

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-Specific Method for the Quantification of Oilseed Rape Line RT73 Using Real-Time PCR

*Validation Report and Protocol
Seeds Sampling and DNA Extraction of Oilseed Rape*

Corrected version 1



European Union Reference Laboratory for
Genetically Modified Food and Feed

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The corrections made in the new version are:
In *Seeds Sampling and DNA Extraction of Oilseed Rape* At Page 6

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

Changed to

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

- 20% w/v PEG (MW 8000)
- 2.5 M NaCl

At Page 6: added the storage conditions of the solutions

Note:

Since 01/12/2009 the term "Community Reference Laboratory (CRL)" is changed into "European Union Reference Laboratory (EURL)".

Since 01/03/2009 to 31/06/2016 the JRC-unit that hosts the EURL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

Since 01/07/2016 the JRC-unit that hosts the EURL GMFF is named "Food and feed compliance"



Event-specific Method for the Quantification of Oilseed Rape Line RT73 Using Real-time PCR

Validation Report

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

07 February 2007

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF) (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out an international collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the RT73 transformation event in oilseed rape DNA (unique identifier MON-ØØØ73-7). The collaborative trial was conducted according to internationally accepted guidelines^(1, 2).

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto provided the detection method and the samples (whole oilseed rape seed containing the transformation event and whole conventional oilseed rape seeds), whereas the JRC extracted the DNA and prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved fourteen laboratories from twelve European Countries.

The results of the international collaborative trial met the ENGL performance requirements and the scientific understanding about satisfactory method performance. Therefore, the CRL-GMFF considers the method validated as fit for the purpose of regulatory compliance.

The results of the collaborative study are made publicly available under <http://gmo-crl.jrc.it/>. The method will also be submitted to ISO 21570 for consideration as an international standard.

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for oilseed rape event RT73 (unique identifier MON-ØØØ73-7) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", <http://gmo-crl.jrc.it/guidancedocs.htm>).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.it/guidancedocs.htm>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, six scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event RT73 was positively concluded in September 2006.

In August-October 2006, the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of the samples and methods) by quantifying five blind GM-levels within the range 0.1%-8.0% on a copy number basis. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

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1. Introduction

Monsanto submitted the detection method and control samples for oilseed rape event RT73 (unique identifier MON-ØØØ73-7) in accordance to Articles 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Directorate General-Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC 1829/2003) organised the international collaborative ring trial for the event-specific method for the detection and quantification of RT73 oilseed rape. The study involved fourteen laboratories, each members of the European Network of GMO Laboratories (ENGL).

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3), according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.

The internal in-house experimental evaluation of the method was carried out in August-October 2006.

Following the evaluation of the data and the results of the in-house laboratory tests, the international ring trial was organised (step 4) and took place in October-November 2006.

A method for DNA extraction from oilseed rape seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at <http://gmo-crl.jrc.it/>.

The operational procedure of the collaborative study included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event RT73 DNA to total oilseed rape DNA. The procedure is a simplex system, in which an oilseed rape (OSR) *CruA* (*Cruciferin A*) endogenous assay (reference gene) and the target assay (RT73) are performed in separate wells.

The international collaborative ring-trial was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. List of participating laboratories

As part of the international collaborative ring trial the method was tested in fourteen ENGL laboratories to determine its performance. Clear guidance was given to the laboratories with regards to the standard operational procedures to follow for the common execution of the protocol. The participating laboratories are listed in alphabetical order in Table 1.

Table 1. ENGL laboratories participating in the validation study of oilseed rape RT73.

Laboratory	Country
AGES- Austrian Agency for Health and Food Safety	Austria
Central Science Laboratory (CSL)	UK
Centro Nacional de Alimentación - Agencia Española de Seguridad Alimentaria	Spain
Chemisches und Veterinäruntersuchungsamt Freiburg	Germany
Finnish Customs Laboratory - Tullilaboratorio	Finland
GIP-GEVES	France
Institute of Public Health	Belgium
Istituto Superiore di Sanità (ISS)	Italy
Laboratoire National de la Protection des Végétaux d'Orléans	France
LGC	UK
National Food Administration	Sweden
National Institute for Food Safety and Nutrition	Hungary
National Institute of Biology	Slovenia
Research Institute of Crop Production	Czech Rep.

3. Materials

For the validation of the quantitative event-specific method, genomic DNA was extracted from samples consisting of:

- i)* seeds of oilseed rape harbouring the RT73 event in homozygous state (Line "SW RaideRR", Lot Number RDR-0103-11058-S) and
- ii)* seeds of conventional oilseed rape (Line "Defender", Lot Number CON-0103-11061-S) in accordance to the provisions of Regulation (EC) No1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of 100% RT73 and non-GM oilseed rape genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the DNA extracted from control samples provided, in a constant amount of total oilseed rape DNA.

