

**EU-RL GMFF Guidance on the Application of P-35S, T-NOS and CryIAb/Ac
Methods for the Detection of Genetically Modified Rice Originating from China
Using Real-Time PCR**

21 December 2011

**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Executive Summary

In support to the repeal of Commission Decision 2008/289/EC of 3 April 2008, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, was requested to carry out an in-house verification study to assess the performance of P-35S, T-NOS and CryIAb/Ac methods as a screening approach for the detection of genetically modified rice in food products. For this, P-35S, T-NOS and CryIAb/Ac SYBR[®] Green Real-time PCR methods and a P-35S/T-NOS duplex Taqman[®] Real-time PCR method were assessed by the EURL-GMFF for their performance in detecting these markers in rice material.

The present verification report documents the results of this EURL-GMFF in-house verification study and confirms that all three SYBR[®] Green and the duplex Taqman[®] Real-time PCR methods can effectively detect the respective GM rice genetic elements in control materials of the Bt63, Kefeng6 and KMD1 genetically modified rice events.

In addition this document provides further guidance on the correct use of these methods. It is destined to control laboratories at the point of export as well as at the point of import.

This document should be used only for the screening for the presence of unapproved rice events and cannot be considered as guidance for other cases of possible contamination. It should be applied by the laboratories in conjunction with standard practices for testing for the presence of GMOs (e.g. use of appropriate controls).

Drafted by:

M. Van den Bulcke (Scientific Officer)

Report review:

1) C. Savini (Scientific Officer)

2) M. Querci (Scientific Officer)

Scientific and technical approval:

M. Mazzara (Competence Group leader)

Compliance with EURL Quality System:

S. Cordeil (Quality Manager)

Authorisation to publish:

G. Van den Eede (Head of MBG Unit)

po

po

po

po

po

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)

Institute for Health and Consumer Protection (IHCP)

Molecular Biology and Genomics Unit

European Union Reference Laboratory for Genetically Modified Food and Feed

Via E. Fermi 2749, I-21027 Ispra (VA)

Italy

Content

1. INTRODUCTION	4
2. PREMISES	4
3. GENERAL RECOMMENDATIONS FOR THE EXPERIMENTAL SETUP, THE SELECTION OF METHODS AND THE EXECUTION OF THE TESTS.....	4
4. MATERIALS AND METHODS.....	5
4.1 Materials	5
4.2 Methods.....	5
5. EXPERIMENTAL DESIGN.....	6
5.1 Specificity testing of the Real-time methods for GM rice originating from China.....	6
5.2 Sensitivity testing of the Real-time methods on GM rice originating from China.....	8
6. METHOD	8
7. DEVIATIONS REPORTED.....	11
8. SUMMARY OF RESULTS	11
8.1 Testing of the specificity of Real-time methods on GM rice samples.....	11
8.2 Testing of the sensitivity of the Real-time methods on GM rice samples	11
9. GUIDANCE TO CERTIFYING LABORATORIES.....	12
10. CONCLUSIONS	13
11. QUALITY ASSURANCE	13
12. REFERENCES	13

1. Introduction

In support to the repeal of Commission Decision 2008/289/EC of 3 April 2008, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the fitness of the P-35S, T-NOS and CryIAb/Ac SYBR®Green and a P-35S/T-NOS duplex Taqman® Real-time PCR methods for the detection of genetically modified (GM) rice in food products. The SYBR®Green methods have been developed by the Scientific Institute of Public Health (Brussels, Belgium) and tested on a broad range of crops (including rice) and in a broad range of matrices (Barbau-Piednoir *et al.*, 2010 and 2011). The P-35S/ T-NOS duplex Taqman® Real-time PCR method was developed at the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL, Germany) and their performance has been reported (Waiblinger *et al.*, 2008 and 2010; Van den Eede, 2010). The respective methods were assessed at the EURL-GMFF for their specificity and the SYBR®Green methods also for the sensitivity in detecting these GM markers in genomic DNA isolated from GM rice materials originating from China.

2. Premises

The EURL-GMFF has attempted to compile an overview of GM rice varieties that may be a source of contamination in rice products imported from China. The EURL-GMFF has listed more than 25 of such GM varieties. However, essential details on their molecular structure and nucleotide sequence information data are currently not available and no certified reference materials are obtainable. Consequently, the routine recommended approaches for detection and identification cannot be used for this specific case. In addition, for the same reasons, laboratories accredited under ISO 17025 cannot include these test procedures into the scope of their accreditation. It is not possible to set up proficiency tests in order for the laboratories to assess their performance. However it is required that the analytical results obtained are of the highest possible quality and that control laboratories use the same, or identically performing analytical procedures.

The first responsibility lies with the Chinese control authorities; the certificates they deliver should provide all necessary information to illustrate the quality of the tests executed.

3. General recommendations for the experimental setup, the selection of methods and the execution of the tests

In order to avoid accidental DNA contamination, the laboratory should follow the requirements of ISO 24276 as regards to laboratory design, apparatus and equipment and use of appropriate controls and their correct interpretation.

A rice taxon-specific method should be used to confirm the suitability of the DNA extracted from the sample in terms of PCR efficiency. The choice of the rice taxon-

specific assay should fall on a fully documented method. Suitable examples are the sucrose-phosphate synthase (SPS) qualitative PCR method (Jiang *et al.*, 2009), the SYBR[®] Green PCR method (Mbongolo Mbella *et al.*, 2011) and the Taqman[®] phospholipase D PCR method validated by the EU-RL GMFF (Mazzara *et al.*, 2006).

