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Revised Guidance on the Detection of Genetically Modified Rice Originating from China Using Real-Time PCR for the detection of P-35S, T-nos and Cry1Ab/Ac

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Revised Guidance on the Detection of Genetically Modified Rice Originating from China Using Real-Time PCR for the detection of P-35S, T-nos and Cry1Ab/Ac

8 May 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In support to the Commission Implementing Decision 2013/287/EU[1], amending Decision 2011/884/EU[2], the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) prepared a revision of the previously[3] published guidance document. This document provides further guidance on the correct use of the methods indicated in the Decision, including measures aimed at improving the specificity of the detection approach.

This revised guidance, as its previous version, is exclusively meant for the implementation of Decision 2013/287/EU and should not be used for other screening activities. Laboratories should apply it only in conjunction with good standard practices for testing for the presence of GMOs (e.g. use of appropriate controls).

[1] COMMISSION IMPLEMENTING DECISION of 13 June 2013 amending Implementing Decision 2011/884/EU on emergency measures regarding unauthorised genetically modified rice in rice products originating from China. 2013/287/EU

[2] COMMISSION IMPLEMENTING DECISION of 22 December 2011 on emergency measures regarding unauthorised genetically modified rice in rice products originating from China and repealing Decision 2008/289/EC. 2011/884/EU

[3] 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. Available at http://gmo-crl.jrc.ec.europa.eu/doc/RiceChinaEmer-P35S-T-NOS-CRYIABAC.pdf

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Content

1. Background

Issues with rice products originating in or consigned from China, contaminated with the unauthorised genetically modified rice Bt 63 date back to the first RASFF (Rapid Alert System for Food and Feed) notifications in 2006. The Commission subsequently adopted Commission Decision 2008/289/EC[4].

In addition to Bt63, other insect resistant rice lines e.g. Kefeng6, Kemingdao1 were found on the market along with several notifications to the RASFF. As a result of these findings, the Commission and the Member States decided to adopt Decision 2011/884/EU on emergency measures regarding unauthorised genetically modified rice in rice products originating from China.

The EU-RL GMFF has attempted to compile an overview of GM rice varieties that could be a source of contamination in rice products imported from China. 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 recommended approaches for detection and identification, depending on the exact knowledge of the GM event(s) and the availability of (certified) reference material, cannot be used for these specific cases. In addition, for the same reasons, laboratories accredited under ISO/IEC 17025[5] cannot include related test procedures into the scope of their accreditation. It is also impossible to set-up proficiency tests for assessing the performance of laboratories. However, it is required that the analytical results obtained are of the highest possible quality and that control laboratories use the same, or identical analytical procedures.

In support to Commission Implementing Decision 2011/884/EC, the EU-RL GMFF had previously carried out an in-house verification study to assess the performance of the P-35S, T-nos and Cry1Ab/Ac SYBR®Green real-time PCR methods and a P-35S/T-nos duplex TaqMan® real-time PCR method and their suitability to detect these markers in rice material. The results of this assessment are published by Kluga *et al*. 2013 [1)].

Based on these results, the EU-RL GMFF prepared a guidance document[6] to assist laboratories on the correct implementation of the detection methods listed in the Decision.

From the experience gained by Member States in Implementing Decision 2011/884/EU and from information collected from stakeholders by the Commission, it appeared necessary to adjust some of the requirements set out in that Decision. Decision 2013/287/EU arose from these amendments. This new Decision does not modify the list of detection methods to be used but introduced some modifications in the sampling protocol and in the number of analytical samples to be analysed. The new Decision also

[4] COMMISSION IMPLEMENTING DECISION of 22 December 2011 on emergency measures regarding unauthorised genetically modified rice in rice products originating from China and repealing Decision 2008/289/EC. 2011/884/EU

[5] ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories.

[6] 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. Available at http://gmo-crl.jrc.ec.europa.eu/doc/RiceChinaEmer-P35S-T-NOS-CRYIABAC.pdf

clarifies certain other aspects not related to the detection methods. Updates include measures aiming at minimising the occurrence of false positive results.

2. 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 [7] as regards 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 [2)], the SYBR®Green PCR method [3)] and the TaqMan® phospholipase D (PLD) PCR method validated by the EU-RL GMFF [4)].

