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



Report on the In-House Validation of a Detection Method for Event Bt 10 Maize using a Qualitative PCR Assay

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In-house validation and reporting:
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EXECUTIVE SUMMARY

Following the Commission Decision of 18 April 2005 (2005/317/EC) "*on emergency measures regarding the non-authorised genetically modified organism Bt10 in maize products*", the JRC as Community Reference Laboratory (CRL) for the GM Food and Feed, Regulation EC 1829/2003), has carried out an in-house validation of the event-specific detection method proposed by GeneScan on Bt10 maize developed by Syngenta Crop Protection AG. The validation was conducted according to internationally accepted guidelines.

Syngenta Crop Production AG provided the DNA samples (genomic DNA extracted from the Bt10 maize line and from a control wild type maize line).

GeneScan provided the event-specific detection method based on a qualitative PCR assay.




The results of the JRC validation demonstrated that the method reliably detects an amplification product specific for Bt10 maize, and therefore allows discriminating event Bt10 from other GM-events in maize lines. The sensitivity of the method is below 0.1%

The absolute LOD of the method is below 20 copies. This corresponds to less than 0.1% in relative terms when 50 ng are analyzed in PCR.

Bt10 can be selectively detected also when present in low concentration in mixture with Bt11.

The method is therefore considered by the CRL as fit for the purpose of Bt10 detection and it is the only accepted to certify the presence of Bt10 in maize commodities in accordance with the Commission Decision 2005/317/EC.

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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) carried out the in-house validation of the event-specific detection method developed by GeneScan on Bt10 maize originated at Syngenta Crop Protection AG.

Upon reception of the protocol and control samples on April 21st, the JRC performed the validation of the method on April 21-22, 2005.

2. Experimental Validation and Results

The following performance characteristics were determined on Bt10 specific-event:

- DNA concentration
- Fragmentation state of DNA
- DNA Purity / absence of PCR-inhibitors
- Specificity
- LOD
- Selectivity

2.1 Bt10 genomic DNA and wild type genomic DNA

One sample of Bt10 genomic DNA was received by Syngenta Crop Protection AG, together with control wild type DNA (WT).

2.2 Control genomic DNA samples

Control genomic DNA to be used in specificity tests from the following samples was extracted according to Nucleospin Food method (Macherey-Nagel GmbH, cat n. 740 945.250) and is presented in Table 1.

Table 1. List of gDNA extracted by the JRC

GM EVENTS		NON GM EVENTS	
Sample DNA	Plant species	Sample DNA	Plant species
Bt10	Maize	Wt Syngenta	Maize
¹ Bt11	Maize	W79A	Maize
Bt11	Maize	W85	Maize
¹ MON810	Maize	B14	Maize
DAS-59122-7	Maize	CM7	Maize
MIR 604	Maize	EM	Maize
¹ GA21	Maize	IA153	Maize
¹ MON863	Maize	W117	Maize
T25	Maize	F64	Maize
TC1507	Maize		
¹ NK603	Maize		
STARLINK	Maize		
H7-1	Sugar beet		
¹ RoundUp Ready	Soy		
No Template Control			

¹Certified Reference Material (JRC, IRMM, Belgium)

2.3 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.

The concentration of Bt10 and wild type control provided by Syngenta Crop Protection AG are reported below:

Bt10 gDNA 70 ng/μl

Wt gDNA 87 ng/μl

Coefficient of variation $[(SD/mean)*100]$ of the measurements was 2.7% and 2.6%, respectively. Each gDNA was diluted to a working stock of 10 ng/μl.

2.4 Integrity check of Bt 10 and wt control DNA

A visual inspection on Bt10 and wt control DNA was performed by agarose gel (1.2%) electrophoresis with 3 μ l of each sample loaded and compared to lambda DNA as marker of molecular weight (M).

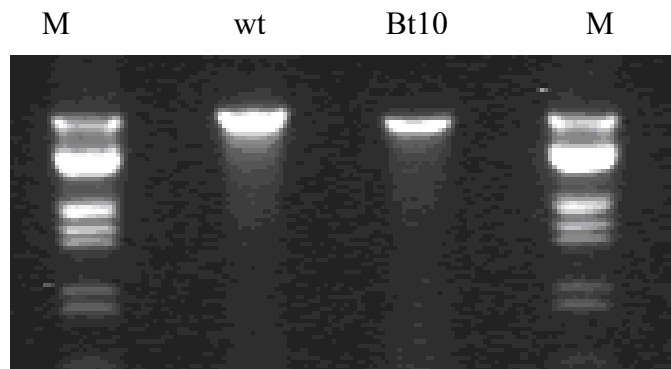


Fig.1. Agarose gel electrophoresis on Bt 10 and wild type DNA

Both DNA samples (Bt10 and wild-type) showed the major fraction being high molecular weight indicating high integrity of extracted DNA.

The same analysis was carried out for the other DNA samples and similar integrity was observed (data not shown).