When performing GMO screening tests that are derived from naturally infecting organisms (such as viruses or bacteria), like the P-35S and the T-NOS targeted in the Decision, it is highly recommended to verify that any positive signal for this GM rice genetic element does not emerge from the presence of the natural organism. For this, parallel testing for the presence of a genetic element of the infecting organisms that is absent from the GMO should be performed. A common example in GMO analysis is testing for Cauliflower Mosaic virus (CaMV) absence to ascertain that positive signals obtained for the CaMV 35S promoter are derived from GMO and not from Cauliflower Mosaic virus particles.

4. Materials and methods

4.1 Materials

The EURL-GMFF tested the following control materials in this study:

- Genomic DNA from GM Bt63 rice (source: EURL-GMFF)
- Genomic DNA from GM Kefeng6 rice (source: EURL-GMFF)
- Genomic DNA from GM KMD1 rice (source: EURL-GMFF)
- Genomic DNA from Minghui non-transgenic rice (source: EURL-GMFF)
- Genomic DNA from GM LLRICE 62 (source: EURL-GMFF and AOCS)
- Genomic DNA from GM LLRICE 601 (source: EURL-GMFF)
- Genomic DNA from GM Bt11 GM maize (source: EURL-GMFF)
- Genomic DNA from GM MON 810 maize (source: EURL-GMFF)

All materials were obtained as genomic DNA except Bt11 and MON 810 GM maize materials which were control materials. Genomic DNA was extracted from the latter using the NucleoSpin[®] Plant II Maxi kit (Machery-Nagel, Germany).

4.2 Methods

DNA quantification

Total DNA concentration was determined by fluorescence detection using the PicoGreen[®] dsDNA Quantification kit (Catalogue Number P7589, Molecular Probes). Suitable dilutions of each genomic DNA sample were prepared in 2 replicates and mixed with the PicoGreen[®] reagent. The DNA concentration was determined on the basis of a five-point standard curve ranging from 0 to 500 ng/mL using a Bio-Rad VersaFluor Fluorometer as fluorescence detector.

Agarose Gel-electrophoresis

Intactness of the genomic DNA was assessed by 1.0% agarose gel electrophoresis and the DNA content was visualised by Ethidium Bromide staining.

PCR primers and reagents

All primers and probes were purchased from Microsynth at desalted purification grade for primers and PAGE purification grade for probes (See table 1 for specifications). In the Real-time PCR reactions, *Power SYBR[®]Green PCR Master Mix* (Part Number 4367659; Applied Biosystems) or *Taqman[®] Universal PCR Master Mix* (Part Number 4318157; Applied Biosystems) was used.

Real-time PCR analysis

SYBR[®]Green PCR analyses were performed according to Barbau-Piednoir *et al.* (2010) with minor adaptations. For all PCR analyses, a standard 20 μ L reaction volume was applied containing 5 μ L of template DNA, 1X *Power SYBR[®]Green PCR Master Mix*, and 250 nM of each primer. The thermal program consisted of a single cycle of DNA polymerase activation for 10 min at 95°C, followed by 40 amplification cycles of 15 sec at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step). After completion of the run, a melting curve analysis was performed by stepwise temperature increase ($\pm 1.75^\circ\text{C}/\text{min}$) from 60°C to 95°C.

Taqman[®] PCR analyses were performed according to Waiblinger *et al.* (2008). In these duplex PCR reactions, 25 μ L final reaction volume was applied containing 5 μ L of template DNA, 1X *Taqman[®] Universal PCR Master Mix* and primers and probes as indicated by Waiblinger *et al.*, 2008 (0.1 μ M of each primer and probe for the CaMV P-35S genetic element; 1 μ M of each primer and 0.2 μ M of probe for the T-NOS genetic element). The thermal program consisted of a single cycle decontamination (UNG) for 2 min at 50°C, single cycle of DNA polymerase activation for 10 min at 95°C, followed by 45 amplification cycles of 15 sec at 95°C and 1 min at 60°C.

In both the *SYBR[®]Green* and the *Taqman[®] PCR* analyses, the threshold cycle (C_t) for each sample was calculated in automatic mode according to the manufacturer's specifications. If considered necessary (e.g. due to highly diverging baseline values close to the exponential phase), the threshold and baseline were adjusted manually.

Positive Control materials and acceptance range setting

For both the *SYBR[®]Green* and the *Taqman[®] PCR* methods, control materials of maize Bt11 and MON 810 were used as positive controls (PC). For the *SYBR[®]Green* analyses the acceptance range for positives in a sample was set at the T_m -value of the respective PC(s) $\pm 1.5^\circ\text{C}$.

5. Experimental design

5.1 Specificity testing of the Real-time methods for GM rice originating from China

As to date no official DNA sequence data are available on the complete GM inserts of any GM rice events originating from China, the presence of a particular GM genetic element in a consignment was defined as follows:

A: For the SYBR® Green PCR methods:

1. A GM genetic element is considered as being present when detected by a particular method meaning that both the measured T_m -value and the C_t -value fall within the criteria of a positive result. In all other cases (thus, when T_m & C_t values are either pos-neg, or neg-pos, or neg-neg), the genetic element is considered as 'not detected' by the applied method.
2. For the T_m -values, a positive result is obtained when the measured T_m -value corresponds with the T_m ($\pm 1.5^\circ\text{C}$) of the corresponding genetic element in the positive control, while a negative result is obtained when the measured T_m -value differs more than $\pm 1.5^\circ\text{C}$ from the T_m -value of the corresponding genetic element in the positive control.