When performing GMO screening tests that target elements that are derived from naturally infecting organisms (such as viruses or bacteria), like the P-35S and the T-nos targeted in the Decision 2013/287/EU, it is standard practice to verify that any positive signal for any of these genetic elements 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 would be absent in the GMO, should be performed in case of a positive signal. A common example in GMO analysis is testing for CaMV presence to ascertain that positive signals obtained for the CaMV 35S promoter are derived from GMO and not from the Cauliflower Mosaic Virus. However, as long as no event-specific methods are available for GM rice events originating from China, the presence of a naturally infecting organism in an analytical sample taken from a consignment originating from China cannot be taken as confirmation of the absence of GM rice in that consignment. The naturally infecting organism could mask the presence of GM rice.

[7] ISO 24276, Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products – general requirements and definitions.

3. Sampling and analysis of the laboratory sample

3.1 Analysis of the laboratory sample in case of grain samples

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

Note: In line with Article 11(5) of Regulation (EC) No 882/2004 [8], a second laboratory sample shall be constituted.

- From the homogenised laboratory sample four analytical samples of 240 g (equivalent to 10,000 rice grains each) are taken.

- The four analytical samples are ground and further analysed separately (see figure 1)

- DNA is extracted from two test portions (e.g. between 100 mg and 1 g) of each of the four analytical samples, and the total DNA content is determined by e.g. PicoGreen® or using an equivalent DNA quantification method.

- From each of the extracted DNA isolates a suitable amount (preferentially at least 20 ng/reaction) is analysed once for the presence of each of the three genetic elements, using the methods specified by Decision 2011/884/EU and applying the decision criteria detailed below (see 4.1 and 4.2). Additionally, the rice-specific phospholipase D (PLD) target sequence has to be analysed.

3.2 Analysis of the laboratory sample in case of processed products

- A laboratory sample of 2.5 kg (that can be reduced to 500 g) is to be provided to the analysing laboratory.

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

- From the homogenised laboratory sample, one analytical sample of 125 g is prepared. This is regarded sufficient because by definition a processed product is considered to be homogenous.

- The analytical sample is ground and analysed (see figure 2)

- DNA is extracted from two test portions (e.g. between 100 mg and 1 g) of the analytical sample and the total DNA content is determined by e.g. PicoGreen®, or using an equivalent DNA quantification method.

- From each of the extracted DNA isolates a suitable amount (preferentially at least 20 ng/reaction) is analysed once for the presence of each of the three genetic elements, using the methods specified by Decision 2011/884/EU and applying the decision criteria

[8] REGULATION (EC) No 882/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules

detailed below (see 4.1 and 4.2). Additionally, the rice-specific phospholipase D (PLD) target sequence has to be analysed.

All PCR analyses should be performed as follows:

Targets
For the detection of GM rice originating from China in food and feed products, PCR methods targeting the following DNA sequences are used:

- the phospholipase D (PLD) for rice,
- the 35S promoter (P-35S) from Cauliflower Mosaic Virus,
- the nopaline synthase terminator (T-nos) from *Agrobacterium tumefaciens*
- the Cry1Ab/Ac toxin from *Bacillus thuringiensis* (Cry1Ab/Ac) (see annex II Decision 2011/884/EU).

Details of the methods targeting the DNA sequences listed above are provided in table 1.

Table 1. Genetic elements targeted, corresponding primer/probe sequences and size of the amplicons.

Genetic element (method)	Primer name	Primer sequence	Amplicon size [bp]	References
PLD	PLD 3959F	GCTTAGGGAACAGGGAAGTAAAGTT	80	3)
	PLD 4038R	CTTAGCATAGTCTGTGCCATCCA		
P-35S	35S_N3Fwd	AAAGCAAGTGGATTGATGTGATA	75	5)
	35S_N3Rev	GGGTCTTGCGAAGGATAGTG		
T-nos	tnos_NN_Fwd	GATTAGAGTCCCGCAATTATACATTTAA	69	5)
	tnos D REV	TTATCCTAGKTTGCGCGCTATATTT		
Cry1Ab/Ac	CryIAb_Bt.Cott_Fwd	ACCGGTTACACTCCCATCGA	73	6)
	CryIAb_Bt.Cott_Rev	CAGCACCTGGCACGAACTC		
P-35S (duplex)	35S-FTM	GCCTCTGCCGACAGTGGT	82	7)
	35S-RTM	AAGACGTGGTTGGAACGTCTTC		
	35S-TMP-FAM	FAM-CAAAGATGGACCCCCACCCACG-BHQ1		
T-nos (duplex)	180-F	CATGTAATGCATGACGTTATTTATG	84	7)
	180-R	TTGTTTTCTATCGCGTATTAAATGT		
	TM-180YY	YY-ATGGGTTTTTATGATTAGAGTCCCGCAA-BHQ1		

Positive controls
As a positive control for the P-35S, T-nos and Cry1Ab/Ac real-time PCR methods the Bt11 maize CRM, purchased from IRMM, must be used. To identify a broader range of Cry1Ab/Ac targets detected by the Cry1Ab/Ac SYBR®Green real-time PCR method, also the IRMM CRM ERM-BF413k (maize MON 810) must be included. As a positive control for rice, any rice material can be used as a genomic DNA source.

