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Event-specific Method for the Quantification of Oilseed Rape Line Rf3 Using Real-time PCR v. 1.01

Validation Report,
Validated Method,
Seeds Sampling and DNA Extraction

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Validated Method:

Page 4 §1 :

For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 5' flanking region) changed by
For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 3' flanking region)

Page 8 Table 2 :

KVM084 For primer
DPA165 Rev primer
changed by
KVM084 **Rev** primer
DPA165 **For** primer

Note:

Since 01/12/2009 the term "Community Reference Laboratory (CRL) " is changed into "European Union Reference Laboratory (EURL)".
Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

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



Event-specific Method for the Quantification of Oilseed Rape Line Rf3 Using Real-time PCR

Validation Report

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

11 January 2007

Executive Summary

The JRC as Community Reference Laboratory for the GM Food and Feed (CRL-GMFF) (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the Rf3 transformation event in oilseed rape DNA (unique identifier ACS-BNØØ3-6). The collaborative trial was conducted according to internationally accepted guidelines (1, 2).

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA extracted from the wild-type and 100% oilseed rape Rf3 event). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved thirteen laboratories from eleven European Countries.

The results of the 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/>.

Note :

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

Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

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Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for oilseed rape event Rf3 (unique identifier ACS-BNØØ3-6) 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, four 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 Rf3 was positively concluded in September 2005.

In March-June 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%-3.6% 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.

Content

1.	INTRODUCTION	5
2.	LIST OF PARTICIPATING LABORATORIES	6
3.	MATERIALS	7
4.	EXPERIMENTAL DESIGN	8
5.	METHOD	8
	DESCRIPTION OF THE OPERATIONAL STEPS FOLLOWED	8
6.	DEVIATIONS REPORTED	9
7.	SUMMARY OF RESULTS	9
	PCR EFFICIENCY AND LINEARITY	9
	GMO QUANTIFICATION	10
8.	METHOD PERFORMANCE REQUIREMENTS	12
9.	CONCLUSIONS	13
10.	QUALITY ASSURANCE	13
11.	REFERENCES	13
12.	ANNEX 1: METHOD ACCEPTANCE CRITERIA AND METHOD PERFORMANCE REQUIREMENTS AS SET BY THE EUROPEAN NETWORK OF GMO LABORATORIES (ENGL)	14

1. Introduction

Bayer CropScience submitted the detection method and control samples for oilseed rape event Rg3 (unique identifier ACS-BNØØ3-6) 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 the GM Food and Feed (see Regulation EC 1829/2003) organised the international collaborative ring trial of the event-specific method for the detection and quantification of Rf3 oilseed rape. The study involved thirteen 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 March-June 2006.

Following the evaluation of the data and the results of the laboratory tests, the international ring trial was organized (step 4) and took place in July 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 Rf3 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 (Rf3) 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 thirteen 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 Rf3.

Laboratory	Country
Behoerde fuer Wissenschaft und Gesundheit	Germany
Bundesinstitut fuer Risikobewertung (BfR)	Germany
Danish Plant Directorate - Laboratory for diagnostics in Plants, Seed and Feed	Denmark
Dr E Wessling Chemical Laboratory	Hungary
Ente Nazionale Sementi Elette/ Laboratorio Analisi Sementi	Italy
Finnish Customs Laboratory	Finland
Institute for Agricultural & Fisheries Research (ILVO)	Belgium
Scientific Institute of the Flemish Community	Belgium
National Food Administration	Sweden
National Institute of Biology	Slovenia
National Institute of Food Hygiene and Nutrition GMO lab	Hungary
Scottish Agricultural Science Agency	United Kingdom
Swiss Federal Research Station for Animal Production and Dairy Products	Switzerland
Umweltbundesamt GmbH	Austria

3. Materials

For the validation of the quantitative event-specific method, control samples consisting of a DNA stock solution homozygous for the GM-event Rf3 (Lot Number 32RRMM0007-1) and a non-GM DNA stock solution (Lot Number 32RRMM0100) extracted from a genetically similar wild-type line was provided by the applicant in accordance to the provisions of Regulation (EC) No 1829/2003, 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% Rf3 and non-GM oilseed rape genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total oilseed rape DNA.