Participants received the following materials:

- ✓ Five calibration samples (160 µl of DNA solution each) for the preparation of the standard curve, denominated from S1 to S5.
- ✓ Twenty unknown DNA samples (80 µl of DNA solution each), denominated from U1-2604 to U20-2604.
- ✓ Amplification reagent control for use on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *CruA* reference gene and for the RT73 specific systems as follows:
 - Universal PCR Master Mix 2X, 3 vials: 5 ml each
 - Sterile distilled water: 8.5 ml
- ✓ Primers and probes (1 tube each) as follows:
 - CruA reference system*
 - BncruA primer 1(10 µM): 160 µl
 - BncruA primer 2(10 µM): 160 µl
 - BncruA probe (10 µM): 160 µl
 - RT73 oilseed rape system*
 - RT73 primer 1 (10 µM): 240 µl
 - RT73 primer 2 (10 µM): 240 µl
 - RT73 probe (5 µM): 160 µl

Table 2 shows the GM contents of the unknown samples (denominated from U1-U20) distributed to ring trial participants.

Table 2. RT73 GM contents

RT73 GM % (GM copy number/OSR genome copy number *100)
0.1
0.4
0.9
4.0
8.0

4. Experimental design

Twenty unknown samples (U1-U20), representing five GM levels, were used in the validation study. On each PCR plate, samples were analysed in parallel with both the RT73 and *CruA* specific system. In total, two plates were run per participating laboratory, with two replicates for each GM level analysed on each run. In total, four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

5. Method

Description of operational steps followed

For specific detection of event RT73 genomic DNA, a 108-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM is used as the reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event RT73 DNA, an OSR-specific reference system amplifies a 101-bp fragment of *CruA* (*Cruciferin A*) oilseed rape endogenous gene (GenBank X14555), using a pair of *CruA* gene-specific primers and a *CruA* gene-specific probe labelled with VIC and TAMRA.

Standard curves are generated both for the RT73 and the *CruA* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For relative quantification of event RT73 DNA in a test sample, the RT73 copy number is divided by the copy number of the oilseed rape reference gene (*CruA*) and multiplied by 100 to obtain the percentage value ($GM\% = RT73 / CruA * 100$).

Calibration sample called S1 was prepared by mixing the appropriate amount of RT73 DNA in control non-GM oilseed rape DNA to obtain 10% RT73 in a total of 200 ng oilseed rape DNA. Samples called S2 and S3 were prepared by 1:4 serial dilutions of the S1 sample and samples S4 S5 were prepared by 1:3 serial dilutions of the S3 sample.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for oilseed rape genome (1.15)⁽³⁾. The copy number values used in the quantification are provided in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µl)	200	50	12.5	4.2	1.4
Oilseed rape genome copies	173910	43480	10870	3623	1208
RT73 oilseed rape genome copies	17391	4348	1087	362.3	120.8

6. Deviations reported

Twelve laboratories reported no deviations.

One laboratory ran the samples in twenty microliters, as only a 384-well plate machine was available; final concentrations of the supplied primers/probe, buffer and the DNA amount were maintained according to the original protocol.

One laboratory prepared the reaction master-mix in a 15 ml tube and did not centrifuge it.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula $((10^{(-1/\text{slope})} - 1) * 100)$] of the standard curves and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both PCR systems and runs (reference and GM specific systems, plates A and B) are summarised in Table 4.

Table 4. Values of standard curves' slopes, PCR efficiency and linearity (R^2)

Lab	Plate	RT73			CruA		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.37	98.14	0.99	-3.39	97.23	0.99
	B	-3.22	95.36	0.99	-3.42	96.12	0.99
2	A	-3.30	99.12	1.00	-3.92	79.83	0.99
	B	-3.46	94.45	0.98	-3.21	95.21	0.98
3	A	-3.49	93.38	0.98	-3.18	93.49	0.99
	B	-3.35	98.74	0.98	-3.55	91.37	0.99
4	A	-3.47	94.02	0.99	-3.49	93.44	1.00
	B	-3.36	98.63	1.00	-3.45	94.82	0.99
5	A	-3.61	89.08	0.99	-3.44	95.46	0.99
	B	-3.40	96.99	0.99	-3.33	99.59	0.99
6	A	-3.43	95.56	1.00	-3.35	98.98	1.00
	B	-3.39	97.12	1.00	-3.32	99.91	1.00
7	A	-3.77	84.16	0.99	-3.44	95.40	0.99
	B	-3.51	92.63	0.98	-3.49	93.59	0.99
8	A	-3.38	97.58	0.99	-3.14	91.58	0.99
	B	-3.43	95.63	0.99	-3.30	99.07	0.99
9	A	-3.57	90.74	1.00	-3.43	95.72	0.99
	B	-3.45	95.08	1.00	-3.47	94.35	0.99
10	A	-3.53	91.91	1.00	-3.59	89.80	1.00
	B	-3.60	89.52	0.99	-3.51	92.67	0.99
11	A	-3.45	94.87	0.99	-3.30	99.28	1.00
	B	-3.39	97.23	0.99	-3.30	98.97	1.00
12	A	-3.38	97.58	0.99	-3.51	92.58	0.99
	B	-3.44	95.35	1.00	-3.52	92.31	0.99
13	A	-3.16	92.86	0.98	-3.36	98.45	1.00
	B	-3.21	95.07	0.98	-3.26	97.56	1.00
14	A	-3.23	96.13	0.98	-3.29	98.51	0.99
	B	-3.43	95.81	0.99	-3.38	97.52	1.00
Mean		-3.42	94.7	0.99	-3.40	95.1	0.99