Note: In the case of the P-35S and the T-NOS genetic elements, only a single amplicon variant is generated by the respective SYBR® Green PCR methods. In this case, the T_m acceptance range falls within $\pm 1.5^\circ\text{C}$ of the T_m -value obtained with either the maize Bt11 or maize MON 810 as a PC.

In the case of the CryIAb/Ac genetic element, two amplicon variants have been reported which result in the generation of amplification products with a different T_m -value: one variant generated with the maize Bt11 as PC, a second variant generated with the maize MON 810 as PC. In the CryIAb/Ac case, a positive result is obtained when the measured T_m -value in the sample using the SYBR® Green CryIAb/Ac PCR method, falls within $\pm 1.5^\circ\text{C}$ of the T_m -value obtained with either the maize Bt11 and the maize MON 810 as PCs.

3. In case the T_m and the C_t -value obtained with a particular method indicate the occurrence of an amplification product with a T_m -value falling out of the acceptance limits of the positive control for that test (= measured T_m -value is more than 1.5°C different from the T_m -value obtained with the positive control in the analysis), the result should be scored as 'not detected'

Note: In some cases, the PCR kinetics and melting profile of such amplification product might be similar to the positive control with however a distinct T_m -value. In order to be taken into consideration in the conclusion of the analysis, the nature of such amplification products is to be confirmed and shown to be related to the target of the method (e.g. by DNA sequence analysis).

4. For the C_t -values, a measurement is positive when an (exponential) amplification is obtained and the measurable fluorescence is above the threshold level. In all other cases, the measurement is considered negative.

B: For the Taqman® PCR methods:

1. A GM genetic element is considered as being present when detected by a particular method meaning that the measured C_t -value falls within the criteria of a

positive result. Otherwise, a genetic element is considered as 'not detected' by the applied method.

2. A measurement is considered positive when an (exponential) amplification is obtained and the measurable fluorescence is above the threshold level. In all other cases, the measurement is considered negative.

For the verification of the specificity of the respective Real-time PCR methods, all tests were performed in triplicate using: the phospholipase D (PLD-rice) for rice, and the 35S promoter (P-35S) from Cauliflower Mosaic Virus, the nopaline synthase terminator (T-NOS) from *Agrobacterium tumefaciens* and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac) as GM genetic elements.

5.2 Sensitivity testing of the Real-time methods on GM rice originating from China

No Limit of Detection (LOD) value was determined for the detection of these genetic elements in the GM rice material as neither the exact copy number of the respective GM genetic elements in the control samples nor the purity of these control samples were known to the EURL-GMFF.

The SYBR[®]Green methods tested for their sensitivity using 0.05, 2.5, 5, 10 and 20 pg genomic DNA of control materials from Bt63 and Kefeng6 rice as template DNA respectively. Taking the rice diploid C-value equalling 0.97 (Arumuganathan *et al*, 1991), the corresponding estimated haploid genome equivalents (HGE) would be 0.1, 5, 10, 20 and 40 HGE. All measurements were performed using six replicates.

The sensitivity of the Taqman[®] methods has been assessed by the method developers and reported by Van den Eede (2010).

6. Method

A description of the use of the SYBR[®]Green Real-time PCR methods in a screening analysis for the presence of GMO in rice consignments at the EURL-GMFF and the interpretation of the obtained results is given below. A schematic representation of all steps in the analysis is shown in figure 1. The case of a rice grain consignment is presented as an example.

Note: The Taqman[®] P-35S/T-NOS duplex PCR method can effectively be used to replace the corresponding SYBR[®]Green Real-time PCR methods.

- A laboratory sample of 2.5 kg is to be provided to the analysing laboratory (or as indicated in the decision).

Note: For the purpose of Article 11(5) of Regulation (EC) No 882/2004 (2), a second laboratory sample shall be constituted.

- From the homogenised laboratory sample four analytical samples of 240 grams (equivalent to 10,000 rice grains) are collected (or as indicated in the decision).
- The four analytical samples are ground and further analysed separately.
- From twice an appropriate amount (between 100mg to 1g) of each of the four analytical samples, DNA is extracted and the total DNA content is determined by PicoGreen[®] as described above or using an equivalent DNA quantification method (e.g. NanoDrop spectrophotometric analysis).
- From the extracted DNA, a suitable amount (e.g. 20 ng/reaction) is analysed once for the presence of the respective genetic elements.
- All PCR analyses are performed as described above. For the detection of GM rice originating from China in food and feed products, PCR methods for the following genetic elements are to be included: the phospholipase D (PLD-rice) for rice and the 35S promoter (P-35S) from Cauliflower Mosaic Virus, the nopaline synthase terminator (T-NOS) from *Agrobacterium tumefaciens* and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac).

As a positive control for the P-35S, T-NOS and CryIAb/Ac Real-time PCR methods the Bt11 and MON 810 GM maize produced by IRMM CRM could be used (see table 2). As a positive control for rice, any rice material (e.g. rice grain) can be used as a source of rice genomic DNA.