2.5 Inhibition test

All samples were tested for the presence of PCR inhibitors in solution. A PCR amplification was conducted targeting the maize endogenous reference gene Alcohol dehydrogenase 1 (*Adh1*). To each reaction 5 μ l were added, and duplicate reactions were performed for each sample.

All samples tested positive, indicating that the DNA solutions did not contain inhibitors of the PCR at the tested concentration.

2.6 Specificity

In preliminary tests, DNA samples from GM and non-GM events, as shown in Table 1, were amplified with event Bt10 specific primers according to Genescan's instructions to determine the specificity of the method. Tests were performed in duplicate x 2 runs on two different GeneAmp PCR system 9700, Applied Biosystems.

2.6.1 Reagents and solutions

Table 2 lists the reagents, the final concentrations and volumes per each reaction of the specificity test.

Table 2. Reagents and concentration

Reagent	Concentration Stock	Final concentration	µl per 1 rxn
H ₂ O			15.38
MgCl ₂	25mM	1.5mM	1.5
Buffer	10x	1x	2.5
dNTPs	25mM each	160µM	0.16
forward primer	100µM	0.6µM	0.15
Reverse primer	100µM	0.6µM	0.15
Taq	5U/µl	0.032U/µl	0.16
DNA	10ng/µl	50 ng/rxn	5
Total volume (µl)			25

2.6.2 Primer sequence

Forward primer (JSF3) 5'- CAC ACA GGA GAT TAT TAT AGG G -3'

Reverse primer (JSR3) 5'- GGG AAT AAG GGC GAC ACG G -3'

Primers were synthesized at Proligo France SAS.

2.6.3 Amplification conditions

Table 3 shows the amplification conditions used in accordance with the detection method developed by GeneScan.

Table3. Amplification conditions

Step1	Step2				Step3	Step4
10 min	25 sec	30 sec	45 sec	Cycles	7 min	∞
94°C	94°C	62°C	72°C	40	72°C	4°C

2.6.4 Amplicon length in event Bt10

When amplifying Bt10 gDNA with the primer pair JSF3/JSR3 at the conditions detailed in Table 3, the expected amplicon length is 130 bp.

2.6.5 Results on agarose gel electrophoresis

Fifty nanograms of gDNA from different GM lines of maize (Bt11 certified reference material, Bt11 used in method validation at the Community Reference Laboratory, MON810, DAS 59122-7) plus 50 ng of Bt10 and wild-type (WT) gDNA were amplified with event Bt10 specific primers and analyzed on 2.7% agarose gel electrophoresis (Figure 2). The molecular weight of the resulting amplicons was compared to a ladder marker of molecular weight (M).

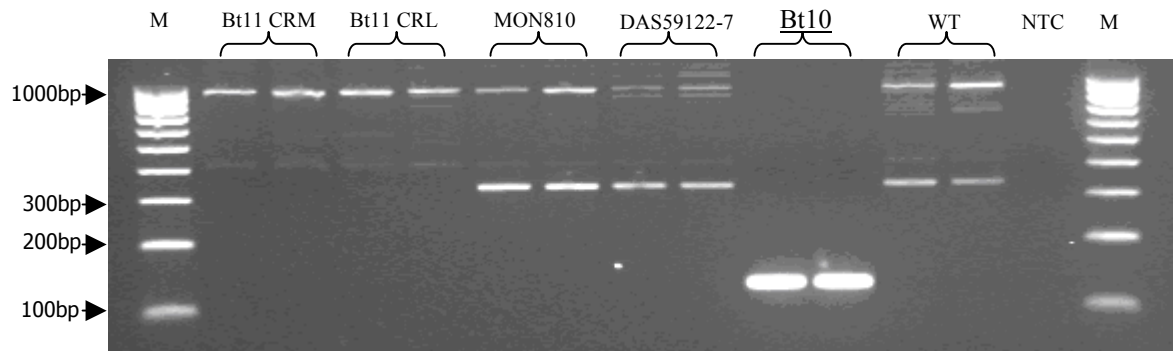


Fig. 2 Specificity test.

As shown in Fig. 2, a 130 bp band is amplified in event Bt10 gDNA, and it is not present in the amplification reactions of the other GM or non-GM lines.

Higher molecular weight amplicons are visible in the other GM-lines of maize and in wild-type maize DNA, but not in Bt10, indicating that unspecific amplicons are frequently synthesized. This suggests that the method can be further optimized.

The specificity of the assay was further tested on several GM-maize and non-GM maize lines, as well as on GM-soy and GM-sugar beet. The test was carried out according to the conditions outlined in Tables 2 and 3, using the primer pair JSF3/JSR3, in duplicate reactions in GeneAmp PCR system 9700, Applied Biosystems. Table 4 shows the obtained results. Presence of amplicon at 130 bp was classified as positive (+) and absence as negative (-) response.

