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



Report on the Verification of an Event-specific Detection Method for Identification of Rice GM-event LLRICE601 Using a Real-time PCR Assay

Method development:

Bayer CropScience

Verification and reporting:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit

EXECUTIVE SUMMARY

Following the Commission Decision of 23/08/2006 (2006/578/EC) "*on emergency measures regarding the non-authorised genetically modified organism LLRICE601 in rice products*", the JRC as Community Reference Laboratory (CRL) for GM Food and Feed, (Regulation EC 1829/2003), has carried out a verification of an event-specific detection method developed by Bayer CropScience and validated by USDA to detect LLRICE601 event. The verification was conducted according to internationally accepted guidelines.

The present verification report confirms that the LOD of the method on LLRICE601 event is at least 0.01% in the conditions described in the report and that the method does not show cross-reactivity with the LLRice62 event and other GM-events.

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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 GM food and feed (see Regulation EC 1829/2003), having regard to the Commission Decision 2006/578/EC carried out the verification of key performance characteristics of the event-specific detection method developed by Bayer CropScience and validated by USDA to detect GM-rice containing the LLRICE601 event.

Upon reception of the protocol and control samples, the JRC performed the verification of the method in the period 24-31 August, 2006.

2. Experimental design

The in-house verification included two modules: genomic DNA extraction and subsequent Real-time semi-quantitative assay.

The CRL received and extracted genomic DNA (gDNA) from the here below samples received from Bayer CropScience following the procedure validated by the CRL in the context of the LLRice62 validation (<http://gmo-crl.jrc.it/statusofdoss.htm>):

- "1% LLRICE601", rice seeds containing 1% seeds homozygous for the event LLRICE601
- "Conventional rice", seeds of conventional rice.

In addition, gDNA from 100% LLRice62 was available as provided by Bayer CropScience in the frame of the validation of event LLRice62.

After the extraction, the following characteristics of the gDNA samples were determined:

- DNA concentration

- DNA Purity / absence of PCR inhibitors

The performance characteristics of the detection method assessed in this study were the following:

- Limit of detection (LOD) for event LL601
- Specificity of the method;

2.1 DNA concentration

The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Suitable dilutions of each DNA extract were prepared in 5 replicates and mixed with the PicoGreen reagent.

DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/ml to 500 ng/ml using a Bio-Rad VersaFluor™ Fluorometer as fluorescence detector.

Table 1. DNA Concentration of the samples

Sample	Concentration ng/μl	RSDr*
1%LLRice 601	89.4	6.07
LLRice62 100%	401.6	2.01
Conventional rice	69.8	4.92

* based on a minimum of five readings

2.2 DNA Purity / absence of PCR inhibitors

The DNA extracted was tested for the presence of PCR inhibitors in solution. Real-time amplification was conducted targeting the rice endogenous reference gene "*phospholipase D'*" already validated by the CRL (http://gmo-crl.jrc.it/summaries/LLRICE62_val_report.pdf).

The DNA extracts were diluted to a level corresponding to the working concentration of the real-time tests (e.g. 40 ng/ μ l). From this sample (named "undiluted"), a 1:4 dilution series was prepared.

To assess the absence of inhibitors in the gDNA extracts, the Ct values of the diluted samples were plotted against the logarithm of the dilution factor, and the Ct values for the undiluted samples were extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct figures for each sample were compared with the measured Ct (Δ Ct).

A triple composite acceptance criterion was used to evaluate the purity of gDNA , based on *i)* Δ Ct values below 0.5, and *ii)* slope between -3.1 and -3.6, *iii)* linearity above 0.98 - as per ENGL acceptance criteria (1). Table 2 reports the results of the evaluation:

Table 2. Δ Ct, slopes and linearity for gDNA extracts

Sample	Δ Ct	Slope of the dilution series	Linearity
1% LLRice 601	0.47	-3.40	0.9967
Conventional rice	0.05	-3.37	0.9994

2.3 Limit of Detection (LOD)

A serial dilution of LLRICE601 gDNA in wild type rice gDNA was analyzed with the event-specific method (Protocol PGS0505) and with the rice reference system (Protocol PGS0476) to estimate the LOD of the method for this event. Total gDNA/reaction was 200ng. Amplifications were performed according to the conditions described in the Methods (Protocols PGS0505 and PGS0476 at <http://gmo-crl.jrc.it/LLRICE601update.htm>). All runs have been carried out on ABI7500 Real-time PCR systems. Results are reported in Table 3.

Samples	% final of GM	Number of replicates	Average Ct	Positive/total amplifications
LL601 1% diluted in wild type rice gDNA	1.000%	10	24.24	10/10
	0.100%	10	27.96	10/10
	0.050%	21	29.12	21/21
	0.010%	21	31.61	21/21
	0.005%	21	32.52	21/21
Conventional rice	null	3	null	0/3
NTC	null	3	null	0/3

The event-specific method detected LLRICE601 in the experimental conditions described. The method can detect LLRICE601 at levels down to 0.005% of GM-DNA in a total of 200 ng rice gDNA. Conventional rice does not result in amplification.

Data presented on the sensitivity of the event-specific method in LLRICE601 event are in line with the report of USDA-GIPSA <http://gmo-crl.jrc.it/LLRICE601update.htm>.

2.4 Specificity

Specificity tests were carried out on ABI7500 on a total of 200 ng gDNA per well, under the conditions described in the Methods.

Table 4. Results of specificity test

GM event	% GM	replicates	Positive GM Amplifications	Average GM Ct
LL62	100	3	-	-
LL601	1	3	3	27.13
LL25 cotton	3.6	3	-	-
Bt176 maize*	2	3	-	-
Ms8xRf3	3.6	3	-	-
T45 oilseed rape	3.6	3	-	-
A2704-12 soy	100	3	-	-
Conventional rice seeds	-	3	-	-
NTC	-	3	-	-

*Certified Reference Material (JRC, IRMM, Belgium)

Table 4, reports the number of positive amplifications observed with the event-specific method and the average Ct values.

GM-events other than the rice positive control LLRICE601, namely LLRice62, LL25 cotton, Bt176 maize, A2704-12 soy, Ms8xRf3 and T45 oilseed rape did not react with the event-specific method.

3. Conclusions

Following the Commission Decision of 23/08/2006 (2006/578/EC) "*on emergency measures regarding the non-authorized genetically modified organism LLRICE601 in rice products*", the JRC as Community Reference Laboratory (CRL) for the GM Food and Feed, (see Regulation EC 1829/2003), has received the samples and the method developed by Bayer CropScience and validated by USDA-GIPSA to identify LLRICE601 event.

The present report confirms that the LOD of the method on LLRICE601 event is at least 0.01%.

In addition, the CRL observed that the event-specific method does not react with other GM events. Additional specificity tests, however, are being carried out at the time of the publication of the present report.

4. Literature

1. European Network of GMO Laboratories (ENGL). Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. 2005 (<http://gmo-crl.jrc.it/guidancedocs.htm>)