Comments:

- Several GMOs authorized for food and feed use under Regulation EC/2003/1829 contain the P-35S, T-NOS and CryIAb/Ac GM genetic elements. Thus, positive signals in rice consignments may originate also from the presence of these GMOs. Consequently, the presence of an approved GMO may mimic the presence of a non-approved GMO. However, for the specific scope of this control measure, the presence or absence of approved GMOs is considered irrelevant and it is not requested that laboratories should carry out any form of ingredient determination.
- The P-35S and the T-NOS GM genetic elements are naturally occurring in Cauliflower Mosaic Viruses and *Agrobacterium* strains, respectively. In line with ISO 21569, when a sample tests positive for P-35S, the method described by Wolff *et al.*, (2000) may be used to rule out the possibility that a detected signal is due to the presence of CaMV. Other methods that perform as efficiently may be applied (e.g. Cankar *et al.*, 2005, Chaouachi *et al.*, 2008). No information on PCR methods aiming specifically at detecting pathogenic *Agrobacterium* strains is available. A generic *Agrobacterium* method has been published (Weller *et al.*, 2002). In any case, rice products are unlikely to contain traces of these pathogenic organisms as the species is not considered a natural host of these micro-organisms.
- The assignment of positive signals for the respective genetic elements in rice consignments aimed at in this regulation to a GMO authorized under EU legislation can be performed according to the current standard laboratory practices for GMO detection.

- The CryIAb/Ac genetic element detected by the SYBR[®]Green method is a synthetic sequence that does not naturally occur in *Bacillus* strains.

Interpretation of the SYBR[®]Green PCR results per subsample per GM genetic element:

The T_m- and C_t-values obtained from each genetic element are to be scored applying the beforehand established acceptance ranges for both the T_m- and the C_t-value of each method (see above).

In SYBR[®]Green Real-time PCR analysis two parameters, the T_m and the C_t-value, determine the presence of a genetic element in a sample. The ten possible combinations for the outcome of analysis based on these 2 parameters are shown in table 2.A.

In all cases, a negative T_m-value results in a negative outcome, notwithstanding a positive C_t-value as the T_m-value is the confirmatory parameter for the presence of the target.

Interpretation of the Taqman[®] PCR results per subsample per GM genetic element:

In Taqman[®] Real-time PCR only the C_t-value determines the presence of a genetic element in a sample, only three combinations for this parameter (++; +--; --) are possible (see Table 2.B).

In all cases where dissimilar PCR outcomes are obtained between both extracts for a GM genetic element of an analytical sample, an additional PCR analysis on the extract with the positive outcome for the presence of this particular genetic element is to be performed. When this PCR is negative, the genetic element is to be scored as 'Not detected'; on the other hand when this PCR is again positive, the genetic element is to be scored as 'Detected'.

Four examples of possible outcomes of the analysis of a rice consignment originating from China are shown in Figure 1.

The detection of any of these three genetic elements (P-35S, T-NOS and CryIAb/Ac) in an analytical sample of a rice consignment indicates that this product contains GM material. Compliance with the certification documents accompanying the consignment needs to be verified. Any non-compliance should be reported.

7. Deviations reported

No deviations have been reported during the course of these analyses.

8. Summary of results

8.1 Testing of the specificity of Real-time methods on GM rice samples

The SYBR[®]Green and the Taqman[®] Real-time PCR methods were tested for their specificity on non-GM rice, the LibertyLink GM rice events LLRICE 601 and LLRICE 62 and on the GM events Bt63, Kefeng6 and KMD1 originating from China. The results of the analysis are shown in Table 3.

All methods are shown to detect the expected genetic elements in each of the GM events and do not give background signals with non-GM rice.

In separate studies, these methods were shown not to generate false positive signals in genomic DNA from wild-type soya, maize, cotton, rapeseed, potato and sugar beet (Barbau-Piednoir *et al.*, 2010 and 2011; Waiblinger *et al.*, 2008 and 2010).

8.2 Testing of the sensitivity of the Real-time methods on GM rice samples

The integrity of the respective genomic DNA samples of all tested materials was verified by agarose gel-electrophoresis. All materials contained high-molecular weight genomic DNA and no visual traces of degradation could be observed (see Figure 2).

As the exact insert(s) present in the respective GM rice events originating from China has not been officially reported to the EURL-GMFF, the precise number of GM genetic elements in terms of expected haploid genome copies could not be determined. For this, a minimal performance sensitivity test was performed to estimate the sensitivity of the SYBR[®]Green PCR methods for detection of GM rice Bt63 and Kefeng6. The sensitivity of the Taqman[®] methods has been assessed by the method developers and reported by Van den Eede (2010).

For this, a dilution series ranging from an estimated 40 to 0.1 HGE was analysed in six repetitions with the three SYBR[®]Green PCR methods (see table 4).

All methods are shown to detect reproducibly (6/6 positives) at least an estimated five haploid genome copies in both GM events. For all methods, the estimated 0.1 HGE level still resulted in relatively frequent numbers of positive results (up to 3/6 positives). Considering that the no-template controls and the wild-type rice controls do not give any positive signal, this unexpected higher frequency is presumably due to the presence of multiple copies of the respective genetic elements at different chromosomal locations in both GM rice events.

