In-house Validation of an Event-specific Method for the Quantification of Oilseed Rape RF2 Using Real-Time PCR

Validation Report

Joint Research Centre
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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house validation study to assess the performance of a quantitative, event-specific method for RF2 event in oilseed rape (unique identifier ACS-BN∅∅2-5). The study 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, Bayer CropScience provided the detection method and the control samples (genomic DNA extracted from the wild-type and 100% oilseed rape RF2 event). The EURL-GMFF prepared the in-house validation samples (calibration samples and blind samples at unknown GM percentage).

The results of the in-house validation study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The results of this EURL-GMFF in-house validation study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

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Report on Steps 1-3 of the Validation Process

Bayer CropScience provided the detection methods and control samples of the oilseed rape event RF2 (unique identifier ACS-BN∅∅2-5) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-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 EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/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 (ENGL) and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, three 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 RF2 was positively concluded in September 2005.

Following the withdrawal from the market of MS1xRF2 hybrid oilseed rape (ACS-BN∅∅4-7xACS-BN∅∅2-5) and its derived products (Commission Decision 2007/306/EC of 25 April 2007), the event-specific method for the quantification of oilseed rape RF2 event did not undergo a full validation process. However, in order to enforce Article 2 of the above Commission Decision, the EURL-GMFF performed an in-house validation of the detection method.

In July-August 2008, the EURL-GMFF assessed the method characteristics by quantifying five GM levels within the range 0.15%-3.3% on a DNA copy number basis. The experiments were performed under repeatability conditions in two different PCR platforms, 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 EURL-GMFF (step 3) is available on request.

Content

1. Introduction

Bayer CropScience provided the detection method and control samples for the oilseed rape event RF2 (unique identifier ACS-BN∅∅2-5) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) No 1829/2003 carried out an in-house validation of the event-specific method for the detection and quantification of RF2 oilseed rape.

Upon reception of methods, samples and related data (step 1), the ERL-GMFF carried out the assessment of the documentation (step 2) and the in-house validation of the method (step 3), according to the requirements of Regulation (EC) No 641/2004. The in-house method validation was performed in July-August 2008.

The in-house validation aimed at assessing the performance of the quantitative real-time PCR (Polymerase Chain Reaction) method. The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event Rf2 DNA to total oilseed rape DNA. The procedure is a simplex system, in which an oilseed rape *cruA (cruciferin A)* endogenous assay (reference gene) and the target assay (Rf2) are performed in separate wells.

The study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725:1994 [(1)].
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" [(2)].

2. Materials

For the validation of the quantitative event-specific method, control samples consisting of a DNA stock solution (Lot number 32RRMM0023) extracted from leaves of plants harbouring the RF2 event in homozygous state, and genomic DNA (Lot number 32RRMM0101) from leaves of wild type plants genetically similar to the GM line were 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 RF2 and non-GM oilseed rape genomic DNA at different GMO content were prepared by the EURL-GMFF, using the control samples provided, in a constant amount of total oilseed rape DNA.

Table 1 shows the five samples used in the validation of the RF2 method.

Table 1. RF2 GM contents

RF2 GM % (GM copy number/oilseed rape genome copy number *100)
0.15
0.45
0.9
2.0
3.3

3. Experimental design

The method was tested in a total of eight runs. In each run, samples were analysed in parallel with both the GM specific system and the *cruA* reference system. Five GM levels per run were used and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. On the whole, quantification of the five GM levels was performed as an average of sixteen replicate samples per GM level.
The method was tested in two different real-time PCR platforms: ABI 7900HT and ABI Prism® 7700.

4. Method

For the detection of event RF2, a 104 bp fragment of the region spanning the junction between the construct inserted and the plant genome is amplified using 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 (6-carboxyfluorescein) is used as reporter dye at its 5′ end and TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For relative quantification of event Rf2 DNA, a reference system amplifies a 101 bp fragment of the *cruA* (*cruciferin A*) oilseed rape endogenous gene (GenBank X14555), using *cruA* specific primers and a *cruA* specific probe labelled with VIC and TAMRA.

For relative quantification of event RF2 DNA in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of RF2 event DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of RF2 event DNA is estimated.

Calibration samples for the preparation of the standard curve, denominated from S1 to S5, were prepared by mixing the appropriate amount of RF2 DNA in non-GM control oilseed rape DNA to obtain the following relative contents of RF2: 3.60%, 1.80%, 0.90%, 0.45% and 0.09%. The total DNA amount was 200 ng, when 5 µL per reaction/well were used (40 ng/µL).

The GM contents of the calibration samples and the total DNA quantity used in the PCR reactions are provided in Table 2 (GM% calculated considering the 1C value for oilseed rape genomes as 1.15 pg)[3].

