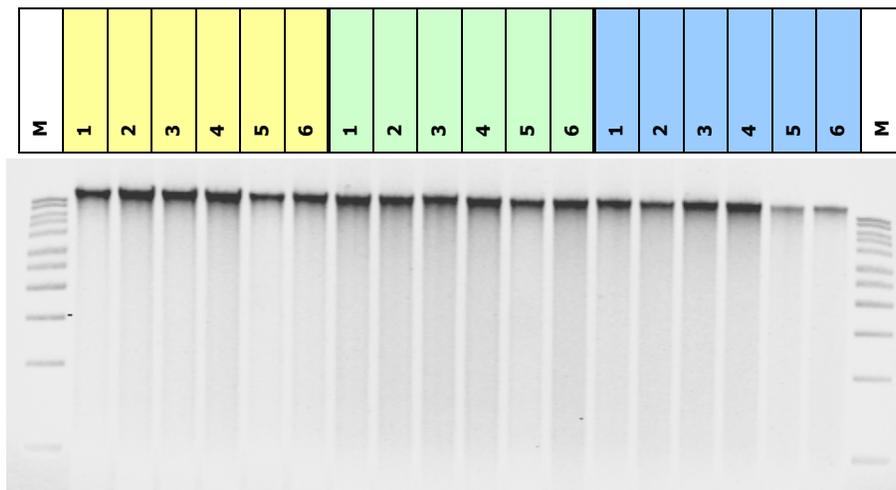
Yield (total volume of DNA solution: 100 μ l)

Overall Average of all samples	8.01 μ g
Standard deviation	1.44 μ g
Coefficient of variation	18.0 %

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. The picture of the agarose gel is shown below. Yellow boxes: samples from 1 to 6 extracted on 28.03.2006; green boxes: samples from 1-6 extracted on 04.04.2006; blue boxes: samples extracted on 06.04.2006.

A DNA ladder 1kb (M) was used.



High molecular weight DNA distribution was observed for all samples.

5.5 Purity / Absence of PCR inhibitors

In order to assess quality, in terms of suitability for PCR amplification, the DNA extracts were adjusted to a concentration of 40 ng/µl (below referred as "undiluted samples"). From these samples, fourfold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with 0,1x TE buffer.

All DNA samples were analyzed applying a real-time PCR system targeting the rice specific reference sequence *Phospholipase D* (for a validation report on the PLD system see <http://gmo-crl.jrc.it/statusofdoss.htm>).

The Ct values of the diluted 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. Subsequently the extrapolated Ct for the undiluted sample was compared with the measured Ct.

The quality of DNA is considered acceptable if difference between the extrapolated Ct value of the undiluted sample and the measured Ct value for the same sample is < 0.5.

The Ct values obtained for undiluted and diluted samples are reported in the table below (yellow boxes: samples from 1 to 6 extracted on 28.03.2006, green boxes: samples from 1 to 6 extracted on 04.04.2006; blue boxes: samples from 1 to 6 extracted on 06.04.2006).

Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of rice reference gene *Phospholipase D*

DNA extract	Undiluted (40 ng/ μ l)	diluted			
	1:1	1:4	1:16	1:64	1:256
1	18.60	20.86	22.88	24.90	26.91
2	18.80	20.65	22.71	24.82	26.83
3	18.75	20.79	22.73	24.73	26.93
4	18.79	20.65	22.67	24.42	26.48
5	18.89	20.83	22.72	24.47	26.80
6	18.77	20.75	22.72	24.49	26.46
1	18.13	20.39	22.44	24.28	26.61
2	18.44	20.49	22.38	24.51	26.71
3	18.29	20.34	22.62	24.61	26.90
4	18.36	20.40	22.53	24.45	26.48
5	18.14	20.47	22.52	24.61	26.93
6	18.42	20.45	22.59	24.69	27.19
1	18.00	20.25	21.84	24.27	26.43
2	18.11	20.09	22.05	24.50	26.29
3	17.92	19.82	22.10	24.19	26.16
4	17.91	19.86	22.01	24.10	26.46
5	18.07	20.27	22.22	23.91	26.31
6	19.14	22.08	23.56	25.41	26.85

The table below summarises the comparison of extrapolated CT values versus measured Ct values for all samples, as well as reporting the values of linearity (R^2) and slope of all measurements.

Comparison of extrapolated Ct values versus measured Ct values (amplification of rice reference gene *Phospholipase D*)

DNA extract	R^2	Slope*	C_T extrapolated	C_T measured	delta C_T^{**}
1	0.9982	-3.350	18.85	18.60	0.25
2	0.9983	-3.431	18.59	18.80	0.21
3	0.9982	-3.392	18.69	18.75	0.06
4	0.9951	-3.197	18.74	18.79	0.04
5	0.9951	-3.269	18.78	18.89	0.11
6	0.9959	-3.140	18.88	18.77	0.11
1	0.9976	-3.408	18.30	18.13	0.17
2	0.9982	-3.456	18.32	18.44	0.12
3	0.9974	-3.603	18.19	18.29	0.09
4	0.9991	-3.347	18.43	18.36	0.07
5	0.9986	-3.569	18.26	18.14	0.12
6	0.9974	-3.708	18.15	18.42	0.27
1	0.9928	-3.479	17.96	18.00	0.05
2	0.9937	-3.497	17.97	18.11	0.14
3	0.9982	-3.506	17.79	17.92	0.13
4	0.9969	-3.639	17.63	17.91	0.29
5	0.9938	-3.291	18.22	18.07	0.16
6	0.9814	-2.686	20.43	19.14	1.28

*The expected Slope for a PCR with 100% efficiency is -3.32

**delta C_T = abs (C_T extrapolated - C_T measured)

Note: yellow boxes represent samples from 1 to 6 extracted on 28.03.2006, green boxes samples from 1-6 extracted on 04.04.2006 and blue boxes samples extracted on 06.04.2006

All except one delta C_T values of extrapolated versus measured C_T < 0.5.
 R^2 of linear regression is > 0.99 for all DNA samples, except one (0.9814).

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. Abbreviations

EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
RNase A	ribonuclease A
TE	tris EDTA
Tris	tris(hydroxymethyl)aminomethane