Note:

Several GMOs authorised for food and feed use under Regulation (EC) No 1829/2003, contain the P-35S, T-nos and Cry1Ab/Ac 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 mask the presence of a non-approved GMO containing similar targets. 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 genetic elements are naturally present in a limited number of micro-organisms (respectively Cauliflower mosaic viruses (CaMV) and Agrobacterium strains). In line with ISO 21569 [9], when a sample tests positive for P-35S, the method described by Wolf et al.[8)] may also be used to rule out the possibility that a detected signal is due to the presence of DNA of the Cauliflower Mosaic Virus in the sample. Other methods that perform as efficiently may be applied (e.g. Cankar et al.[9], Chaouachi et al.[10)]). No information on PCR methods aiming specifically at detecting pathogenic Agrobacterium strains is available but a generic Agrobacterium method has been published (Weller et al. [11]).
In any case, the presence of a naturally infecting organism in an analytical sample cannot be taken as confirmation of the absence of GM rice in that consignment because the naturally infecting organism could mask the presence of GM rice and it is not requested that laboratories should carry out any form of micro-organism determination.

The Cry1Ab/Ac GM rice genetic element detected by the SYBR®Green method is targeting two different synthetic sequences (one similar to the synthetic sequence present in Bt11 and the other similar to the synthetic sequence present in MON 810) not known to naturally occur in Bacillus strains.

4. Interpretation of analytical results

As to date no official DNA sequence data are available on the complete GM inserts of any GM rice event originating from China, the presence of one of the above indicated genetic elements in a consignment of rice or rice products originating from China is regarded to be sufficient to assume presence of GM rice in the material.

In all cases where dissimilar PCR outcomes are obtained between both DNA extracts from the same analytical sample taken from rice or rice products originating from China for a GM genetic element of the same analytical sample, an additional PCR analysis on the extract with the positive outcome for this particular GM genetic element is to be performed. When this PCR is negative, the presence of this GM genetic element is to be scored as 'Not detected'; on the other hand, when this PCR is again positive, the GM genetic element is to be scored as 'Detected'. In case of rice, the laboratory sample is declared "Positive" if one of the 4 analytical samples is positive; in case of rice

[9] ISO 21569, Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – qualitative nucleic acid based methods

products, assumed to be homogenised, the laboratory sample is declared "Positive" if the GM genetic element is detected in the analytical sample (see figure 1 and 2).

The detection of any one of these three GM genetic elements in any of the analytical samples of a rice or rice product consignment from China indicates that this consignment contains GM rice material.

Three examples of possible outcomes of the analysis of a rice consignment originating from China are included in figures 1 and 2.

To establish presence or absence of any of the three target GM elements in an analytical sample the following criteria should be used:

4.1 SYBR®Green PCR methods (for P-35S, T-nos and Cry1Ab/Ac)

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_q-value fall within the criteria of a positive result, defined in point 2 and 3, below.

In all other cases (thus, when any of these two values is outside the range for a positive result = $T_m - C_q$ values are either pos-neg, or neg-pos, or neg-neg), the GM genetic element is considered as "not detected" by the applied method (see table 1).

2. For the C_q values, a measurement result is defined as positive only when an (exponential) amplification is observed and the fluorescence signal of the reaction is above the PCR threshold. In all other cases the measurement result is considered negative.

3. For the T_m values, a positive result is obtained only if the following conditions are met:

(A) The measured T_m value corresponds to the T_m of the corresponding GM genetic element in the positive control (± 1°C)[10].

(B) For reactions with $C_q \geq 33$ with the correct T_m value, the GM target is considered detected if the height of the T_m peak is ≥ 20% of the height of the T_m peak of the corresponding GM genetic element in the positive control[11].

[10] In the case of Cry1Ab/Ac the T_m should be compared to both positive controls (maize Bt11 and maize MON 810) as outlined in the corresponding technical guidance document.

[11] The T_m peak height should be established when the amplification curve has reached its plateau and, if possible, be averaged over repeated reactions; and the value of 20 % should only be used in case the amplification of the control reaction has reached its plateau.