The participants received the following materials:

- ✓ Five calibration samples (200 µl of DNA solution each) for the preparation of the standard curve, denominated from S1 to S5.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), denominated from U1 to U20.
- ✓ Amplification reagent control for use on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *CruA* reference gene and for the Rf3 specific systems as follows:
 - Universal PCR Master Mix 2X, 2 vials: 5 ml each
 - Distilled sterile water: 4 ml
- ✓ Primers and probes (1 tube each) as follows:
 - CruA reference system*
 - MDB510 primer (10 µM): 320 µl
 - MDB511 primer (10 µM): 320 µl
 - TM003 TaqMan® probe (10 µM): 160 µl
 - Rf3 oilseed rape system*
 - KVM084 primer (10 µM): 160 µl
 - DPA165 primer (10 µM): 160 µl
 - TM010 TaqMan® probe (10 µM): 160 µl

Table 2 shows the GM contents of the unknown samples distributed to the participants.

Table 2. Rf3 GM contents

Rf3 GM % (GM copy number/OSR genome copy number *100)
0.1
0.4
0.9
1.8
3.6

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 Rf3 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 ad using the electronic tool provided (Excel spreadsheet).

5. Method

Description of the operational steps followed

For specific detection of event Rf3 genomic DNA, a 139-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 reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event Rf3 DNA, a 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.

For relative quantification of event Rf3 DNA in a test sample, the normalised ΔC_t values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔC_t values against the logarithm of the amount of Rf3 event DNA). The normalised ΔC_t values of the unknown samples are measured and, by means of the regression formula, the relative amount of Rf3 event DNA is estimated.

Calibration samples from S1 to S5 were prepared by mixing the appropriate amount of Rf3 DNA from the stock solution in control non-GM oilseed rape DNA to obtain the following relative contents of Rf3: 3.6%, 1.8%, 0.9%, 0.45% and 0.09%. Total DNA amount per reaction was 200 ng, when 5 μ l of a DNA solution at the concentration of 40 ng/ μ l were loaded.

The GM contents of the calibration samples and total DNA quantity used in PCR are provided in Table 3 (%GM calculated considering the 1C value for oilseed rape genomes as 1.15 pg) (3).

Table 3. % GM values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µl)	200	200	200	200	200
% GM (DNA/DNA)	3.6	1.8	0.9	0.45	0.09

6. Deviations reported

Nine laboratories reported no deviations from the protocol.

One laboratory used the Hex instead of the VIC detection channel to detect the specific fluorescence from the VIC probe. However, the laboratory declared that HEX wavelengths both for excitation and emission spectra are similar to the VIC and the filter used is the same.

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 used commercially available nuclease-free water instead of the water batch provided.

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 curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories are summarised in Table 4.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R²)

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R ²)
1	A	-3.73	85.4	1.00
	B	-3.60	89.4	1.00
2	A	-2.98	83.2	0.98
	B	-3.96	79.0	0.99
3	A	-3.29	98.6	0.99
	B	-3.38	97.7	0.99
4	A	-4.17	73.7	0.99
	B	-3.83	82.3	0.98
5	A	-3.67	87.4	1.00
	B	-3.36	98.6	1.00
6	A	-3.72	85.8	0.98
	B	-3.25	96.8	1.00
7	A	-3.60	89.6	1.00
	B	-3.61	89.2	1.00
8	A	-3.67	87.3	1.00
	B	-3.75	84.9	1.00
9	A	-3.49	93.5	1.00
	B	-3.54	91.6	1.00
10	A	-3.47	94.0	1.00
	B	-3.45	94.7	1.00
11	A	-3.57	90.5	1.00
	B	-3.62	88.9	1.00
12	A	-3.69	86.5	1.00
	B	-3.89	80.8	1.00
13	A	-3.09	89.3	0.99
	B	-3.15	92.3	0.97
Mean		-3.56	88.9	0.99

The mean PCR efficiency was 88.9% 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).