The mean PCR efficiency was around 95% for both systems and the linearity of the method was on average 0.99.

Data reported confirm the appropriate performance characteristics of the method tested.

GMO quantification

Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for all unknown samples (U1-U20)

LAB	Sample GMO content (GM% = GM copy number/OSR genome copy number *100)																			
	0.10				0.40				0.90				4.00				8.00			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.06	0.08	0.09	0.04	0.33	0.35	0.32	0.39	0.87	1.03	1.08	0.78	4.24	4.25	4.43	3.04	9.66	9.69	8.15	7.00
2	0.05	0.06	0.04	0.03	0.18	0.29	0.25	0.26	0.64	1.04	0.45	0.55	1.88	5.25	3.42	1.99	6.30	3.27	4.29	4.90
3	0.08	0.13	0.11	0.08	0.48	0.46	0.51	0.53	1.14	1.10	0.84	0.82	4.70	4.07	5.73	5.62	8.36	9.50	11.82	9.07
4	0.07	0.12	0.08	0.07	0.29	0.43	0.38	0.28	0.66	0.83	0.91	0.92	3.44	4.62	4.96	3.95	9.99	9.00	6.84	6.82
5	0.06	0.06	0.11	0.06	0.38	0.69	0.28	0.34	0.72	0.88	1.01	1.00	4.82	5.59	5.86	4.07	7.38	7.22	6.63	10.05
6	0.11	0.06	0.09	0.08	0.39	0.32	0.34	0.41	0.88	0.89	1.01	0.88	5.32	4.62	3.74	5.10	7.89	9.20	8.70	8.47
7	0.05	0.10	0.09	0.06	0.32	0.41	0.54	0.31	0.74	1.00	0.79	1.02	3.69	4.41	4.11	4.18	9.59	7.26	8.02	8.93
8	0.07	0.07	0.07	0.04	0.24	0.37	0.29	0.24	0.67	0.58	0.46	0.83	3.74	3.12	3.46	3.17	6.52	5.58	5.85	5.93
9	0.09	0.10	0.06	0.08	0.28	0.33	0.32	0.28	0.91	1.05	0.69	0.85	3.88	4.12	5.84	3.47	8.91	9.45	7.03	8.22
10	0.10	0.10	0.11	0.08	0.35	0.41	0.38	0.43	0.86	0.78	0.79	1.17	3.77	3.39	4.29	4.42	6.41	7.26	9.18	7.28
11	0.07	0.07	0.07	0.08	0.23	0.45	0.36	0.42	0.90	0.92	0.85	0.84	4.22	4.01	3.90	4.49	8.42	9.05	5.98	8.61
12	0.08	0.10	0.08	0.07	0.32	0.40	0.38	0.24	0.74	0.95	0.80	0.95	4.69	3.77	4.19	4.52	8.73	8.58	8.42	9.90
13	0.05	0.06	0.04	0.07	0.25	0.26	0.25	0.27	0.80	0.64	0.65	0.60	3.26	3.76	3.69	3.49	8.89	9.76	8.60	8.08
14	0.09	0.09	0.07	0.05	0.39	0.40	0.36	0.47	0.94	1.00	0.78	0.87	3.97	4.24	4.35	4.31	9.57	8.36	10.01	10.74

