EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
**COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED**

# Report on the Verification of a Construct-specific Detection Method for Identification of Rice GM-Events containing P35S::BAR 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 a construct-specific detection method developed by Bayer CropScience and validated by USDA to detect GM-rice events containing the P35S::BAR elements. The verification was conducted according to internationally accepted guidelines.

The present verification report confirms that the relative limit of detection (LOD) of the method on LLRice62 and on LLRice601 events is at least 0.01% in the conditions described in the report. In addition, it provides evidence that a difference in length exists between the amplicons generated upon amplification of DNA of LLRice62 and the LLRice601 events.

The CRL has furthermore verified that the P35S::BAR method is not specific to GM-rice events but it reacts with other GM-events (e.g. LLCotton25, Bt176 maize, Starlink maize, Ms8 and Rf3 oilseed rape), thus including some authorized GM lines. As such, when tested on complex food and feed matrixes this method could result in the generation of false positive signals with respect to LLRICE601 or LLRICE62.

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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 a verification of key performance characteristics of the a detection method developed by Bayer CropScience and validated by USDA to detect GM rice containing the P35S::BAR elements.
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 CRL extracted genomic DNA (gDNA) from the samples listed below, received from Bayer CropScience and following the method validated by the CRL and available at http://gmo-crl.jrc.it/LLRICE601update.htm :

- "SSC" (sensitivity sample control), flour originated from 6000 grains spiked with one single homozygous grain containing LLRice62 event;
- "PSC" (positive sample control), flour originated from 3000 grains spiked with one single homozygous grain containing LLRice62 event;
- "Conventional rice", seeds of conventional rice.
- "1% LLRice601", rice seeds containing 1% seeds homozygous for the event LLRice601

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

Genomic DNA from other GM events (see Table 4) were available as control samples provided by applicants to the CRL..

After the extraction the following characteristic 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 LL62 and LL601
- Specificity of the method;
- Molecular size of the amplified product.

## 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.

Samples PSC and SSC were extracted in duplicate. The concentration of one duplicate is provided in Table 1.

**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 |
| PSC | 77.0 | 2.74 |
| SSC | 72.6 | 5.83 |
| Conventional rice | 69.8 | 4.92 |

* based on a minimum of five readings

## 2.2 DNA Purity / absence of PCR inhibitors

The DNA extracted were 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/μl). 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 (ΔCt).

A triple composite acceptance criterion was used to evaluate the purity of gDNA , based on *i*) ΔCt 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. ΔCt, slopes and linearities for gDNA extracts**

| Sample | ΔCt | Slope of the dilution series | Linearity |
|---|---|---|---|
| 1% LLRice 601 | 0.47 | -3.40 | 0.9967 |
| PSC | 0.23 | -3.56 | 0.9998 |
| SSC | 0.21 | -3.25 | 0.9992 |
| Conventional rice | 0.05 | -3.37 | 0.9994 |

## 2.3 Limit of Detection (LOD)

A serial dilution of LLRICE62 and LLRICE601 gDNA in wild type rice gDNA was analysed with the P35S::BAR (Protocol PGS0494) and with the rice reference system (Protocol PGS0476) to estimate the LOD of the method for both events. Total gDNA/reaction was 200ng. Amplifications were performed according to the conditions described in the Methods (Protocols PGS0494 and PGS0476 at http://gmo-crl.jrc.it/statusofdoss.htm. All runs on ABI7500 Real-time PCR systems.

Results are reported in Table 3.

**Table 3. Plate A**

| Samples | % final of GM | Number of replicates | Average Ct | Positive/total amplifications |
|---|---|---|---|---|
| LL62 100% diluted in wild type rice DNA | 1.000% | 10 | 25.89 | 10/10 |
| | 0.100% | 10 | 29.48 | 10/10 |
| | 0.050% | 21 | 30.55 | 21/21 |
| | 0.010% | 21 | 33.20 | 21/21 |
| | 0.005% | 21 | 34.29 | 21/21 |
| PSC | 0.033% | 3 | 31.89 | 3/3 |
| SSC | 0.016% | 3 | 33.34 | 3/3 |
| NTC | null | 3 | null | 0/3 |

| Table 3. Plate B | | | | |
|---|---|---|---|---|
| **Samples** | **% final of GM** | **Number of replicates** | **Average Ct** | **Positive/total amplifications** |
| LL601 1% diluted in wild type rice gDNA | 1.000% | 10 | 28.32 | 10/10 |
| | 0.100% | 10 | 32.78 | 10/10 |
| | 0.050% | 21 | 33.78 | 21/21 |
| | 0.010% | 21 | 36.45 | 21/21 |
| | 0.005% | 21 | 37.78 | 21/21 |
| Conventional rice | null | 3 | null | 0/3 |
| NTC | null | 3 | null | 0/3 |

