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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED



Event-specific method for the quantitation of rice line LLRICE62 using real-time PCR

Validation Report

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

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (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 LLRICE62 transformation event in rice DNA (unique identifier ACS-OSØØ2-5). The collaborative trial was conducted according to internationally accepted guidelines.

Bayer CropScience provided the method-specific samples (genomic DNA extracted from the 0% and 100% event LLRICE62 rice), whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved eleven laboratories from eight Countries of the European Union.

The results of the collaborative trial met the ENGL performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as Community Reference Laboratory 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 for consideration as international standard.

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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) organised the collaborative trial of the event-specific method for the detection and quantification of LLRICE62 rice. The study involved eleven laboratories, members of the European Network of GMO Laboratories (ENGL).

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

The internal tests were carried out in September-October 2005.

Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in October 2005.

The operational procedure of the collaborative study comprised 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 LLRICE62 DNA to total rice DNA. The procedure is a simplex system, in which a rice *PLD* (*Phospholipase D*) endogenous assay (reference gene) and the target assay (LLRICE62) are performed in separate wells. The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels.

The 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 Participants

The method was tested in eleven ENGL laboratories to determine its performance. Each laboratory was requested to carefully follow the protocol provided. The participating laboratories are listed in Table 1 in alphabetical order.

Table 1. ENGL laboratories participating in the validation study of rice LLRICE62.

Laboratory	Country
AGES-Institute for Food Control Vienna (CC Biochemistry)	Austria
Centro Nacional de Alimentación – Agencia Española de Seguridad Alimentaria	Spain
Danish Institute for Food and Veterinary Research	Denmark
Ente Nazionale Sementi Elette (central office in Milano) / Laboratorio Analisi Sementi	Italy
General Chemical State Laboratory, Food Division	Greece
Istituto Superiore di Sanità, ISS	Italy
Laboratoire de la DGCCRF	France
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen Amtliche Lebensmittelüberwachung	Germany
LAV Sachsen-Anhalt	Germany
LSGV Saarland	Germany
RIKILT - Institute of Food Safety	Netherlands

3. Materials

For the validation of the quantitative event-specific method, a LLRICE62 DNA stock solution, extracted from LLRICE62 line (Bayer BioScience, Lot Number 32Ref0062) and non-GM near-isogenic line (Bayer BioScience, Lot Number 32Ref0060 e 32Ref00632) was provided by the applicant.

Samples containing mixtures of 0% and 100% LLRICE62 rice genomic DNA at different GMO concentrations were prepared by the JRC in a constant amount of total rice DNA.

The participants received the following materials:

- ✓ Five calibration samples (200 µl of DNA solution each) from S1 to S5.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), from U1 to U20.
- ✓ Amplification reagent control was used on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *PLD* reference gene and for the LLRICE62 specific systems as follows:
 - Universal PCR Master Mix 2X, 2 bottles: 5 ml each
 - Distilled sterile water: 4 ml
- ✓ Primers and probes (1 tube each) as follows:
 - PLD* system
 - KVM159 primer (10 µM): 160 µl
 - KVM160 primer (10 µM): 160 µl
 - TM013 TaqMan[®] probe (10 µM): 160 µl
 - LLRICE62 rice system*
 - MDB616 primer (10 µM): 320 µl
 - MDB694 primer (10 µM): 320 µl
 - TM019 TaqMan[®] probe (10 µM): 160 µl

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

Table 2. LLRICE62 GM contents

LLRICE62 GM % (GM copy number/rice genome copy number *100)
0.15
0.40
0.90
2.00
3.30

4. Experimental design

Twenty unknown samples, representing five GM levels, were used in the validation study. On each PCR plate, samples were analyzed in parallel with both the LLRICE62 and *PLD* specific system. Two plates in total were run, with two replicates for each GM level analysed on each run. The PCR analysis was triplicated for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

5. Method

Description of the operational steps

For specific detection of event LLRICE62 genomic DNA, an 88-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 as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event LLRICE62 DNA, a rice-specific reference system amplifies a 64-bp fragment of *PLD* (phospholipase D) a rice endogenous gene (GenBank AB001919), using a pair of *PLD* gene-specific primers and a *PLD* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of event LLRICE62 DNA in a test sample, the normalized ΔC_t values of calibration samples are used to calculate, by linear regression, a reference curve (plotting ΔC_t values against the logarithm of the amount of LLRICE62 event DNA). The normalized ΔC_t values of the unknown samples are measured and, by means of the regression formula, the relative amount of LLRice62 event DNA is estimated.