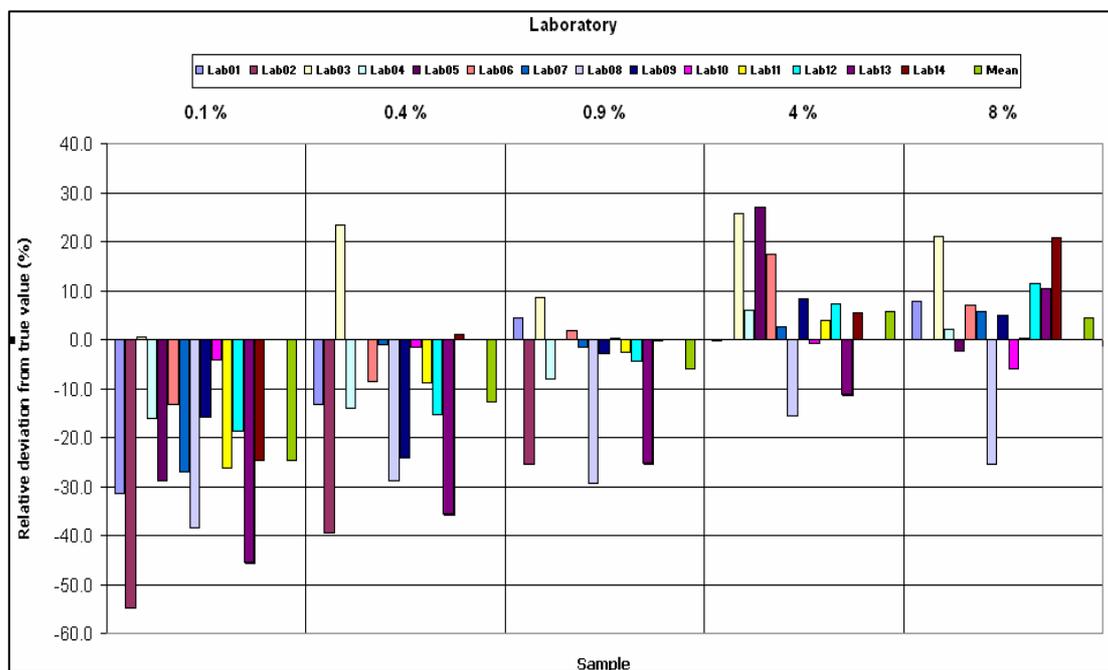
In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories; green bars represent the overall mean.

As observed in Figure 1, the majority of laboratories underestimated the true value at 0.1%, 0.4% and 0.9%, whereas GM levels of 4% and 8% were overestimated by the majority of participants.

In three laboratories the underestimation was over 25% both at 0.1% and at 0.4%. However, the corresponding values were not indicated as outliers according to Cochran and Grubbs tests (ISO 5725-2).

Overall, the average relative deviation (represented by the green bar) was clearly acceptable at all GM levels tested, indicating a satisfactory accuracy of the method.

Figure 1. Relative deviation (%) from the true value of RT73 for all laboratories



8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (<http://gmo-crl.jrc.it/guidancedocs.htm>, see also Annex 1), repeatability and reproducibility were assessed through an international collaborative trial, carried out with the support of ENGL laboratories (see Table 1). Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The *relative reproducibility standard deviation* (RSD_R), that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As can be observed in Table 6, the method fully satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 28% at the 0.1% level, well within the acceptance criterion.

Table 6. Oilseed rape RT73: summary of validation results.

Unknown sample GM%	Expected value (GMO %)				
	0.1	0.4	0.9	4.0	8.0
Laboratories having returned results	14	14	14	14	14
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	0	1	1
Reason for exclusion	-	1 C	-	1 C	1 DG
Mean value	0.08	0.35	0.85	4.23	8.36
Relative repeatability standard deviation, RSD_r (%)	23	17	17	14	14
Repeatability standard deviation	0.02	0.06	0.14	0.58	1.13
Relative reproducibility standard deviation, RSD_R (%)	28	24	19	17	16
Reproducibility standard deviation	0.02	0.09	0.16	0.71	1.37
Bias (absolute value)	-0.02	-0.05	-0.05	0.23	0.36
Bias (%)	-25	-13	-6.0	5.8	4.5

C = Cochran's test; DG= Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Bias is estimated according to ISO 5725 data analysis protocol.

Table 6 further documents the *relative repeatability standard deviation* (RSD_r), as estimated for each GM level. In order to accept methods for collaborative ring trial evaluation, the CRL requires that RSD_r values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [<http://gmo-crl.jrc.it/guidancedocs.htm>]).

As can be observed from the values reported in Table 6, the method satisfies this requirement throughout the whole dynamic range tested.

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be $\pm 25\%$ across the entire dynamic range. In this case the method satisfies this requirement; in fact, the highest value of bias (%) is -25% at the 0.1% level, and the bias (%) for the remaining levels is well within the acceptance criterion.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under <http://gmo-crl.jrc.it/guidancedocs.htm>). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative ring trial (please see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria. The method is therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all 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)]

11. References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Royal Botanic Garden, Kew. Plant DNA C-values Database (release 4.0, October 2005). MD Bennett and IJ Leitch (<http://www.rbgekew.org.uk/cval/homepage.htm>)

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of -3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(1/\text{slope})} - 1]$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of $(-3.1 \geq \text{slope} \geq -3.6)$

R² Coefficient

Definition: The R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R² should be ≥ 0.98 .

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an $RSD_r \leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{\text{th}}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq 5\%$ false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm 30\%$. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the $1/10$ and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An $RSD_R < 50\%$ is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.