4.2 TaqMan® PCR methods (for P-35S, T-nos)

1. A GM genetic element is considered as being detected by a particular method only if the measured C_q value falls within the criteria of a positive result.

2. A measurement is considered positive when an (exponential) amplification is obtained and the measured C_t is above the PCR threshold.

4.3 Interpretation of the SYBR®Green PCR results per subsample per GM genetic element (P-35S, T-nos, and Cry1Ab/Ac)

The T_m and C_q values obtained from the tests for each GM genetic element are to be scored applying the above established acceptance ranges for both the T_m and the C_q value of each method (see 4.1 above).

In SYBR®Green real-time PCR analysis two parameters, the T_m and the C_q value, determine the presence of a genetic element in a sample. The possible combinations for the outcome of the 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_q value, as the T_m value is the confirmatory parameter for the presence of the target. The possible combinations of these parameters and their interpretation are given in table 2.A.

4.4 Interpretation of the TaqMan® PCR results per analytical sample or subsample per GM genetic element (P-35S and T-nos)

As in TaqMan® real-time PCR only the C_q value determines the presence of a GM genetic element in a sample, only three combinations for this parameter (++; +-; --) are possible (see table 2.B).

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Table 2: Decision tables for the outcome of a real-time PCR analysis of DNA extracts (E) from a rice analytical samples

2.A: SYBR®Green PCR methods

The rules are:
1) If T_m and C_q are both positive, the measurement results is = (+) >> positive
2) If T_m or C_q are negative, the measurement result is negative

And:

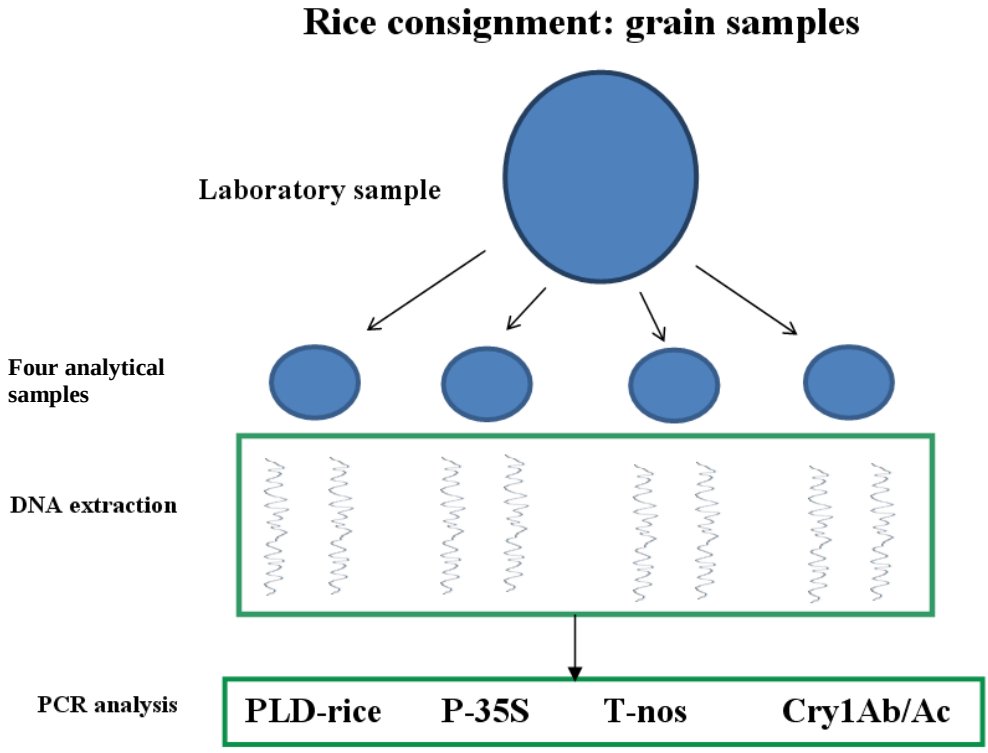
3) If E1 & E2 are positive, the measurement result is positive
4) If E1 & E2 are negative, the measurement result is negative
5) If E1 or E2 are positive and E2 or E1 are negative, repeat the positive extract. If the repetition is again positive, the final outcome is positive, too. If the repetition is negative the sample is negative.