Calibration samples from S1 to S5 were prepared by mixing the appropriate amount of LLRICE62 DNA from the stock solution in control non-GM rice DNA to obtain the following relative contents of LLRICE62: 3.6%, 1.8%, 0.9%, 0.45% and 0.09%. The total DNA amount was 200 ng, when 5 μ l per reaction/well are used (40 ng/ μ l).

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 rice genomes as 0.45 pg [Arumuganathan *et al.*, 1991]).

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µl)	200	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 deleted a standard S4 in one plate.

One laboratory performed the setting of baseline and threshold according to Bio-Rad iCycler IQ 3.1 instructions.

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 (R^2)

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)
1	A	-3.27	98	1.00
	B	-3.32	100	1.00
2	A	-3.52	92	1.00
	B	-3.50	93	1.00
3	A	-3.31	100	1.00
	B	-3.34	99	0.99
4	A	-3.23	96	1.00
	B	-3.35	99	1.00
5	A	-3.45	95	0.99
	B	-3.33	99	0.98
6	A	-3.32	100	0.99
	B	-3.36	99	1.00
7	A	-3.26	97	1.00
	B	-3.22	96	1.00
8	A	-3.31	100	1.00
	B	-3.38	98	1.00
9	A	-3.32	100	0.99
	B	-3.43	96	0.98
10	A	-3.28	98	1.00
	B	-3.32	100	1.00
11	A	-3.30	99	0.99
	B	-3.21	95	0.98
Mean		-3.33	98	0.99

Data reported in Table 4 confirm the good performance characteristics of the method tested. The mean PCR efficiency was 98% and the linearity of the method was on average 0.99.

GMO quantitation

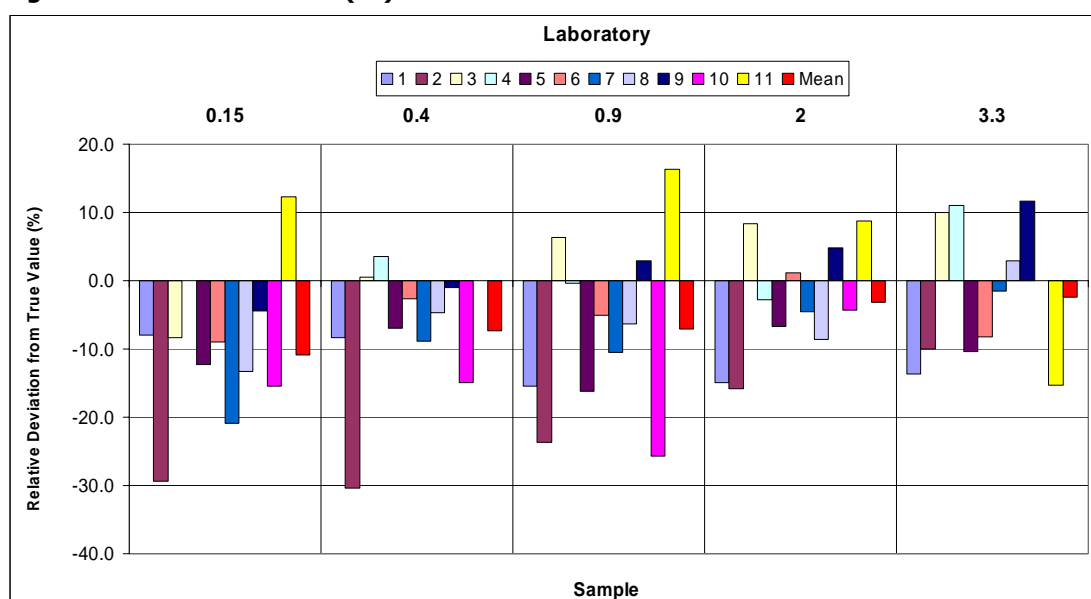
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. Replicates' mean value by laboratories and by all unknown samples.