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



Event-specific Method for the Quantification of Oilseed Rape Line RT73 Using Real-time PCR

Protocol

07 February 2007

Method development:

Monsanto Company

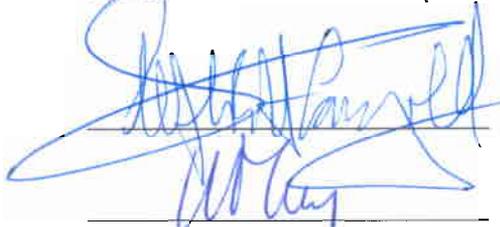
Collaborative trial:

Directorate General Joint Research Centre
European Commission
Biotechnology & GMOs Unit

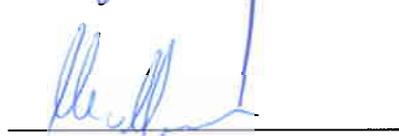
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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event RT73 DNA to total oilseed rape (OSR) DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For specific detection of event RT73 genomic DNA, a 108-bp fragment of the recombination region between the insert and the plant genome (located at the 3' flanking region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantitation of event RT73 DNA, an oilseed rape-specific reference system amplifies a 101-bp fragment of the *Cruciferin A* gene (*CruA*), an oilseed rape endogenous gene, using a pair of specific primers and a *CruA* gene-specific probe labelled with VIC and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event RT73 DNA in a test sample, event RT73 and *CruA* Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event RT73 DNA to total oilseed rape DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for suitable DNA extracted from oilseed rape leaves, grains or seeds.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories in October-November 2006.

Each participant received twenty unknown samples containing RT73 oilseed rape genomic DNA at five GM contents, between 0.1 % and 8.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM RT73 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found under <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.04% in 200 ng of total oilseed rape DNA.

The relative LOD was not assessed in the collaborative trial.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is 0.085% in 200 ng of total oilseed rape DNA.

The lowest relative GM content of the target sequence included in the international collaborative trial was 0.1 %.

2.5 Molecular specificity

The method utilises a unique DNA sequence of the recombination region between the insert and the plant genome. The sequence is specific to RT73 event and thus imparts event-specificity to the method.

The specificity of the event-specific system was analysed by the RT73-specific real-time PCR against DNA extracted from plant materials containing the specific GM targets of oilseed rape RT73, RT200, maize GA21, NK603, MON810, MON863, wheat MON71800, soybean 40-3-2, cotton 15985 and non-transgenic oilseed rape, maize, wheat, soybean, cotton, amaranth, rice, barley, lentil, millet, oat, peanut, pine nuts, rye berries and sunflower.

None of the non-transgenic plant lines and none of the GM lines, except the positive control RT73, provided positive signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “flow direction” during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean laboratory benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of RT73 oilseed rape

3.2.1 General

The PCR set-up for the taxon specific target sequence (*CruA*) and for the GMO (RT73) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated. The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µl for the taxon specific reaction mixture and for a total volume of 50 µl for the RT73 specific reaction mixture, with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% RT73 in non-GM oilseed rape DNA for a total of 200 ng of DNA (corresponding to 173910 oilseed rape genome copies with one genome assumed to correspond to 1.15 pg of haploid oilseed rape genomic DNA) ⁽¹⁾.

A calibration curve is produced by plotting the Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice.**
2. In two reaction tubes (one for RT73 system and one for the *CruA* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the OSR *CruA* reference system.

Component	Final concentration	µl/reaction
TaqMan [®] Universal PCR Master Mix (2x)	1x	12.5
MDB510 For primer (10 µM)	200 nM	0.5
MDB511 Rev primer (10 µM)	200 nM	0.5
TM003 probe (10 µM)	200 nM	0.5
Nuclease free water	#	7
Template DNA (max 200 ng)	#	4
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the RT73 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
RT73 primer 1 (10 µM)	150 nM	0.75
RT73 primer 2 (10 µM)	150 nM	0.75
RT73 Probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the RT73 and one for the *CruA* master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 21 x 3 = 63 µl master mix for three PCR repetitions for each sample of the *CruA* reference system and 46 x 3 = 138 µl master mix for three PCR repetitions for each sample of the RT73 system). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µl in each well of the *CruA* system and 50 µl in each well of the RT73 system. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for oilseed rape RT73/*CruA* systems

Step	Stage	T °C	Time (sec)	Acquisition	Cycles	
1	UNG	50 °C	120	No	1	
2	Initial denaturation	95 °C	600	No	1	
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. RT73) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the update button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).
- c) Save the settings
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *CruA* system).
- e) Save the settings and export all the data into an Excel file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct values for each reaction.