The P35S::BAR method detected both LLRice62 and LLRice601 in the experimental conditions described. The method can detect both events 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 P35S::BAR method in LLRice62 and LLRice601 events are in line with the report of USDA-GIPSA.**

## 2.4 Molecular weight of the LLRice62 and LLRice601 amplification products

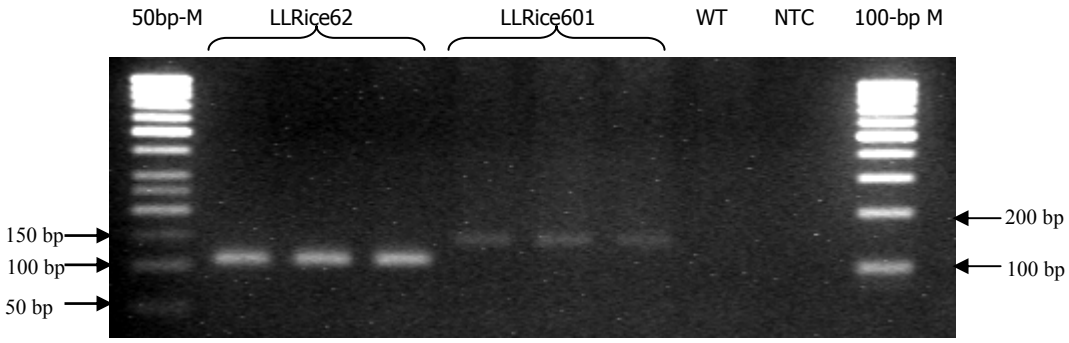
An end-point PCR was run in triple on LLRice601 and LLRice62 gDNA with the primers of the P35S::BAR method. The PCR was performed in 50 µl reaction volume containing the following: 1x Buffer Gold (Applied Biosystems), 2.5 mM MgCl2 (Applied Biosystems), 0.2mM dNTPs, 400 nM primers of the P35S::BAR method, 0.025 U/µl of AmpliTaq Gold polymerase (Applied Biosystems ) and 200 ng of template DNA. PCR amplification reactions were run in GeneAmp PCR system 9700, according to the following conditions: 10 min at 95 ºC, 40 cycles of 25 s at 95 ºC, 45 s at 60 ºC, and 45 s at 72 º C, with a final extension step of 7 min at 72 ºC.

The samples were subsequently loaded on a 2.5% agarose gel and run at 70V.

Figure 1 shows the different amplicon size produced on LLRice62 and LLRice601

gDNA.

**Figure 1.**




50-bp M: 50-bp ladder; 100-bp M: 100 bp ladder; WT: conventional rice; NTC: no template control

From this it can be seen that the amplicon generated with LLRice601 is slightly larger (approximately 40 bp) than the one generated with LLRice62, suggesting that although the same construct has been used for the transformation of both rice varieties, some post transformation rearrangements have been occurred, particularly in LLRice601. The CRL will investigate this in more detail.

## 2.5 Specificity

Specificity tests were carried out on ABI7500 on a total of 200ng gDNA per well, under the conditions described in the Methods. Results are shown in Tables 4 and 5. Species specific reference systems used in the specificity experiment are shown in Table 6.

**Table 4. Results of specificity test**

| GM event | % GM | Replicates | Positive GM amplifications/ Positive ref. amplifications | Ct value for the GM target (PGS0494) | Ct value for the Reference system (PGS0476) | ΔCt |
|---|---|---|---|---|---|---|
| LL62 | 100 | 3 | 3/3 | 19.12 | 19.07 | 0.05 |
| LL601 | 1 | 3 | 3/3 | 29.33 | 19.76 | 9.57 |
| LL25 cotton | 100 | 3 | 3/3 | 23.03 | 21.12 | 1.91 |
| Bt176 maize* | 2 | 3 | 3/3 | 34.71 | 22.23 | 12.48 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Starlink maize ** | 5 | 3 | 3/3 | 29.70 | 24.16 | 5.54 |