Table 4. Results of specificity test

GM EVENTS		NON-GM EVENTS	
Sample DNA	Specificity Event Bt10	Sample DNA	Specificity Event Bt10
Bt10	+	Wt Syngenta	-
¹ Bt11	-	W79A	-
Bt11	-	W85	-
¹ MON810	-	B14	-
DAS-59122-7	-	CM7	-
MIR 604	-	EM	-
¹ GA21	-	IA153	-
¹ MON863	-	W117	-
T25	-	F64	-
TC1507	-	No Template Control	-
¹ NK603	-		
STARLINK	-		
H7-1 sugar beet	-		
¹ RoundUp Ready	-		

¹Certified Reference Material (JRC, IRMM, Belgium)

Only Bt10 gDNA provided a positive response, **indicating a very high level of specificity.**

2.7 Limit of Detection (LOD)

The LOD was calculated by amplifying Bt10 gDNA at defined copy numbers per reaction. Acceptance criterion was defined as the lowest copy number at which the presence of the amplicon could be detected at least 95% of the time, ensuring a $\leq 5\%$ false negative rate (ENGL, 2005). In the model, one copy of maize haploid genome is considered to correspond to 2.72 pg (Arumuganathan, K. *et al.* 1991).

Amplification conditions were as detailed above (Tables 2 and 3 with primer pair JSF3/JSR3).

Table 5. LOD test results

Copy numbers/rxn	Number of replicates	Positive results	Negative results
500	10	10	0
100	21	21	0
20	21	21	0
4	21	18	3
0	21	0	21

The absolute LOD of the event Bt10 specific method is <20 copies. This corresponds to <0.1% in relative terms when 50 ng are analysed in PCR.

2.8 Selectivity test

A selectivity test was performed by spiking Bt10 gDNA in Bt11 gDNA at the following final GM concentrations (DNA/DNA): 0%, 0.1% and 1% Bt10.

For each concentration, 21 replicates have been tested as described (Table 2 and 3, primer pairs JSF3/JSR3). The amplification reactions were run on a 2.7% agarose gel. Results are reported in Table 6. Figure 3 shows selective amplification of Bt10 maize at 0.1% and 1% concentrations.

Table 6. Selectivity test results

Bt10 concentration in Bt11	Number of replicates	Positive results	Negative results
1%	21	21	0
0.1%	21	21	0
0%	21	0	21

This result indicates that Bt10 can be selectively detected also when present in low concentration in mixture with Bt11.

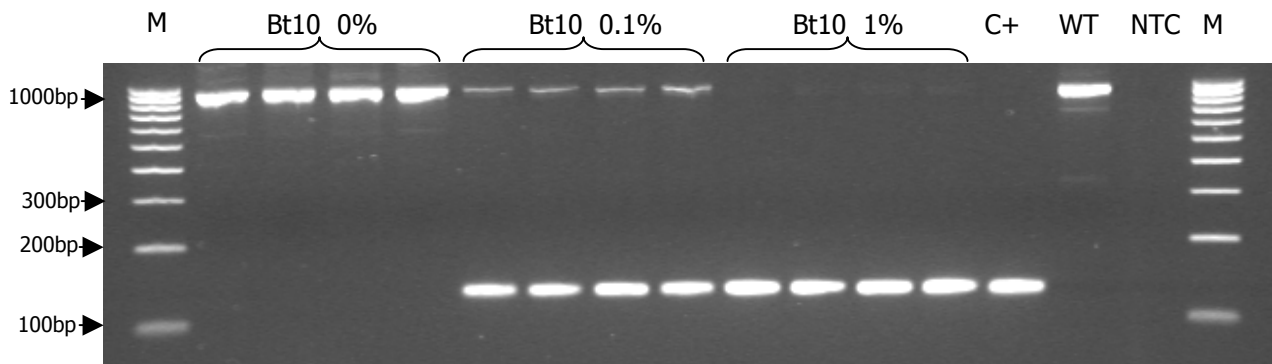


Fig.3. Selective amplification of Bt10 at 0.1% and 1% concentrations.
C+: positive control Bt10 100%

3. Conclusions

A 130 bp amplicon is detected in event Bt10 maize DNA following PCR assay performed according to the PCR-assay method provided by Genescan. The same amplicon band is not detected when analyzing either even Bt11 maize DNA or a wide range of GM and non-GM maize lines, in addition to GM soy and GM sugar beet.

Therefore, the CRL concludes that this amplicon is specifically detected in Bt10 and can discriminate between Bt10 and other GM-events in maize, including Bt11.

The absolute LOD of the event Bt10 specific method is <20 copies. This corresponds to <0.1% in relative terms when 50 ng are analyzed in PCR.

Bt10 can be selectively detected also when present in low concentration in mixture with Bt11.

The method is therefore fit for its intended purpose. However, at this stage of testing, the method produces a higher molecular-weight multi-band pattern in GM and non-GM maize which requires additional efforts in its optimization.

4. Literature

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. *Plant Mol Biol Reporter* 9, 208-218.

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>)