The standard curves are generated both for the *CruA* and the RT73 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of RT73 DNA in the unknown sample, the RT73 copy number is divided by the copy number of the oilseed rape reference gene (*CruA*) and multiplied by 100 to obtain the percentage value (GM% = oilseed rape/*CruA* * 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/15 ml tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>RT73 target sequence</i>	
RT73 primer 1	5' – CCA TAT TGA CCA TCA TAC TCA TTG CT-3'
RT73 primer 2	5' –GCT TAT ACG AAG GCA AGA AAA GGA-3'
RT73 probe	FAM 5'-TTC CCG GAC ATG AAG ATC ATC CTC CTT-3' TAMRA
<i>Reference gene CruA target sequence</i>	
MDB510	5' – GGC CAG GGT TTC CGT GAT -3'
MDB511	5' – CCG TCG TTG TAG AAC CAT TGG -3'
TM003 (probe)	VIC 5' – AGT CCT TAT GTG CTC CAC TTT CTG GTG CA -3' TAMRA

5. References

1. Royal Botanic Garden, Kew. Plant DNA C-values Database (release 4.0, October 2005). MD Bennett and IJ Leitch (<http://www.rbgekew.org.uk/cval/homepage.htm>)



EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance



Seeds Sampling and DNA Extraction of Oilseed Rape

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

07 February 2007
Corrected version 1 - 25 July 2017

JOINT RESEARCH CENTRE
Health, Consumers and Reference Materials
Food & Feed Compliance

Method development and single laboratory validation:

Monsanto Company

Method testing and confirmation:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Modification from the previous version:

At page 6:

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

Changed to

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

- 20% w/v PEG (MW 8000)

- 2.5 M NaCl

At Page 6: added the storage conditions of the solutions

Note:

Since 01/12/2009 the term "Community Reference Laboratory (CRL)" is changed into "European Union Reference Laboratory (EURL)".

From 01/03/2009 until 31/06/2016 the JRC-unit that hosts the EURL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

Since 01/07/2016 the JRC-unit that hosts the EURL GMFF is named "Food and feed compliance"

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR) and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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1 Introduction

A plant DNA extraction protocol is described here as derived from the publicly available "CTAB" method ⁽¹⁾. The modified protocol can be used for extraction of DNA from leaves, seeds and grains of soybean 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 Materials (Equipment/Chemicals/Plasticware)

2.1. Equipment

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

1. Centrifuge (Beckman Coulter Avanti J-251)
2. Shaker (LabLine Enviro 3527)
3. Thermometer (VWR Cat. No. 61222-504)
4. Vacufuge (Eppendorf 5301 22 82 010-9)
5. Water bath (Precision Cat. No. 51220046)
6. Micro-centrifuge (Any appropriate model)

2.2. Chemicals

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

1. 24:1 chloroform:isoamyl alcohol (Sigma Cat. No. C-0549)
2. 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma Cat. No. P-3803)
3. Ammonium acetate 7.5 M (Sigma Cat. No. A-2706)
4. CTAB (Sigma Cat. No. H-6269)
5. 0.5 M EDTA, pH 8.0 (GibcoBRL Cat. no. 15575-038)
6. 100% ethanol (AAPER)
7. NaCl (Sigma Cat. No. S-5150)
8. 2-mercaptoethanol (Bio-Rad Cat. no. 161-0710)
9. RNase A (Roche Cat. No. 10 109 196 001)
10. Isopropanol (EM Science Cat. No. PX1835-9)
11. 1 M Tris HCl pH 8.0 (Sigma Cat. No. T-3038)
12. Proteinase K (Roche Cat. No. 03 115 836 001)
13. Polyethylene Glycol (MW 8000) (Sigma Cat. No. P2139)

2.3. Solutions

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

- 1. CTAB Extraction Buffer (2%) (store at room temperature)**
 - 2% w/v CTAB
 - 100 mM Tris HCl pH 8.0
 - 20 mM EDTA pH 8.0
 - 1.4 M NaCl
- 2. Tris-EDTA buffer (TE 1X) (store at room temperature)**
 - 10 mM Tris HCl pH 8.0
 - 1 mM EDTA pH 8.0
- 3. Proteinase K (10 mg/mL) (store at -20 °C)**
- 4. RNase A (10 mg/mL) (store at -20 °C)**
- 5. PEG Precipitation Buffer (20% w/v) (store at room temperature)**
 - 20% w/v PEG (MW 8000)
 - 2.5 M NaCl
- 6. Ethanol (70% v/v) (store at room temperature)**
- 7. Ethanol (80% v/v) (store at room temperature)**

2.4. Plasticware

- 50 mL conical tubes (Corning Cat. No. 430290)
- 13 mL Sarstedt tubes (Sarstedt Cat. No. 60.540)
- 1.5 mL microcentrifuge tubes
- filter tips

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

3 Description of the methods

3.1 Sampling

For sampling methods, it is referred 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) N. 1830/2003.

3.2 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.

3.3 Principle

The basic principle of the DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further 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, CTAB 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 PEG precipitation and re-suspension in TE-buffer.

Tissues crushing procedure

Tissues should be processed prior to extraction procedure. Possible methods of processing include a mortar and pestle with liquid nitrogen (leaf) or commercial blender (grain or seed).