	E 1	T_m C_q	T_m C_q	T_m C_q	T_m C_q
E 2		+ +	+ -	- +	- -
T_m C_q		Positive	Negative	Negative	Negative
+ +	Positive	Positive	Repeat E2	Repeat E2	Repeat E2
+ -	Negative	Repeat E1	Negative	Negative	Negative
- +	Negative	Repeat E1	Negative	Negative	Negative
- -	Negative	Repeat E1	Negative	Negative	Negative

2.B: TaqMan® PCR methods

GM genetic element	***Case 1***	***Case 2***		***Case 3***
E 1	+	+	-	-
E 2	+	-	+	-
Interpretation	Positive	Repeat E1	Repeat E2	Negative

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



Outcome of real-time PCR tests (3 examples)

Product A	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	-/-	-/-	-/-	-/-
CrylAb/Ac	-/-	-/-	-/-	-/-

Outcome: No positive results — *Conclusion:* GM negative

Product B	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	-/-	+/+	-/-	-/-
CrylAb/Ac	-/-	+/+	-/-	-/-

Outcome: Positive results — *Conclusion:* GM positive

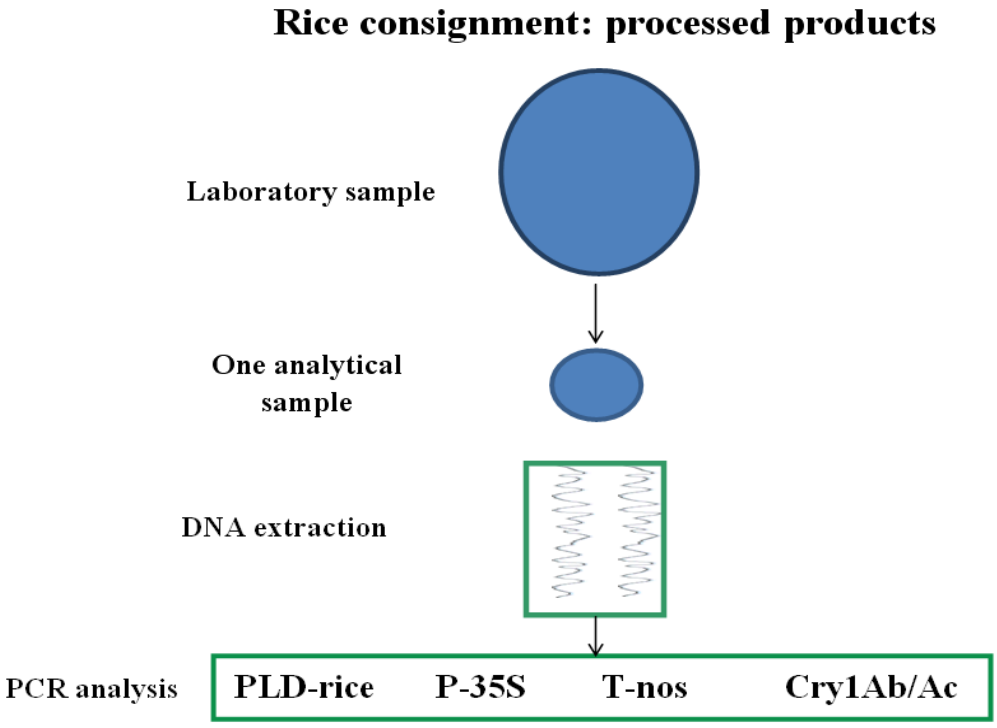
Product C	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	+/-	-/-	-/-	+/+
CrylAb/Ac	-/-	-/-	-/-	+/+

Outcome: Positive results — *Conclusion:* GM positive

Product D	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	+/-	-/-	-/-	-/-
CrylAb/Ac	-/-	-/-	-/-	-/-

Outcome: Dissimilar result — *Conclusion:*
Retest: if " +" GM positive
if " - " GM negative

Figure 2: Analytical scheme of the PCR screening procedure for the presence of GM rice materials in consignments of rice processed products originating from China (detailed description is given in the text)



Outcome of real-time PCR screening (3 examples)

		Conclusion
Product A	**Sample A**	
PLD-rice	+/+	**GM negative**
P-35S	-/-	
T-nos	-/-	
Cry1Ab/Ac	-/-	
Product B	**Sample B**	
PLD-rice	+/+	**GM positive**
P-35S	+/+	
T-nos	-/-	
Cry1Ab/Ac	-/-	
Product C	**Sample C**	
PLD-rice	+/+	**GM positive**
P-35S	-/-	
T-nos	-/-	
Cry1Ab/Ac	+/+	

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Abstract

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