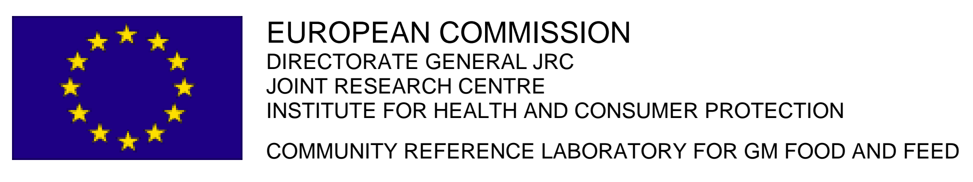

Verification of Performance of Ms8 and Rf3 Event-specific Methods on the Hybrid Ms8xRf3 Using Real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
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Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF) (see Regulation EC No 1829/2003), has carried out an in-house verification study to assess the performance of two quantitative, event-specific methods for use with the hybrid oilseed rape line (unique identifier ACS-BN∅∅5-8xACS-BN∅∅3-6) which combines both the Ms8 and Rf3 transformation events. Both methods have been previously fully validated individually on parental lines, to detect and quantify each insert on extracted DNA. The study 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 methods and the samples (genomic DNA extracted from the wild-type and 100% event Ms8xRf3 hybrid oilseed rape). The JRC prepared the in-house validation samples (calibration samples and blind samples at unknown GM percentage).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.it/doc/Method%20requirements.pdf) and to the validation results for the individual parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

The results of CRL-GMFF in-house verification studies are publicly available at http://gmo-crl.jrc.it/.

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Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection methods and control samples for the hybrid oilseed rape containing the stacked events Ms8xRf3 (unique identifier ACS-BN∅∅5-8xACS-BN∅∅3-6) 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/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and a request of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for the hybrid oilseed rape Ms8xRf3 was positively concluded in October 2005.

The event-specific detection methods for the two oilseed rape lines hosting the single events Ms8 and Rf3 were validated by the CRL-GMFF following the conclusion of the respective international collaborative ring trials and the publication of the respective validation reports (http://gmo-crl.jrc.it/statusofdoss.htm). Hence, the detection methods for the hybrid oilseed rape Ms8xRf3 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on hybrid samples combining both traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In July 2006, the CRL-GMFF experimentally verified 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 DNA copy number basis. The experiments were performed under 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

Bayer CropScience submitted both detection methods (for Ms8 and Rf3) and control samples for the hybrid oilseed rape hosting the stacked events Ms8xRf3 (unique identifier ACS-BN∅∅5-8xACS-BN∅∅3-6) 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 Directorate General Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC 1829/2003) carried out an in-house verification of both event-specific methods for the detection and quantification of Ms8 and Rf3 in the hybrid oilseed rape line combining the two traits derived through traditional breeding techniques between progeny of the parental Ms8 and Rf3 oilseed rape lines. The single methods had been previously validated by international collaborative trial on the single parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3), according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures.
The CRL-GMFF method verification was performed 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 in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of event Ms8 and Rf3 DNA to total oilseed rape DNA in the hybrid line. The procedure is a simplex system, in which an oilseed rape (OSR) *CruA* gene (*Cruciferin A*) endogenous assay (reference gene) and the target assays (Ms8 or Rf3) 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 verification of the quantitative event-specific methods, control samples consisting of a DNA stock solution (Lot number 32RRMM0128) extracted from leaves of plants harbouring the Ms8XRf3 events in hemizygous 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 100% Ms8xRf3 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 protocols (reagents, concentrations, primer/probe sequences, amplification profile) followed in the in-house verification are as those already published as validated methods for the individual Ms8 and Rf3 events.

Table 1 shows the five levels of unknown samples used in the verification of the Ms8 and Rf3 methods on extracted hybrid Ms8xRf3 DNA.

Table 1. Ms8 and Rf3 GM contents in Ms8xRf3

Ms8 GM % (GM copy number/oilseed rape genome copy number *100)	**Rf3 GM %** (GM copy number/oilseed rape genome copy number *100)
0.1	0.1
0.4	0.4
0.9	0.9
1.8	1.8
3.6	3.6

3. Experimental design

Eight runs for each event-specific method were carried out. 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 examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. On the whole, for each method (Ms8 and Rf3), quantification of the five GM levels was performed as an average of sixteen replicate samples/GM-level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of the operational steps

For specific detection of event Ms8 genomic DNA, a 129-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. Similarly, 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. Both 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 Ms8 and Rf3 DNA, an OSR-specific reference system which 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, was used.

For relative quantification of events Ms8 and Rf3 DNA in a test sample, the normalised ∆Ct values of calibration samples (denominated from S1 to S5) are used to calculate, by linear regression, a reference curve (plotting ∆Ct values against the logarithm of the amount of event DNA). The normalised ∆Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of Ms8 or Rf3 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 Ms8xRf3 DNA from the stock solution in non-GM control oilseed rape DNA to obtain the following relative contents of Ms8xRf3: 3.60%, 1.80%, 0.90%, 0.45% and 0.09%. The total DNA amount was 200 ng, when 5 µl per reaction/well was used (40 ng/µl).