3.4 Oilseed rape seed DNA extraction protocol

1. Weight out 5-6 g of processed tissue into a 50 mL conical tube appropriate for centrifugation.
Note: For unprocessed tissue, weighing may occur prior to processing as long as entire processed sample is transferred to the conical tube.
2. For each 5-6 g sample add 25 mL of a solution consisting of 24.25 mL, pre-warmed CRAB extraction buffer, 0.5 mL 2-mercaptoethanol (2-ME), and 0.25 mL of 10 mg/mL proteinase K for a final concentration of 2% (2-ME) and 100 μ g/mL (proteinase K).
3. Incubate the tube for 60 minutes at 55 °C. Cool the tube briefly on bench (10 minutes)
4. Add 20 mL of phenol:chloroform:isoamyl alcohol (PCI, 25:24:1). Cap the tube and mix vigorously by vortex or inversion.
5. Centrifuge for 10 minutes at 13000 x g and 20-25 °C to separate the aqueous and organic phases. Transfer upper aqueous phase to a clean 50 mL conical tube.
6. Repeat extraction two times for a total of three extractions (step 4-5).
7. Transfer upper aqueous phase to a new tube and add approximately 2/3 volume of -20 °C isopropanol and gently invert the tube several times to mix.
8. To precipitate the DNA place the tube at -20 °C for 30 minutes. DNA may be stored as an isopropanol precipitate at -20 °C for up to 1 year.
9. To pellet the DNA centrifuge the tubes at approximately 13000 x g for 20 minutes at 4 °C.
10. Re-dissolve the pellet in 4 mL of TE pH 8.0. Transfer to a 13-mL Sarstedt tube and add approximately 40 μ L of 10 mg/mL RNase, then incubate at 37 °C for 30 minutes.
11. To extract the DNA add 4 mL of chloroform:isoamyl alcohol (CIA, 24:1). Centrifuge for 10 minutes at approximately 13000 x g at room temperature. Transfer the upper aqueous phase to a clean Sarstedt tube.
12. Repeat step 11 twice, then add half volume of 7.5 M ammonium acetate, gently mix by inversion/pipetting and add 2 volumes of 100% ethanol. Mix by inversion/pipetting and place at -20 °C for 30 minutes. DNA may be stored as ethanol precipitate at -20 °C for up to 1 year.

13. Centrifuge at 13000 x g for 20 minutes at 4 °C to pellet the DNA.
14. Rinse the DNA pellet twice with 70 % ethanol and remove residual ethanol by vacuum.
15. Re-suspend DNA in 1 mL TE, pH 8.0 and incubate at 65 °C for 1 hour with periodic gentle mixing.
16. Centrifuge the DNA solution at 16000 x g for 10 minutes at 4 °C. Transfer the aqueous portion to a clean tube without disturbing the pellet and store at 4 °C.
17. Add equal volume of 20% PEG precipitation buffer to the extracted DNA solution. Mix well by pipetting or inversion.
18. Incubate the PEG/DNA mixture for 15 minutes at 37 °C.
19. Centrifuge the PEG/DNA mixture for 15 minutes at approximately 15000 x g at room temperature.
20. Pour off supernatant or remove by pipetting. Wash the walls of the tube and DNA pellet with 80% ethanol (1.25 times volume of the original PEG/DNA mixture). Pour off ethanol or remove by pipetting.
21. Repeat wash once for a total of two washes (step 20).
22. Completely dry any residual ethanol by vacuum at low heat (4-6 minutes).
23. Re-suspend the pellet in TE or H₂O using approximately equal volume as original DNA solution.
24. Centrifuge the re-suspended DNA solution at 15000 x g for 15 minutes.
25. Transfer DNA solution to a clean tube without disturbing the pellet.

Abbreviations:

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

4 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 "Recommended Procedure for DNA Extraction from Plant Tissues" 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.

4.1 Preparation of samples

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

4.2 DNA extraction

DNA was extracted following the "Recommended Procedure for DNA Extraction from Plant Tissues" 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.

4.3 DNA concentration, yield and 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 for all samples is reported in the Table 1 below:

Table 1. DNA concentration (ng/ μ L) of eighteen samples extracted in three days: 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.

