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

# Seeds Sampling and DNA Extraction of Oilseed Rape

# Report on the Validation of an Oilseed Rape DNA Extraction Method from Seeds

**Biotechnology & GMOs Unit**
**Institute for Health and Consumer Protection**
**DG Joint Research Centre**

**Method development and single laboratory validation:**
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**Method testing and confirmation:**
European Commission, Directorate General-Joint Research Centre
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# Content

# 1. Introduction

A plant DNA extraction protocol is described here as derived from the publicly available “Dellaporta” method (1). The modified protocol can be used for extraction of DNA from seeds and grains of oilseed rape ground to powder using a mortar and pestle.

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

# 2. Description of the methods

**Sampling:**
Sampling approaches for seeds and grains are referred to in 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 “Oilseed rape seed DNA extraction protocol” method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of oilseed rape tissues and derived matrices. However, validation data presented here are restricted to ground oilseed rape seeds. Application of the method to other matrices may require adaptation and possible further specific validation.

**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 “Oilseed rape seed DNA extraction protocol” method starts with a lysis step (thermal lysis in the presence of Tris HCl, EDTA, SDS and β-mercaptoethanol) followed by removal of contaminants such as lipophilic molecules and proteins by extraction with phenol and chloroform.

A DNA precipitate is then generated by using isopropanol. The pellet is dissolved in TE-buffer. Remaining inhibitors are removed by an anion exchange chromatography step using the DNA Clean & Concentrator 25 kit (Zymo Research).

**Seed crushing procedure:**

- Collect 11 grams of seeds (+/- 3000 seeds)
- Crushed the seeds in a 200 ml mortar using a pestle
- Grind thoroughly till all seeds are pulverized and a fine powder is obtained

**Oil Seed Rape seed DNA extraction protocol**

1. Transfer 11 grams of grounded seeds into a 250 ml centrifuge bucket
2. Add 150 ml Extraction Buffer
3. Add 10.5 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 10 ml filtered supernatant to a new 15 ml Falcon tube
8. Add 3 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 30 minutes at 3.000 x *g*
11. Transfer 8 ml supernatant to a new 15 ml Falcon tube using a 10 ml pipette
12. Add 6 ml isopropanol, mix gently for one minute
13. Incubate on ice for 5 minutes
14. Centrifuge for 15 minutes in a table centrifuge at 3.000 x *g*
15. Remove supernantant 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 15 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 microcentrifuge 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 microcentrifuge 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 microcentrifuge at maximum speed to pellet the DNA
30. Remove all supernantant
31. Add 1 ml 70% ethanol to wash the DNA pellet. Make sure the pellet is not attached to the bottom. Shake the samples for 1 hour
32. Centrifuge for 5 minutes in a microcentrifuge at maximum speed
33. Remove supernatant and air-dry the pellet at 37 °C until all ethanol residue is evaporated
34. Add 100 µl TE 0.1X 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 microcentrifuge at maximum speed
38. Purify the DNA samples using the DNA Clean & Concentrator 25 kit according to the manufacturer's instruction
39. Elute the DNA from the column twice using two times 100µl TE 0.1X

# 3. Equipment / Chemicals / Plasticware

### 3.1. Equipment

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

1. 200 ml mortar and pestle
2. Sorval RC-5B Superspeed Centrifuge with SLA-1500 rotor (or equivalent) for 250 ml centrifuge buckets
3. Miracloth filtration membrane (Calbiochem Cat. No. 475855)
4. Microcentrifuge with 18.000 x g for Eppendorf tubes
5. Table centrifuge (swinging buckets) with 3000 x g for 15 ml Falcon tubes
6. Water bath adjustable to 65 °C ± 1 °C
7. UV spectrophotometer for DNA quantification

### 3.2. Chemicals

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

1. $Na_2$-EDTA; Titriplex III (Merck Cat. No. 1.08418.1000)

2. Tris-HCl; Tris(hydroxymethyl)aminomethane hydrochloride (USB Cat. No. 22676)
3. NaCl; sodium chloride (Duchefa Cat. No. S0520)
4. KAc; potassium Acetate (Merck Cat. No. 1.04820.1000)
5. NaAc; sodium acetate (Merck Cat. No. 1.06268.1000)
6. SDS; sodium dodecyl sulphate(BDH Cat. No. 442444H)
7. β-mercaptoethanol (Sigma Cat.No. M6250)
8. RNAse A (Roche Cat. No. 0109-142)
9. Ethanol p.a. (Merck Cat. No. 1.00983.1000)
10. Isopropanol p.a. (Merck Cat. No. 1.09634.2500)
11. Phenol-chloroform-isoamylalcholol (25/24/1) (Sigma Cat. No. P-3803)
12. Chloroform p.a. (Merck Cat. No. 1.02445.2500)