| GM event | % GM | Replicates | Positive GM amplifications/ Positive ref. amplifications | Ct value for the GM target (PGS0494) | Ct value for the Reference system (PGS0476) | ΔCt |
|---|---|---|---|---|---|---|
| Ms1 oilseed rape | 100 | 3 | 0/3 | - | 20.28 | - |
| Ms8 oilseed rape | 5 | 3 | 3/3 | 35.18 | 20.95 | 14.23 |
| Rf1 oilseed rape | 100 | 3 | 2/3 | 42.09 | 21.49 | 20.60 |
| Rf2 oilseed rape | 100 | 3 | 0/3 | - | 21.19 | - |
| Rf3 oilseed rape | 100 | 3 | 3/3 | 33.81 | 20.04 | 13.77 |
| Ms8xRf3 | 100 | 3 | 1/3 | 40.27 | 21.03 | 19.24 |
| Ms1xRf1 | 100 | 3 | 3/3 | 37.61 | 20.17 | 17.44 |
| T45 oilseed rape | 100 | 3 | 0/3 | - | 20.19 | - |
| A2704-12 soy | 100 | 3 | 1/3 | 41.05 | 21.12 | 19.93 |
| Conventional rice seeds | - | 3 | 0/3 | - | 19.50 | - |
| NTC | - | 3 | 0/0 | - | - | - |

*Certified Reference Material (JRC, IRMM, Belgium)
**provided by GIPSA as sample for proficiency test scheme

**Table 5. Genetic elements present in samples used**

| GM event | Genetic construction present | Source |
|---|---|---|
| LL62 | Contains the *bar* gene under the regulation of the CAMV 35S promoter | CRL |
| LL601 | Contains the *bar* gene under the regulation of the CAMV 35S promoter | Bayer |
| LL25 cotton | Contains the *bar* gene under the regulation of the CAMV 35S promoter | CRL |
| Bt176 maize | Contains the *bar* gene under the regulation of the CAMV 35S promoter | AgBios database |
| Starlink maize | Contains the *bar* gene under the regulation of the CAMV 35S promoter | Patent WO 92/09696 |
| Ms1 oilseed rape | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Ms8 oilseed rape | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Rf1 oilseed rape | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Rf2 oilseed rape | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Rf3 oilseed rape | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Ms8xRf3 | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| Ms1xRf1 | Contains the *bar* gene but not the CAMV 35S promoter | CRL |
| T45 oilseed rape | Contains the *pat* gene under the regulation of the CAMV 35S promoter | CRL |
| A2704-12 soy | Contains the *pat* gene under the regulation of the CAMV 35S promoter | CRL |

**Table 6. Species-specific reference systems**

| Plant | Species-specific reference system | Reference |
|---|---|---|
| Rice | Phospholipase D | (2) |
| Maize | Adh | (3) |
| Cotton | Adh C | (4) |
| Oilseed rape | Cruciferin | Provided by Bayer CropScience |
| Soybean | Lectin | Provided by Bayer CropScience |

Table 4, reports the number of positive amplifications observed with the P35S::BAR method and with the species-specific reference systems as control. The average Ct values obtained with the P35S::BAR method and with the reference systems are shown as well as the corresponding $\Delta$Ct figures.

GM-events other than rice positive controls LLRice62 and LLRice601 reacted with the P35S::BAR method, namely LL25 cotton, Bt176 maize, Starlink maize, Rf3, Ms8 and,Rf1 oilseed rape.

# 3. Conclusions

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 received the samples and the method developed by Bayer CropScience and validated by USDA-GIPSA to detect GM-rice events LLRice601 and LLRice62 containing the P35S and BAR elements.

The present report confirms that the LOD of the method on LLRice62 and on LLRice601 events is at least 0.01%. In addition, it provides evidence of a difference in length of the amplicons generated upon amplification of DNA of LLRice62 and the LLRice601 events.

In addition, the CRL observed that the P35S::BAR method is not specific to GM-rice events but it reacts with other GM-events (e.g. LLCotton25, Bt176 maize, Starlink maize, Ms8 and Rf3 oilseed rape), including some authorised ones. As such, when tested on complex food and feed matrixes this method could result in generation of false positive signals with respect to LLRICE601 or LLRICE62.

# 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)
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