Sample	Concentration (ng/ μ L)
1	231
2	228
3	251
4	249
5	269
6	285
1	262
2	297
3	215
4	241
5	290
6	338
1	406
2	361
3	402
4	379
5	380
6	350

According to the data reported in Table 1, the following figures are calculated:

DNA concentration (ng/ μ L)

Overall average of all samples:	302 ng/ μ L
Standard deviation of all samples	64.3 ng/ μ L
Coefficient of variation	21.3 %

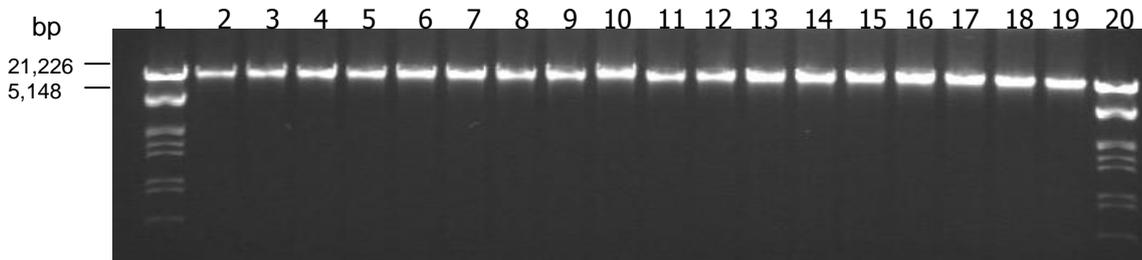
Yield (total volume of DNA solution: 1000 μ L)

Overall average of all samples:	302 μ g
Standard deviation	64.3 μ g
Coefficient of variation	21.3 %

4.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 μ L of the DNA solution were analysed on a 1.0% agarose gel (Figure 1).

Figure 1. Agarose gel electrophoresis of genomic DNA samples extracted from oilseed rape seeds. Lanes 2-7: samples extracted on day 1; lanes 8-13 samples extracted on day 2; lanes 14-19 samples extracted on day 3; lanes 1 and 20: Lambda DNA/EcoRI+HindIII Marker.



The 18 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 DNA samples showed indications of significant degradation ('smearing').

4.5 Purity / Absence of PCR inhibitors

In order to assess the purity and confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 40 ng/ μ L (hereafter referred as "undiluted" samples). Subsequently fourfold serial dilutions of each extract were prepared with 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the target sequence of the oilseed rape endogenous gene *CruA* (*Cruciferin A*). The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 2 below:

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of rapeseed *Cruciferine* gene, *cruA*

DNA extract	Undiluted (40 ng/ μ L)	Diluted			
		1:4	1:16	1:64	1:256
1	21.50	23.06	25.26	27.27	29.43
2	21.26	23.24	24.97	26.87	29.38
3	21.32	23.02	24.99	27.09	29.20
4	21.38	23.09	24.97	27.04	29.21
5	21.42	23.14	24.90	27.06	29.10
6	21.28	23.05	24.95	27.16	29.40
1	21.23	23.21	25.02	26.84	28.99
2	21.49	23.00	25.06	26.85	28.85
3	21.17	23.16	25.03	27.22	29.14
4	21.25	23.28	24.99	26.91	29.56
5	21.37	23.15	24.94	27.14	29.30
6	21.25	23.19	25.00	26.79	29.28
1	21.19	22.80	24.73	27.02	29.55
2	21.06	23.05	24.94	27.19	29.48
3	21.29	23.01	24.89	26.74	29.49
4	21.21	23.16	25.04	27.11	29.27
5	21.23	23.10	24.90	26.85	29.34
6	21.13	22.97	24.77	26.85	29.06

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.

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of rapeseed *Cruciferine* gene, *cruA*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	Δ Ct**
1	0.997	-3.51	20.97	21.50	0.53
2	0.991	-3.38	21.03	21.26	0.23
3	0.999	-3.43	20.91	21.32	0.41
4	0.997	-3.40	20.97	21.38	0.41
5	0.997	-3.33	21.04	21.42	0.38
6	0.998	-3.53	20.83	21.28	0.45
1	0.996	-3.18	21.22	21.23	0.01
2	0.998	-3.22	21.10	21.49	0.39
3	0.996	-3.35	21.10	21.17	0.07
4	0.990	-3.48	21.00	21.25	0.25
5	0.997	-3.43	20.97	21.37	0.40
6	0.990	-3.33	21.06	21.25	0.20
1	0.995	-3.74	20.39	21.19	0.80
2	0.994	-3.58	20.78	21.06	0.28
3	0.988	-3.54	20.71	21.29	0.57
4	0.996	-3.39	21.04	21.21	0.17
5	0.991	-3.43	20.88	21.23	0.35
6	0.995	-3.38	20.82	21.13	0.31

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)**

To measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct value for the "undiluted" sample (40 ng/μL) was extrapolated from the equation calculated by linear regression.

Subsequently the extrapolated Ct for the "undiluted" sample was compared with the measured Ct. The evaluation is carried out considering that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is suppressed by > 0.5 cycles from the calculated Ct value.

All Δ Ct values of extrapolated versus measured Ct are < 0.5, with three exceptions: the sample number 1 extracted on day 1, with a value of 0.53, and samples number 1 and 3 extracted on day 3 with a value of 0.80 and 0.57, respectively.

R² of linear regression is > 0.99 for all DNA samples, except one (0.988).

5 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.

6 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)]

7 References

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