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

**1. Extraction buffer (Dellaporta buffer)**

- 100 mM Tris HCl pH 8.0
- 50 mM EDTA pH 8.0
- 500 mM NaCl
- 10 mM β-mercaptoethanol

**2. Tris-EDTA buffer (TE 1X)**

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

**3. Tris-EDTA buffer (TE 0.1X)**

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

**4. RNase A (10 mg/ml)**

**5. SDS 20 %**

**6. Ethanol 70%**

**7. 5M KAc**

**8. 3M NaAc** pH 5.2

### 3.3. Plasticware

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

| Item |
|---|
| 15 ml conical tubes |
| 2 ml microcentrifuge tube |
| filter tips |

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

Experimental data are provided from an in-house validation in which the analytical module has been successfully applied to the relevant matrix in the context of the application for authorisation. Four samples of 11 grams (approximately 3000 Oilseed rape seeds) were grounded to fine powders, and all flour was taken from each ground sample for DNA extraction.

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 concentration, yield, repeatability

The concentration of the extracted DNA was determined spectrophotometrically (Ultrospec 2000, Pharmacia Biotech). 20 µl of extracted DNA was diluted 1:10 in MilliQ water. Absorption was measured for both blank (TE 0.1X diluted in MQ water) and diluted DNA solutions at 260 nm.

DNA concentration was calculated based on the assumption that an OD of 1 corresponds to 50 µg/ml DNA (2).

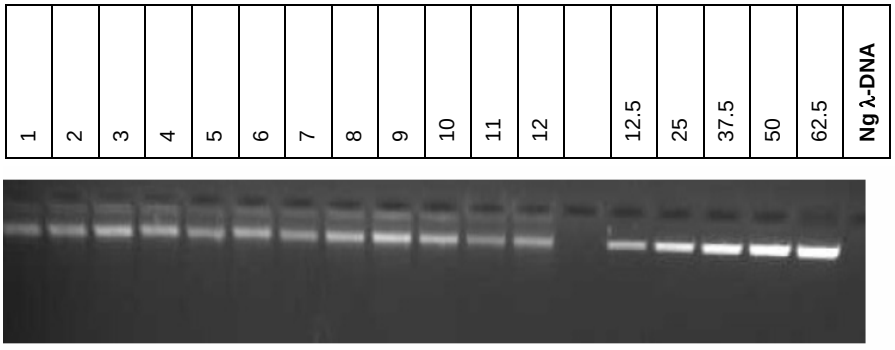
Results are summarised in the following Table:

| DNA extraction | Sample mass (gr) | [DNA] (ng/ul) | DNA extraction efficiency (ug DNA/11 gram meal) |
|---|---|---|---|
| 1A | 11 | 50 | 10.0 |
| 2A | 11 | 65 | 13.0 |
| 3A | 11 | 58 | 11.6 |
| 4A | 11 | 63 | 12.6 |
| 1B | 11 | 69 | 13.8 |
| 2B | 11 | 55 | 11.0 |
| 3B | 11 | 53 | 10.6 |
| 4B | 11 | 59 | 11.8 |
| 1C | 11 | 65 | 13.0 |
| 2C | 11 | 67 | 13.4 |
| 3C | 11 | 55 | 11.0 |
| 4C | 11 | 64 | 12.8 |

The average DNA extraction efficiency was 12.1 $\pm$ 1.2 μg DNA / 11 g sample.

### 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 appeared as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA. None of the 12 genomic DNA samples showed indications of significant degradation ('smearing').



### 4.3. Evidence of the absence of PCR-inhibitory compounds

Presence of PCR-inhibitory compounds in the DNA preparations was tested by real-time PCR using the oligonucleotides directed to the endogenous control gene *Cruciferine* gene (*cruA*) 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 $\Delta$Ct should be approx. 3.32, if the DNA is diluted 2X, the $\Delta C_t$ 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 analyse 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 8 different amounts of DNA (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) of which the Ct values were determined in triplicate in a real-time PCR run with the *cruA* oligonucleotide primers and probe. In total, the analysis was executed three times with DNA samples extracted on different days. To analyse the data, the mean Ct 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 optimal 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 of the presence of PCR-inhibitory compounds. The PCR efficiency has been calculated by the following equation: Efficiency (%) = $100 * [10^{(-1/slope)}]-1$

| DNA | Slope, a | Efficiency (%) | Intercept, b | Linearity, $r^2$ |
|---|---|---|---|---|
| 1A | -3.4400 | 95.3 | 29.469 | 0.9986 |
| 1B | -3.4741 | 94.0 | 29.588 | 0.9978 |
| 1C | -3.4975 | 93.2 | 30.059 | 0.9962 |

# 5. Experimental testing of the DNA extraction method by the Community Reference Laboratory for GM Food and Feed

The aim of the experimental testing was 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 quality and quantity appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.