9. Guidance to certifying laboratories

Laboratories certifying rice consignments destined to the EU market to test for the presence of GM rice should apply the detection methods documented in this report and certification reports should document at least the testing for a rice taxon-specific method and for the P-35S, the T-NOS and the CryIAb/Ac GM genetic elements as described in this report. The pLOD shall also be reported when appropriate. For practical guidance for determining the LOD, reference is made to the ENGL report on "Verification of analytical Methods for GMO testing when implementing interlaboratory validated methods".

Practical limit of detection (pLOD):

Definition = The pLOD is the lowest quantity (number of units; X) of the **target** that can be detected with an acceptable probability, relative to the detected quantity (number of units; Y) of the corresponding species in the specific sample, expressed as concentration (%).

Explanatory comment:

Theoretically, the probability of a false negative test result (type II error) is $< 5\%$ if the nominal (expected) number of units sampled in a single **test** (X) is 5 when the units are randomly distributed in the sample material. Similarly, the probability of a false negative test result (type II error) is $< 1\%$ if the nominal number of units (X) is 7. Experimental evidence from validation of GMO detection methods show that the absolute LOD is in the range of 5-10 copies, and it is therefore reasonable to set as a fixed value $X = 10$ in the absence of detailed experimental evidence for each of the targets relevant to GMO testing.

Notably, X is independent of the type of material (e.g. grains or copies of a specific DNA sequence motif).

$$pLOD = \frac{X}{Y} \times 100\%$$

If the material in question is grains (or other similarly near-homogeneously sized particles) then the pLOD is likely to be a function of the number of grains in the sample. Thus, if 10^4 grains are sampled then the grain (particle) based pLOD is:

$$pLOD_{\text{grain}} = \frac{10 \text{ grains}}{10^4 \text{ grains}} \times 100\% = 0.1\%$$

If the material in question is processed (e.g. flour) or liquid, then it is reasonable to assume that the DNA present in the sample is a more representative estimator than e.g. particles. Then the pLOD is likely to be a function of the number of haploid genome equivalents of the species to which the target belongs. The number of haploid genome equivalents is determined/estimated on the basis of detected number of copies of a reliable single copy species specific DNA sequence motif.

Thus, if the species is rice and 2×10^4 copies of such a rice specific DNA sequence motif are detected then the DNA based pLOD is:

$$pLOD_{\text{DNA}} = \frac{10 \text{ GM target sequence copies}}{2 \times 10^4 \text{ species specific sequence copies}} \times 100\% = 0.05\%$$

Notably, the true pLOD that is to be reported is the highest (inferior) of the pLOD_{grain} and pLOD_{DNA} if the material in question is grains or particles.

10. Conclusions

The P-35S, T-NOS and CryIAb/Ac SYBR® Green Real-time PCR methods and a P-35S/T-NOS duplex Taqman® Real-time PCR method allowed detecting the respective GM genetic elements in control samples of GM rice originating from China. All methods are capable to detect at least an estimated five haploid genome copies of the GM rice Bt63 and Kefeng6 control DNAs.

The P-35S and T-NOS Taqman® methods described in the P-35S/T-NOS duplex Taqman® Real-time PCR method could also be used in a simplex Taqman® format, although they were not tested in this format on the rice control materials.

These Real-time PCR methods can thus be used to screen rice consignments originating from China for the presence of GM materials.

11. Quality assurance

The EURL-GMFF carries out all operations according to ISO 9001:2008 (certificate number: CH-32232) and all technical activities regarding method validation under Regulation.EC1981/2006 according to ISO 17025:2005 (certificate number: ACCREDIA 1172, [Flexible Scope for DNA extraction and qualitative /quantitative PCR] - Reference Methods available at the Accredia WebSite under: http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7).

12. References

- Arumuganathan K, Earle ED (1991) "Nuclear content of some important plant species" *Plant Molecular Biology Reporter* 9:208-218
- Barbau-Piednoir E, Lievens A, Mbongolo Mbella EG, Roosens N, Sneyers M, Leunda-Casi A, Van den Bulcke M. (2010) "SYBR®Green qPCR screening methods for the presence of "35S promoter" and "NOS terminator" elements in food and feed products" *Eur Food Res Technol* 230:383-393
- Barbau-Piednoir E, Lievens A, Mbongolo Mbella EG, Leunda-Casi A, Roosens N, Sneyers M, and Van den Bulcke M. (2011) "SYBR®Green qPCR screening methods for the detection of Roundup Ready®, LibertyLink® and CryIAb traits in genetically modified materials" *Eur Food Res Technol* DOI 10.1007/s00217-011-1605-7
- Cankar K, Ravnikaar M, Zel J, Gruden K, Toplak N (2005) "Real-time polymerase chain reaction detection of Cauliflower mosaic virus to complement the 35S screening assay for genetically modified organisms" *J. AOAC Int.* 88(3): 814-822.