Table 2. 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 (RF2 DNA/total DNA)	3.6	1.8	0.9	0.45	0.09

5. Deviations reported

The oilseed rape endogenous gene used was *cruA* (*Cruciferin A*) (GenBank X14555), instead of *Brassica napus* cruciferin (GenBank X59294) as reported by the applicant.

The accuracy and precision of the RF2 method on ABI Prism® 7700 for the GM level 3.3% was calculated on fifteen replicates instead of sixteen.

6. Results

PCR efficiency and linearity

The values of the slope of the ΔCt curve [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] and of the R^2 (expressing the linearity of the regression) in the eight runs for both real-time PCR platforms used (ABI 7900HT and ABI Prism® 7700), are summarised in Tables 3 and 4.

Table 3. Values of slope, PCR efficiency and R^2 obtained with the ABI 7900HT platform

Run	ABI 7900HT		
	Slope	PCR Efficiency (%)	R^2
1	-3.28	102	0.98
2	-3.12	109	0.99
3	-3.17	107	0.99
4	-3.18	106	0.99
5	-3.23	104	0.99
6	-3.21	105	0.99
7	-3.36	99	0.99
8	-3.27	102	0.99
Mean	**-3.23**	**104**	**0.99**

Table 4. Values of slope, PCR efficiency and R^2 obtained with the ABI Prism® 7700 platform

Run	ABI Prism® 7700		
	Slope	PCR Efficiency (%)	R^2
1	-3.28	102	0.99
2	-3.42	96	0.98
3	-3.44	95	0.99
4	-3.30	101	0.99
5	-3.22	104	0.98
6	-3.22	105	1.00
7	-3.15	108	0.98
8	-3.43	96	0.99
Mean	**-3.31**	**101**	**0.99**

The mean slopes obtained with the two platforms (-3.23 and -3.31) are within the acceptable range of -3.1 and -3.6. The mean PCR efficiencies are close to 100%, with a higher value observed for the ABI 7900HT platform (104% vs. 101% for the ABI Prism® 7700). The R^2 is 0.99 for both platforms.
These results confirm the appropriate performance characteristics of the method tested on the two different real-time PCR platforms.

7. Method performance requirements

The results of the in-house validation study for the method of detection of oilseed rape RF2 are reported in Tables 5 and 6. The results are evaluated with respect to the method acceptance criteria, as established by the ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf, see also Annex 1). Tables 5 and 6

report the accuracy and precision of the method for each GM level, for tests conducted with two instruments.

Table 5. Accuracy and precision of the RF2 method on ABI 7900HT

ABI 7900HT					
Unknown sample GM%	**Expected value (GMO %)**				
	0.15	**0.45**	**0.90**	**2.0**	**3.3**
Mean	**0.14**	**0.48**	**0.98**	**2.14**	**3.46**
SD	0.01	0.04	0.05	0.13	0.21
RSDr (%)	6.8	7.6	5.6	6.2	6.1
Bias (%)	-5.6	6.7	8.7	6.7	4.8

Table 6. Accuracy and precision of the RF2 method on ABI Prism® 7700

ABI Prism® 7700					
Unknown sample GM%	**Expected value (GMO %)**				
	0.15	**0.45**	**0.90**	**2.0**	**3.3**
Mean	**0.13**	**0.45**	**0.92**	**2.08**	**3.31***
SD	0.01	0.05	0.08	0.21	0.29
RSDr (%)	9.7	10	8.5	10	8.9
Bias%	-11	0.3	2.7	3.8	0.1

* mean of 15 replicates

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the accuracy of the quantification, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in Tables 5 and 6, the bias for the two real-time PCR platforms varies between -5.6% and 8.7% for the ABI 7900HT and between -11% and 3.8% for the ABI Prism® 7700. Therefore, the method satisfies this requirement throughout its dynamic range.

Tables 5 and 6 also report the *relative repeatability standard deviation (RSD$_r$)* as estimated for each GM level. In order to accept methods for evaluation, the EURL-GMFF requires that RSD$_r$ values are below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf]). As it can be observed, the RSDr ranges from 5.6% to 7.6% for the ABI 7900HT platform and from 8.5% to 10% in the ABI Prism® 7700. Therefore, the method satisfies the precision requirement across the entire dynamic range.

8. Conclusions

The performance of the event-specific method for the quantitative detection of oilseed rape RF2 event was evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). Its applicability to different real-time PCR instruments was also investigated.

The results obtained during the in-house validation indicate that the method submitted by the applicant complies with ENGL performance criteria with both PCR platforms. 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.

9. Quality assurance

The EURL-GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative /quantitative PCR) - Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

10. 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.
3. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.

11. 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 ± 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 = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6)

R^2 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 Quantification (LOQ)

Definition: The limit of quantification 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 $1/10^{th}$ of the value of the target concentration with an RSD_r ≤ 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 $1/20^{th}$ 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.