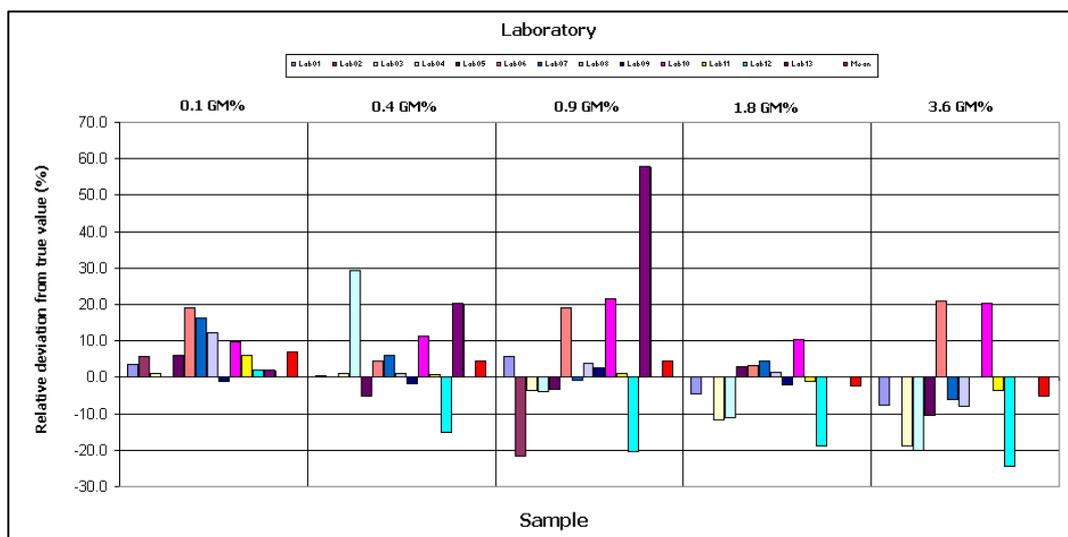
LAB	Sample GMO content (GM% = GM copy number/OSR genome copy number *100)																			
	0.10				0.40				0.90				1.8				3.60			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.11	0.12	0.08	0.10	0.43	0.36	0.41	0.40	0.96	1.04	0.90	0.91	1.83	1.83	1.66	1.57	3.51	3.39	3.42	3.01
2	0.11	0.08	0.10	0.13	0.77	0.83	0.41	0.39	0.87	0.95	0.48	0.52	1.84	4.29	1.45	1.11	6.98	4.84	3.27	1.42
3	0.09	0.11	0.11	0.09	0.37	0.40	0.45	0.39	0.97	0.89	0.83	0.78	1.87	1.28	1.71	1.51	3.40	2.88	2.82	2.61
4	0.17	0.12	0.13	0.14	0.62	0.39	0.58	0.48	0.86	0.91	0.98	0.72	1.44	1.33	1.83	1.81	3.51	2.24	3.22	2.56
5	0.10	0.12	0.10	0.11	0.44	0.32	0.38	0.38	0.63	0.99	0.86	1.00	1.70	1.98	1.77	1.96	2.78	2.99	3.58	3.53
6	0.12	0.11	0.11	0.14	0.35	0.48	0.35	0.49	0.95	0.95	1.17	1.21	1.69	1.73	2.03	1.98	3.35	3.94	4.18	5.97
7	0.11	0.12	0.11	0.12	0.44	0.40	0.46	0.40	0.81	0.88	0.98	0.91	1.81	1.94	1.89	1.88	3.55	2.76	3.54	3.66
8	0.11	0.13	0.10	0.11	0.40	0.44	0.39	0.39	0.96	1.12	0.74	0.92	1.81	2.02	1.71	1.76	3.32	3.69	2.95	3.31
9	0.09	0.09	0.11	0.10	0.39	0.38	0.42	0.38	0.99	0.87	0.89	0.94	1.82	1.74	1.66	1.83	3.59	3.45	3.72	3.67
10	0.11	0.10	0.11	0.11	0.41	0.52	0.42	0.43	1.04	1.12	1.09	1.13	2.06	2.37	1.79	1.72	4.68	4.49	3.94	4.23
11	0.11	0.11	0.09	0.11	0.39	0.40	0.39	0.43	0.86	0.91	0.92	0.95	1.78	1.83	1.74	1.77	3.58	3.47	3.47	3.35
12	0.11	0.07	0.12	0.12	0.35	0.34	0.32	0.35	0.67	0.68	0.77	0.73	1.96	1.29	1.51	1.08	2.78	2.63	2.69	2.80
13	0.12	0.10	0.09	0.10	0.45	0.41	0.53	0.54	1.41	1.33	1.18	1.76	2.19	3.22	2.64	3.99	6.20	4.67	6.92	8.19