Chaouachi M, Fortabat MN, Geldreich A, Yot P, Kerlan C, Kebdani N, Audeon C, Romaniuk M, Berteau Y (2008) "An accurate real-time PCR test for the detection and quantification of cauliflower mosaic virus (CaMV): applicable in GMO screening" *European Food Research and Technology* 227, Number 3, 789-798

ENGL report on "Verification of analytical Methods for GMO testing when implementing interlaboratory validated methods" (2011) EUR 24790 EN doi:10.2788/88038

Jiang L., Yang L, Zhang H, Guo J, Mazzara M, Van den Eede G, Zhang D. (2009) "International Collaborative Study of the Endogenous Reference gene, Sucrose Phosphate Synthase (SPS), Used for Qualitative and Quantitative Analysis of Genetically Modified Rice" *J. Agric. Food Chem. Vol 57*, 3525–3532 3525; DOI:10.1021/jf803166p

Mazzara M, Grazioli E, Savini C, Van den Eede G (2006) "Event-specific Method for the Quantification of Rice line LLRICE62 Using Real-time PCR – Validation Report and Protocol – Sampling and DNA extracton of Rice" EUR22490 EN JRC34091 (ISBN 92-79-03129-5) http://gmo-crl.jrc.ec.europa.eu/summaries/LLRICE62_validated_Protocol.pdf

Mbongolo Mbella EG, Lievens A, Barbau-Piednoir E, Sneyers M, Leunda-Casi A, Roosens N, and Van den Bulcke M. (2011) "SYBR®Green qPCR methods for detection of endogenous reference genes in commodity crops: a step ahead in combinatory screening of Genetically Modified Crops in food and feed products" *Eur Food Res Technol* 232:485–496

Van den Bulcke M, Lievens A, Barbau-Piednoir E, Mbongolo Mbella EG, Roosens N, Sneyers M, Leunda Casi A (2010) "A theoretical introduction to "Combinatory SYBR®Green qPCR Screening", a matrix-based approach for the detection of materials derived from genetically modified plants" *Anal Bioanal Chem* 396:2113-2123

Van den Eede G (2010) "Compendium of reference methods for GMO analyses" <http://publications.jrc.ec.europa.eu/repository/handle/111111111/15068>

Waiblinger, HU, Ernst B, Anderson A, and Pietsch.K (2008) "Validation and collaborative study of a P35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products" *Eur Food Res Technol* 226:1221-1228

Waiblinger HU, Grohmann L, Mankertz J, Engelbert D, Pietsch K (2010) "A practical approach to screen for authorised and unauthorised genetically modified plants" *Anal Bioanal Chem* 396(6): 2065-2072

Weller SA, Simpkins SA, Stead DE, Kurdziel A, Hird H, Weekes R (2002) "Identification of *Agrobacterium* spp. present within *Brassica napus* seed by TaqMan PCR – implications for GM screening procedures" *Arch Microbiol* (2002) 178 :338–343

Table 1: Description of the respective GM rice genetic elements, their corresponding primer sequences, and the molecular weight of the different Real-time PCR amplicons used in this study

Abbreviations: "PLD-rice": coding region of the phospholipaseD of *Oryza sativa* (rice); "P-35S" : promoter (P) and terminator (T) of the 35S gene from Cauliflower Mosaic Virus; "T-NOS": terminator (T) of the nopaline synthase gene of *Agrobacterium tumefaciens*; "CryIAb/Ac": coding region of *Bacillus thuringiensis* CryIAb and/or CryIAC insect resistance genes; 'cosyps' = 'Combinatory SYBR®Green PCR Screening'; bp = basepairs: FAM = 6-carboxy-fluoresceine; BHQ1 = Black Hole Quencher 1; YY = Yakima Yellow.

GM rice genetic element (method)	Primer name	Primer sequence	Amplicon size [bp]	References
PLD-rice (cosyps)	PLD 3959F	GCTTAGGGAACAGGGAAGTAAAGTT	80	Mbongolo Mbella et al., 2011
	PLD 4038R	CTTAGCATAGTCTGTGCCATCCA		
P-35S (cosyps)	35S_N3Fwd	AAAGCAAGTGGATTGATGTGATA	75	Barbau-Piednoir et al, 2010
	35S_N3Rev	GGGTCTTGCGAAGGATAGTG		
T-NOS (cosyps)	tnos_NN_Fwd	GATTAGAGTCCCGCAATTATACATTAA	69	Barbau-Piednoir et al, 2010
	tnos D REV	TTATCCTAGKTTGCGCGCTATATTT		
CryIAb/Ac (cosyps)	CryIAb_Bt.Cott_Fwd	ACCGGTTACACTCCCATCGA	73	Barbau-Piednoir et al, 2011
	CryIAb_Bt.Cott_Rev	CAGCACCTGGCACGAACTC		
P-35S (duplex)	35S-FTM	GCCTCTGCCGACAGTGGT	82	Waiblinger et al., 2008
	35S-RTM	AAGACGTGGTTGGAACGTCTTC		
	35S-TMP-FAM	FAM-CAAAGATGGACCCCCACCCACG-BHQ1		
T-NOS (duplex)	180-F	CATGTAATGCATGACGTTATTTATG	84	Waiblinger et al., 2008
	180-R	TTGTTTTCTATCGGTATTAAATGT		
	TM-180YY	YY-ATGGGTTTTTATGATTAGAGTCCCGCAA-BHQ1		

Table 2: Decision Table for the outcome of a Real-time PCR analysis of DNA extracts from a rice analytical samples (Ext = 'extract')

A: SYBR®Green PCR methods

GM rice genetic element	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6		Case 7		Case8		Case 9		Case 10	
	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t
Extract 1	+	+	-	-	-	+	+	-	+	-	+	-	+	-	-	+	-	+	+	-
Extract 2	+	+	-	-	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+
Interpretation	Positive		Negative		Negative		Negative		Repeat Ext.2		Repeat Ext.2		Repeat Ext.1		Repeat Ext.2		Negative		Negative	

B: Taqman® PCR methods

GM rice genetic element	Case 1		Case 2		Case 3	
Extract 1	+		+		-	
Extract 2	+		-		-	
Interpretation	Positive		Repeat Ext. 1		Negative	

Table 3: Specificity assessment of the four SYBR® Green (A) and the duplex Taqman qPCR (B) methods using gDNA from relevant GM-rice events and testing of two rice consignments originating from China.