For detailed information on the preparation of standard curve calibration samples please refer to the protocols of validated methods at http://gmo-crl.jrc.it/statusofdoss.htm

5. Deviations reported

No deviations from the protocols of the two previously validated methods were introduced.

6. Summary of 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 GM events are summarised in Tables 2 and 3.

Table 2. Values of slope, PCR efficiency and linearity (R^2) of the ΔCt standard curve for the Ms8 method on hybrid Ms8xRf3

Run	Ms8		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.35	98.6	0.997
2	-3.43	95.6	0.989
3	-3.10	89.9	0.997
4	-3.28	98.4	0.991
5	-3.15	92.3	0.994
6	-3.07	88.4	0.999
7	-3.18	93.7	0.995
8	-3.35	98.6	0.993
Mean	**-3.24**	**94.5**	**0.995**

Table 3. Values of slope, PCR efficiency and linearity (R^2) of the Ct standard curve for the Rf3 method on hybrid Ms8xRf3

Run	Rf3		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.184	93.9	0.995
2	-3.342	99.2	0.997
3	-3.685	86.8	0.987
4	-3.433	95.6	0.986
5	-3.508	92.8	0.996
6	-3.448	95.0	0.997
7	-3.458	94.6	0.990
8	-3.471	94.1	0.994
Mean	**-3.441**	**94.0**	**0.993**

The mean PCR efficiencies were higher than 90% and the linearity of both methods (R^2 value) was above 0.99.

Data reported in Table 2 and 3 confirm the appropriate performance characteristics of both methods tested on the hybrid material.

7. Method performance requirements

The results of the in-house verification study for Ms8 and Rf3 against hybrid Ms8xRf3 oilseed rape material are reported in Tables 4 and 5, respectively. Both sets of results are evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1). Further, Tables 4 and 5 detail estimates of accuracy and precision for each GM level and for both methods.

Table 4. Estimates of accuracy and precision for the Ms8 method on hybrid oilseed rape Ms8xRf3.

Ms8					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.4**	**0.9**	**1.8**	**3.6**
Mean	0.09	0.36	0.91	1.86	3.56
SD	0.01	0.04	0.10	0.18	0.27
RSDr (%)	13	11	11	9.5	7.7
Bias%	-9.4	-9.6	0.9	3.5	-1.1

Table 5. Estimates of accuracy and precision for the Rf3 method on hybrid oilseed rape Ms8xRf3.

Rf3					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.4**	**0.9**	**1.8**	**3.6**
Mean	0.10	0.36	0.93	1.75	3.50
SD	0.01	0.04	0.07	0.20	0.32
RSDr (%)	8.9	11	7.7	12	9.1
Bias%	0.0	-10.5	3.5	-2.8	-2.6

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 4 and 5, both methods satisfy such a requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document 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-GMFF 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 Tables 4 and 5, both methods satisfy this requirement across their respective dynamic ranges.

8. Comparison of method performance between hybrid and parental lines

A synoptic comparison of the two method performances on the hybrid oilseed rape and parental lines respectively is shown in Tables 6 and 7.

Table 6. Comparison of accuracy and precision of Ms8 method on the hybrid and parental line.

Accuracy and precision of Ms8 quantification on hybrid Ms8xRf3			Accuracy and precision of Ms8 quantification on parental line Ms8*		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.1	-9.4	13	**0.1**	7.4	22
0.4	-9.5	11	**0.4**	-3.5	18
0.9	0.9	10	**0.9**	-0.9	14
1.8	3.4	9.5	**1.8**	-1.0	17
3.6	-1.1	7.7	**3.6**	-7.5	11

***method validated (http://gmo-crl.jrc.it/statusofdoss.htm)**

Table 7. Comparison of accuracy and precision of Rf3 method on the hybrid and parental line.

Accuracy and precision of Rf3 quantification on hybrid Ms8xRf3			Accuracy and precision of Rf3 quantification on parental line Rf3*		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.1	0.0	8.9	**0.1**	6.9	13
0.4	-10	11	**0.4**	4.4	12
0.9	3.5	7.7	**0.9**	4.5	14
1.8	-2.8	12	**1.8**	-2.4	12
3.6	-2.6	9.1	**3.6**	-5.2	13

***method validated (http://gmo-crl.jrc.it/statusofdoss.htm)**

Overall, the individual Ms8 and Rf3 event-specific methods show comparable performances in terms of accuracy and precision of quantification when applied to the single parental lines or to the hybrid product.

Therefore, the in-house method verification has demonstrated that the Ms8 and the Rf3 methods can be equally applied in the quantification of the respective events in the hybrid oilseed rape product.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events Ms8 and Rf3 combined in the hybrid Ms8xRf3, have 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), and to the validation results for the individual parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are 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.

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 ± 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 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 $1/10^{th}$ 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 ≤ 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 ± 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 ± 25% of the accepted reference value over the whole dynamic range.