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; red bars represent the overall mean.

As observed in Figure 1 there is no under-/over-estimation trend in the quantification of the different GM levels. Only two laboratories, following removal of outliers according to Cochran and Grubbs test (ISO 5725-2), overestimated significantly the true value.

Overall, the average relative deviation (represented by the red bar) is well within the limit of the trueness acceptance level (maximal bias < 7%).

Figure 1. Relative deviation (%) from the true value of Rf3 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 are 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 23% at the 0.9% level, well within the acceptance criterion.

Table 6. Oilseed rape Rf3: summary of validation results.

Unknown sample GM%	Expected value (GMO %)				
	0.1	0.4	0.9	1.8	3.6
Laboratories having returned results	13	13	13	13	13
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	0	2	2
Reason for exclusion	1 G	1 C	-	2 C	1 C/1G
Mean value	0.11	0.42	0.94	1.76	3.41
Relative repeatability standard deviation, RSD_r (%)	13	12	14	12	13
Repeatability standard deviation	0.01	0.05	0.13	0.20	0.46
Relative reproducibility standard deviation, RSD_R (%)	13	15	23	13	19
Reproducibility standard deviation	0.01	0.06	0.21	0.23	0.66
Bias (absolute value)	0.007	0.017	0.041	-0.044	-0.186
Bias (%)	6.9	4.4	4.5	-2.5	-5.2

C = Cochran's test; G= 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 across 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 such requirement throughout the whole dynamic range tested; in fact, the highest value of bias (%) is 6.9 at the 0.1% level, 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 (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. A 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. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218

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: $\text{Efficiency} = [10^{(1/\text{slope})} - 1]$

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 R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 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 $1/10^{\text{th}}$ of the value of the target concentration with an $\text{RSD}_r \leq 25\%$. 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.

Annex 2: Event-specific Method for the Quantification of Oilseed Rape Line Rf3 Using Real-time PCR

Validated Method

11 January 2007

Corrected version 1 - 28/08/2013 (see page 2)

Method development:

Bayer CropScience

Collaborative trial:

Joint Research Centre – European Commission
Unit for Molecular Biology and Genomics

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

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

The EURL GMFF is also ISO 17043:2010 accredited (Proficiency test provider) and apply the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the IHCP Institute provided by CERMET

Correction from the previous version:

Corrected version 1 - 28/08/2013

Page 1 and 2: new layout

Page 4 §1 :

For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 5' flanking region) changed by

For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 3' flanking region)

Page 8 Table 2 :

KVM084 For primer

DPA165 Rev primer

changed by

*KVM084 **Rev** primer*

*DPA165 **For** primer*

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)

Institute for Health and Consumer Protection (IHCP)

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Contents

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY	4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL.....	5
2.3 LIMIT OF DETECTION.....	5
2.4 LIMIT OF QUANTITATION	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURE	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF RF3 OILSEED RAPE	7
3.2.1 General	7
3.2.2 Calibration	7
3.2.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS.....	9
3.4 CALCULATION OF RESULTS.....	10
4. MATERIALS.....	10
4.1 EQUIPMENT	10
4.2 REAGENTS.....	11
4.3 PRIMERS AND PROBES.....	11
5. REFERENCES.....	11

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 Rf3 DNA to total oilseed rape (OSR) DNA in a sample.