The CRL-GMFF tested the "Oilseed rape seed DNA extraction protocol" proposed by the applicant on samples of food and feed consisting of ground oilseed rape seeds provided by the applicant.

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

### 5.1 Preparation of samples

About 300 g of oilseed rape seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

### 5.1 DNA extraction

DNA was extracted following the "Oil Seed Rape seed DNA extraction protocol" described above and in-house validated by the applicant.
The DNA extraction was carried out on 6 test portions (replicates) and repeated over 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.

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

| Sample | Concentration (ng/µl) |
|---|---|
| **1** | 25.4 |
| **2** | 33.7 |
| **3** | 64.4 |
| **4** | 36.2 |
| **5** | 83.6 |
| **6** | 37.5 |
| **1** | 35.4 |
| **2** | 30.1 |
| **3** | 21.0 |
| **4** | 36.2 |
| **5** | 25.5 |
| **6** | 28.1 |
| **1** | 37.2 |
| **2** | 50.8 |
| **3** | 31.6 |
| **4** | 43.8 |
| **5** | 56.1 |
| **6** | 45.1 |

**DNA concentration (ng/µl)**

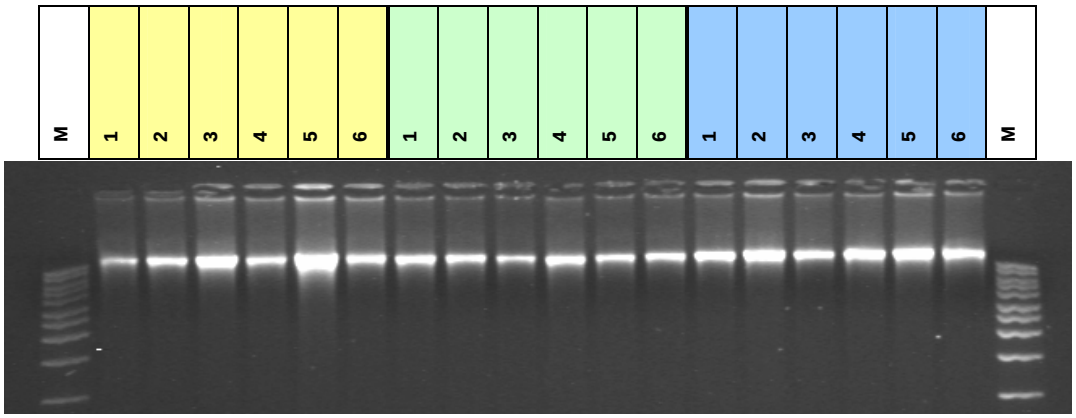
| | |
|---|---|
| Overall average of all samples: | 40.1 ng/µl |
| Standard deviation of all samples | 15.5 ng/µl |
| Coefficient of variation | 38.7 % |

**Yield (total volume of DNA solution: 3600 µl)**

| | |
|---|---|
| Overall average of all samples: | 8.0 µg |
| Standard deviation | 3.1 µg |
| Coefficient of variation | 38.7 % |

### 5.3 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 µl of the DNA solution were analysed on a 1.0% agarose gel. In yellow boxes samples extracted on day 1, in green boxes samples extracted on day 2, and in blue boxes samples extracted on day 3. A DNA ladder 1kb (M) was used.




High molecular weight DNA was observed for all samples

### 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.
The Ct values obtained for the undiluted and diluted samples are reported in the table below.

**Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of rapeseed *Cruciferine* gene, *cru*A**

| | Undiluted (40 ng/µl) | Diluted | | | |
|---|---|---|---|---|---|
| DNA extract | 1:1 | 1:4 | 1:16 | 1:64 | 1:256 |
| 1 | 22.06 | 24.09 | 25.98. | 28.28 | 30.33 |
| 2 | 22.01 | 23.94 | 26.05 | 28.05 | 30.34 |
| 3 | 20.90 | 22.63 | 24.80 | 26.78 | 29.04 |
| 4 | 21.92 | 23.62 | 25.69 | 28.08 | 30.39 |
| 5 | 20.86 | 22.80 | 24.73 | 27.01 | 29.33 |
| 6 | 21.49 | 23.49 | 25.51 | 27.78 | 30.08 |
| 1 | 21.70 | 23.54 | 25.64 | 27.69 | 29.93 |
| 2 | 21.69 | 23.89 | 26.09 | 28.16 | 30.34 |
| 3 | 22.35 | 24.44 | 26.64 | 28.84 | 30.88 |
| 4 | 21.45 | 23.21 | 25.36 | 27.35 | 29.26 |
| 5 | 21.94 | 23.74 | 25.79 | 27.96 | 30.10 |
| 6 | 21.73 | 23.53 | 25.86 | 27.74 | 29.61 |
| 1 | 21.25 | 23.22 | 25.19 | 27.44 | 29.59 |
| 2 | 20.89 | 22.69 | 24.57 | 26.65 | 28.83 |
| 3 | 22.30 | 24.51 | 26.31 | 28.34 | 30.70 |
| 4 | 21.10 | 23.12 | 24.87 | 27.13 | 29.48 |
| 5 | 21.56 | 23.88 | 25.79 | 28.08 | 30.27 |
| 6 | 21.03 | 22.91 | 24.96 | 26.90 | 28.98 |

Note: only samples 3 and 5 from the first extraction (yellow boxes), and samples 2, 4, 5, 6 from the third extraction (blue boxes) were diluted to the concentration of 40 ng/ul. All other samples with a measured DNA concentration lower than 40 ng/ul, were tested undiluted.

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 rapeseed Cruciferine gene, *cru*A)**

| DNA extraction | $R^2$ | Slope | Ct extrapolated | mean Ct measured | ΔCt |
|---|---|---|---|---|---|
| 1 | 0.998 | -3.49 | 21.91 | 22.06 | 0.15 |
| 2 | 0.998 | -3.52 | 21.80 | 22.01 | 0.21 |
| 3 | 0.999 | -3.52 | 20.51 | 20.90 | 0.39 |
| 4 | 0.997 | -3.77 | 21.27 | 21.92 | 0.65 |
| 5 | 0.997 | -3.64 | 20.49 | 20.86 | 0.36 |
| 6 | 0.998 | -3.66 | 21.20 | 21.49 | 0.28 |
| 1 | 0.999 | -3.53 | 21.39 | 21.70 | 0.31 |
| 2 | 0.998 | -3.56 | 21.76 | 21.69 | 0.08 |
| 3 | 0.999 | -3.57 | 22.33 | 22.35 | 0.02 |
| 4 | 0.998 | -3.34 | 21.27 | 21.45 | 0.18 |
| 5 | 0.999 | -3.53 | 21.59 | 21.94 | 0.35 |
| 6 | 0.994 | -3.35 | 21.65 | 21.73 | 0.08 |
| 1 | 0.998 | -3.55 | 21.02 | 21.25 | 0.23 |
| 2 | 0.996 | -3.41 | 20.56 | 20.89 | 0.33 |
| 3 | 0.944 | -3.42 | 22.32 | 22.30 | 0.02 |
| 4 | 0.994 | -3.54 | 20.82 | 21.10 | 0.29 |
| 5 | 0.997 | -3.56 | 21.64 | 21.56 | 0.08 |
| 6 | 0.998 | -3.35 | 20.90 | 21.03 | 0.13 |

Note: In yellow boxes samples from 1 to 6 extracted on day 1; in green boxes samples from 1-6 extracted on day 2; in blue boxes samples from 1-6 extracted on day 3.
***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, with one exception, the sample number 4 of the first extraction with a value of 0.65.
$R^2$ of linear regression is > 0.99 for all DNA samples, except one (0.944).

# 6. Conclusion

The data reported confirm that the extraction method provides DNA of suitable quantity and quality for subsequent PCR based detection applications. The method is therefore applicable to the samples of food and feed provided in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

# 7. Quality assurance

The CRL-GMFF carries out all its operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]

# 8. References

1. Dellaporta S, Wood J and Hicks JB. [1983] Plant DNA mini preparation. Plant Molecular Biology Reporter 1, nr. 4, pp. 19-21.

2. Sambrook J. and D. W. Russell. Molecular Cloning. A laboratory manual. Third edition. 2001. Cold Spring Harbor Laboratory Press.

# 9. Abbreviations

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