Annotations: For the C_t-values: a “+” means (exponential) amplification and a C_t-value above the LOD, “-” means no amplification or amplification below the LOD; For the T_m-values: a “+” means that the observed T_m-value equals the T_m of the positive control $\pm 1^{\circ}\text{C}$, while a “-” means that the observed T_m-value differs more than $\pm 1^{\circ}\text{C}$ from the T_m of the positive control; “PLD-rice”: coding region of the phospholipase D of *Oryza sativa* (rice); “CryIAb/Ac”: coding region of *Bacillus thuringiensis* CryIAb and/or CryIac insect resistance genes; “P-35S”: promoter (P) and terminator (T) of the 35S gene from Cauliflower Mosaic Virus; “T-NOS”: terminator (T) of the nopaline synthase gene of *Agrobacterium tumefaciens*.

Sample Name	Species	GM % [m/m]	Origin	PLD – rice (Sybr)			CryIAb/Ac (Sybr)			P-35S (Sybr)			T-NOS (Sybr)			P-35S (Taq)		T-NOS (Taq)	
				Expected presence	C _t	T _m	Expected presence	C _t	T _m	Expected presence	C _t	T _m	Expected presence	C _t	T _m	Expected presence	C _t	Expected presence	C _t
Bt11	Maize	100	EU-RL GMFF	No	-	-	Yes	25.52	78.2*	Yes	27.43	76.3	Yes	25.52	71.3	Yes	26.02	Yes	27.72
MON 810	Maize	100	EU-RL GMFF	No	-	-	Yes	26.18	79.5	Yes	30.72	76.1	No	-	-	Yes	27.24	No	-
Wt Minghui	Rice	0	EU-RL GMFF	Yes	+	77.2	No	-	-	No	-	-	No	-	-	No	-	No	-
LLRICE 62	Rice	100	EU-RL GMFF	Yes	+	77.2	No	-	-	Yes	27.41	75.9	No	-	-	Yes	26.19	No	-
LLRICE 601	Rice	100	EU-RL GMFF	Yes	+	77.0	No	-	-	Yes	29.56	75.9	No	-	-	Yes	28.44	No	-
Bt63	Rice	Not reported	EU-RL GMFF	Yes	+	77.2	Yes	23.39	78.8	No	-	-	Yes	26.61	71.3	No	-	Yes	27.52
Kefeng6	Rice	Not reported	EU-RL GMFF	Yes	+	77.2	Yes	23.19	78.6	Yes	26.66	75.9	Yes	27.21	71.3	Yes	24.61	Yes	25.90
KMD1	Rice	Not reported	EU-RL GMFF	Yes	+	77.2	Yes	27.4	78.2	Yes	31.46	75.9	Yes	30.21	71.1	Yes	29.28	Yes	29.34

* In bold, the correspondence of the measured T_m-values between the positive controls and the GM rice control samples is indicated. The CryIAb target in the GM maize Mon810 has a different sequence, resulting in a slightly different T_m-value.

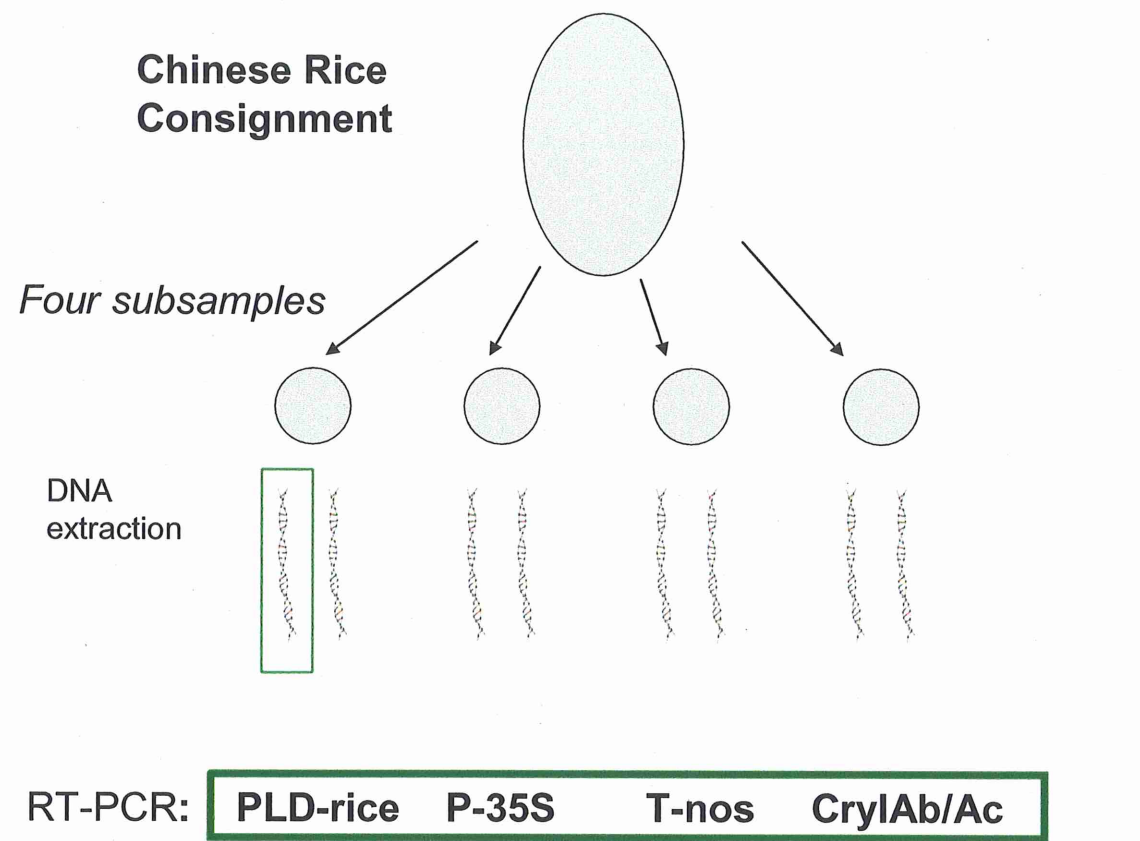
Table 4: Sensitivity testing of the P-35S, T-NOS and CryIAb/Ac SYBR®Green Real-time PCR methods on a dilution series of genomic DNA from the GM rice events Kefeng6 and Bt63. The indicated values of the T_m and C_t are the average of all positives.