The PCR assay was optimised for the 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 the 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 Rf3 genomic DNA, a 139-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 Rf3 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 quantitation of the amount of event Rf3 DNA in a test sample, the normalised Δ Ct values of the calibration samples are used to calculate by linear regression a reference curve Δ Ct-formula. The normalised Δ Ct values of the unknown samples are measured and, by means of the reference Δ Ct-formula, the relative amount of Rf3 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

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

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

2.2 Collaborative trial

The method was validated in an international collaborative trial organized by the Joint Research Centre (JRC) of the European Commission, European Union Reference Laboratory for GM Food and Feed. The study was undertaken with 13 laboratories in July-August 2006.

Each participant received twenty unknown samples containing Rf3 oilseed rape genomic DNA at five GM contents, between 0.1 % and 3.6 %.

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 Rf3 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

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total oilseed rape DNA. The relative LOD was not assessed in a collaborative trial. The lowest relative GM content of the target sequence included in collaborative trial was 0.1%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is at least 0.09% in 200 ng of total oilseed rape DNA. The lowest relative GM content of the target sequence included in 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 Rf3 event and thus imparts event-specificity to the method.

The specificity of the forward and reverse oligonucleotide primer was experimentally tested in duplicated end-point PCRs against DNA extracted from plant materials containing

the specific targets of maize T25, MON810, Bt11, Bt176, GA21, NK603, CBH351, Roundup Ready Soybean, soybean A2704-12, oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, LLCotton25 and LLRice62.

None of the GM-lines, except the positive control Rf3, yielded detectable amplicons in duplicate experiments.

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 and freezer and in equipment 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 shall not 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 Rf3 oilseed rape

3.2.1 General

The PCR set-up for the taxon specific target sequence (*CruA*) and for the GMO (Rf3) 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 per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of Rf3 DNA in a total amount of 200 ng oilseed rape DNA. The GM content of the standard samples ranges from 3.6% to 0.09%.

A calibration curve is produced by plotting the ΔC_t -values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve ($y = ax + b$) are then used to calculate the mean % GM content of the blind samples based on their normalised ΔC_t values.

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 Rf3 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
MBD510 For primer (10 µM)	200 nM	0.5
MBD511 Rev primer (10 µM)	200 nM	0.5
TM003 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the Rf3 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
DPA165 For primer (10 ·M)	400 nM	1
KVM084 Rev primer (10 ·M)	400 nM	1
TM010 Probe (10 ·M)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the Rf3 and one for the *CruA* master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA

(e.g. $5 \times 3 = 15 \mu\text{l}$ 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. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately $250 \times g$ 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 Rf3/*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. Rf3) 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 = $25 - 3 = 22$).

Annex 3: Seeds Sampling and DNA Extraction of Oilseed Rape

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

11 January 2007

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

Method development and single laboratory validation:
Bayer BioScience N.V.

Method testing and confirmation:
European Commission, Directorate General-Joint Research Centre
Biotechnology & GMOs Unit

Note:

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

Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

Address of contact laboratory:

European Commission, Directorate General-Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology & GMOs Unit
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Via Fermi 1, 21020 Ispra (VA) - Italy

Content

1. INTRODUCTION	4
2. DESCRIPTION OF THE METHODS.....	4
3. EQUIPMENT/CHEMICALS/PLASTICWARE	6
4. TESTING OF THE DNA EXTRACTION METHOD BY THE METHOD DEVELOPER .	8
5. EXPERIMENTAL TESTING OF THE DNA EXTRACTION METHOD BY THE EUROPEAN UNION REFERENCE LABORATORY FOR GM FOOD AND FEED.....	11
6. CONCLUSION	15
7. QUALITY ASSURANCE	15
8. REFERENCES	15
9. ABBREVIATIONS.....	15

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 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 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 supernatant
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. Na₂-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-isoamylalcohol (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).