LAB	Sample GMO content (GM% = GM copy number / rice genome copy number * 100)																			
	0.15				0.40				0.9				2.0				3.30			
	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	0.13	0.13	0.14	0.15	0.38	0.36	0.35	0.37	0.77	0.77	0.82	0.68	1.62	1.61	1.83	1.75	3.00	2.77	2.86	2.77
2	0.10	0.10	0.11	0.11	0.20	0.30	0.34	0.28	0.65	0.67	0.70	0.73	1.60	1.79	1.79	1.55	3.43	2.87	2.80	2.78
3	0.14	0.14	0.14	0.14	0.38	0.39	0.42	0.42	1.07	1.02	0.89	0.85	2.12	2.09	2.09	2.36	3.68	3.52	3.41	3.92
4	0.13	0.11	0.11	0.32	0.34	0.38	0.44	0.49	0.75	0.76	1.05	1.02	1.76	1.72	2.15	2.15	3.09	3.00	4.20	4.35
5	0.13	0.13	0.15	0.12	0.41	0.39	0.37	0.32	0.76	0.80	0.81	0.65	2.00	1.55	1.93	1.98	3.41	2.83	2.82	2.77
6	0.13	0.14	0.14	0.13	0.35	0.42	0.38	0.41	0.95	0.86	0.80	0.81	2.01	1.69	2.13	2.26	2.89	2.88	2.87	3.48
7	0.12	0.12	0.13	0.11	0.39	0.37	0.36	0.34	0.79	0.82	0.78	0.83	1.85	1.98	1.86	1.94	3.31	3.22	3.25	3.22
8	0.14	0.13	0.13	0.13	0.41	0.38	0.37	0.37	0.84	0.91	0.87	0.75	1.99	1.81	1.71	1.80	3.60	3.55	3.13	3.30
9	0.14	0.14	0.14	0.16	0.36	0.40	0.40	0.42	0.97	0.92	0.92	0.89	1.94	2.13	2.14	2.17	3.44	3.61	3.80	3.90
10	0.10	0.14	0.15	0.11	0.45	0.34	0.32	0.26	0.62	0.64	0.81	0.60	2.09	1.40	2.20	1.97	4.26	4.40	3.51	2.06
11	0.21	0.14	0.07	0.26	0.37	0.44	0.38	0.66	0.97	0.99	0.98	1.25	1.97	2.10	2.21	2.43	2.13	3.67	3.12	2.25

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; the red bar represents the overall mean. As it can be observed, a general trend of underestimation is visible for most of the laboratories and for all GM levels. All laboratories but one underestimated the true value of LLRICE62 content at 0.15% GM content, although the mean value (represented by the red bar) is well within the limit of the trueness acceptance level (bias = 25%). The same can be observed for the 0.4% level. The average relative deviation at all the other GM levels is definitely modest.

Figure 1. Relative deviation (%) from the true value of LLRice62 for all laboratories



8. Method performance requirements

The results of the collaborative trial are reported in table 6. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 6 estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Table 6. LLRICE62 validation data.

Unknown sample GM%	Expected value (GMO %)				
	0.15	0.40	0.90	2.00	3.30
Laboratories having returned results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	0	0	1
Reason for exclusion	1 C. test	1 C. test	-	-	1 C. test
Mean value	0.134	0.371	0.836	1.937	3.223
Relative repeatability standard deviation, RSD_r (%)	20.99	11.56	10.52	9.82	11.92
Repeatability standard deviation	0.03	0.04	0.09	0.19	0.38
Relative reproducibility standard deviation, RSD_R (%)	21.89	14.46	16.59	12.14	14.97
Reproducibility standard deviation	0.03	0.05	0.14	0.24	0.48
Bias (absolute value)	-0.016	-0.029	-0.064	-0.063	-0.077
Bias (%)	-10.86	-7.37	-7.07	-3.16	-2.35

C. test = Cochran's test; G. test = Grubbs' test

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 it can be observed in table 6, the method fully satisfies this requirement at all GM level tested. In fact, the highest value of RSD_R (%) is 21.89 at the 0.15% level, well within the acceptance criterion.

In the same table the *relative repeatability standard deviation* (RSD_r) values are also reported, as estimated from ring trial results for each GM level. In order to accept methods for collaborative trial evaluation, the CRL requires that RSD_r be below 25%, as indicated by ENGL. As it can be observed from the values reported in table 6, the method satisfies this requirement throughout the whole dynamic range tested.

In table 6 measures of method *bias*, which allow estimating *trueness*, are also shown for each GM level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be $\pm 25\%$ throughout the whole dynamic range. In this case the method satisfies such requirement throughout the whole

dynamic range tested; In fact, the highest value of bias (%) is -10.86 at the 0.15% 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 (available under <http://gmo-crl.jrc.it>). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the collaborative study.

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its intra and inter-laboratory variability, and trueness.

In conclusion, the method is considered complying with the current labeling requirements in Europe.

10. References

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