Kefeng6 rice									
estimated HGE	# pos. (n=6)	P-35S		T-nos		CryIAb/Ac			
		C _t	T _m	# pos. (n=6)	C _t	T _m	# pos. (n=6)	C _t	T _m
40	6	30.15	76.25	6	28.12	71.72	6	27.17	79.40
20	6	31.09	76.08	6	29.16	72.10	6	28.18	79.13
10	6	31.95	76.35	6	30.08	71.77	6	29.24	79.35
5	6	33.12	76.30	6	31.17	71.43	6	30.10	79.10
1	6	35.23	76.05	6	33.19	71.5	6	31.94	79.15
0.1	3	37.91	76.13	3	36.44	71.47	1	34.60	78.80
0.02	0	-	-	1	36.79	72	1	34.18	78.9
0.002	0	-	-	0	-	-	0	-	-

Bt63 rice

estimated HGE	# pos. (n=6)	T-nos		CryIAb/Ac			
		C _t	T _m	# pos. (n=6)	C _t	T _m	
40	6	29.87	71.98	6	27.97	79.10	
20	6	30.95	72.15	6	28.57	78.92	
10	6	32.01	71.87	6	29.91	78.55	
5	6	32.81	71.62	6	30.75	79.23	
1	5	35.33	71.98	5	32.87	78.9	
0.1	3	36.61	71.47	2	34.53	79.2	
0.02	0	-	-	1	34.26	78.5	
0.002	0	-	-	0	-	-	

Figure 1: Analytical scheme of the PCR screening procedure for the presence of GM rice materials in rice consignments originating from China (detailed description is given in the text)



Outcome of RT-PCR screening (3 examples)

Product A	Subsample 1	Subsample 2	Subsample 3	Subsample 4		
PLD-rice	+/+	+/+	+/+	+/+	Outcome: No positive results	Conclusion: GM negative
P-35S	-/-	-/-	-/-	-/-		
T-nos	-/-	-/-	-/-	-/-		
CryIAb/Ac	-/-	-/-	-/-	-/-		
Product B	Subsample 1	Subsample 2	Subsample 3	Subsample 4		
PLD-rice	+/+	+/+	+/+	+/+	Outcome: Positive results	Conclusion: GM positive
P-35S	-/-	-/-	-/-	-/-		
T-nos	-/-	+/+	-/-	-/-		
CryIAb/Ac	-/-	+/+	-/-	-/-		
Product C	Subsample 1	Subsample 2	Subsample 3	Subsample 4		
PLD-rice	+/+	+/+	+/+	+/+	Outcome: Positive results	Conclusion: GM positive
P-35S	-/-	-/-	-/-	-/-		
T-nos	+/-	-/-	-/-	+/+		
CryIAb/Ac	-/-	-/-	-/-	+/+		
Product D	Subsample 1	Subsample 2	Subsample 3	Subsample 4		
PLD-rice	+/+	+/+	+/+	+/+	Outcome: Dissimilar result Retest: if “+” if “-”	Conclusion: GM positive GM negative
P-35S	-/-	-/-	-/-	-/-		
T-nos	+/+	-/-	-/-	-/-		
CryIAb/Ac	-/-	-/-	-/-	-/-		

Figure 2: Agarose gel-electrophoresis of genomic DNA of control samples of GM rice applied in this study. In all assays, 100ng of total genomic DNA was loaded. Sample order: M (= Molecular weight marker); 1: LLRICE 62 (AOCS); 2: Bt63 (EURL-GMFF); 3: Kefeng6 (EURL-GMFF); 4: LLRICE 62 (EURL-GMFF); 5: LLRICE 601 (EURL-GMFF); 6: MingHui (EURL-GMFF).