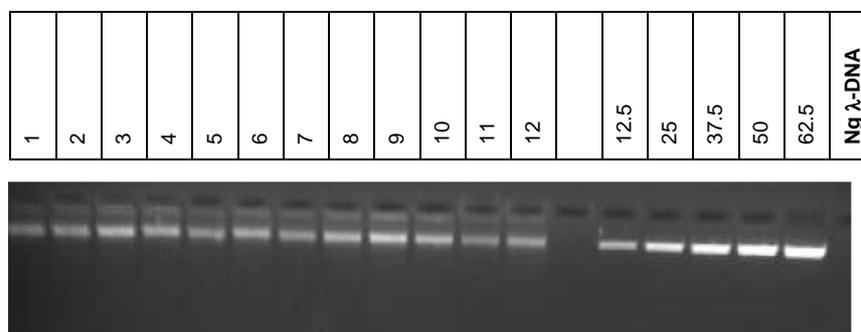
Results are summarised in the following Table:

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 $12.1 \pm 1.2 \mu\text{g DNA} / 11 \text{ g sample}$.

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 ΔC_t should be approx. 3.32, if the DNA is diluted 2X, the ΔC_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 C_t 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 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 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 (%) = $100 * [10^{(-1/\text{slope})}] - 1$

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 European Union 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/ μ l)
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/ μ l)

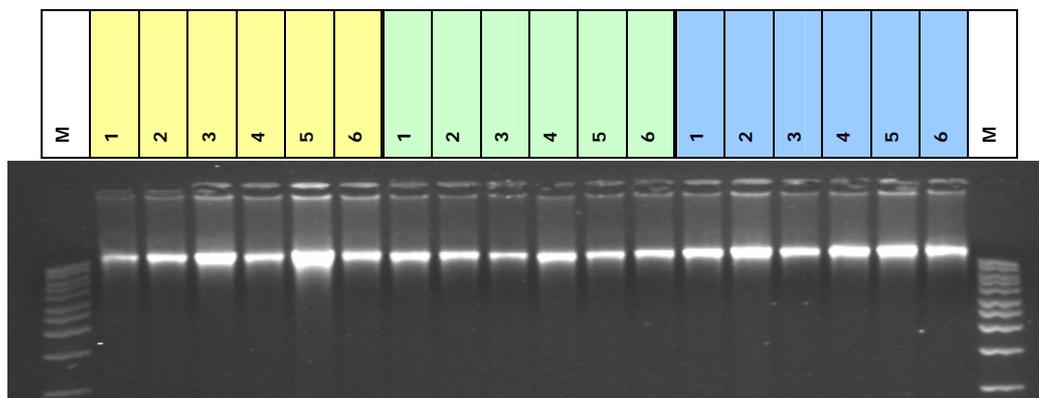
Overall average of all samples:	40.1 ng/ μ l
Standard deviation of all samples	15.5 ng/ μ l
Coefficient of variation	38.7 %

Yield (total volume of DNA solution: 3600 μ l)

Overall average of all samples:	8.0 μ g
Standard deviation	3.1 μ g
Coefficient of variation	38.7 %

5.3 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. 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, *cruA*

DNA extract	Undiluted (40 ng/ μ l)	Diluted			
	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, *cruA*)

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

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9. Abbreviations

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

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Abstract

The JRC as European Union Reference Laboratory for the GM Food and Feed (EURL-GMFF) (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the Rf3 transformation event in oilseed rape DNA (unique identifier ACS-BN003-6). The collaborative trial was conducted according to internationally accepted guidelines (1, 2).

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA extracted from the wild-type and 100% oilseed rape Rf3 event). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved thirteen laboratories from eleven European Countries.

The results of the collaborative trial met the ENGL performance requirements and the scientific understanding about satisfactory method performance. Therefore, the EURL-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.

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

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Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security, including nuclear; all supported through a cross-cutting and multi-disciplinary approach.

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