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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 an event-specific Detection Method for Event Bt 10 using a qualitative PCR assay and verification by restriction analysis

Protocol Version 2

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In-house validation and reporting:
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EXECUTIVE SUMMARY

Following the Commission Decision of 18 April 2005 (317/2005/EC) "*on emergency measures regarding the non-authorised genetically modified organism Bt10 in maize products*", on April 21-22, 2005 the JRC as Community Reference Laboratory (CRL) for the GM Food and Feed, Regulation EC 1829/2003), carried out an in-house validation of an event-specific detection method developed by GeneScan on Bt10 maize originated at Syngenta Crop Protection AG.

In the "Report on the In-House Validation of a Detection Method for Event Bt10 Maize using a Qualitative PCR Assay" (1), the CRL concluded that the method was fit for its intended purpose but pointed out that it required further additional efforts in its optimization.

On June 20th, 2005 GeneScan provided a third version of the event-specific detection method of Bt10 maize originated. In this version, that makes use of a new pair of primers, the robustness of the method has been improved and the multi-band pattern of unspecific amplification, detected in GM and non-GM maize, has been eliminated.

Upon reception of the new protocol, the JRC performed the validation of the method on July 4-8, 2005.

The results of the JRC validation demonstrate that the new method reliably detects an amplification product specific for Bt10 maize, and therefore allows discriminating event Bt10 from other GM-events in maize lines. In addition, no unspecific amplifications products are detected among GM-events in maize and among conventional maize, soybean and sugar beet, thus confirming that the new method is optimized for Bt10 event.

The sensitivity of the method is below 0.05%.

The absolute LOD of the method is 4 copies. This corresponds to 0.022% 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 accepted to certify the absence of Bt10 in maize commodities in accordance with the Commission Decision 317/2005/EC.

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**13-07-2005**	**signed**
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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 an in-house validation of an event-specific detection method developed by GeneScan on Bt10 maize originated at Syngenta Crop Protection AG.

In the "Report on the In-House Validation of a Detection Method for Event Bt10 Maize using a Qualitative PCR Assay" (1), the CRL concluded that the method was fit for its intended purpose but pointed out that it required further additional efforts in its optimization.

On June 20th, 2005 GeneScan provided a revised version of the event-specific detection method of Bt10 maize originated at Syngenta Crop Protection AG.

Upon reception of the new protocol, the JRC performed the validation of the method on July 4-8, 2005.

In this version, that makes use of a new pair of primers, the ruggedness of the method has been optimized and the multi-band pattern of unspecific amplification, detected in GM and non-GM maize, has been eliminated.

2. Experimental Validation and Results

The Bt10 genomic DNA sample had been provided by Syngenta Crop Protection AG and data regarding its concentration, fragmentation state and absence of PCR-inhibitors had been illustrated in the above mentioned Report (1).

In the present in-house validation of the new version of the detection method, the following performance characteristics have been determined on Bt10 specific-event:

- Specificity
- LOD
- Selectivity
- Enzymatic digestion of the Bt10 specific amplicon

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 isogenic Bt10).

2.2 Specificity

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

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 isogenic Bt10 (Syngenta)	Maize
[1]Bt11	Maize	W85	Maize
Bt11	Maize	B14	Maize
[1]MON810	Maize	CM7	Maize
DAS-59122-7	Maize	EM	Maize
MIR 604	Maize	IA153	Maize
Bt176	Maize	W117	Maize
[1]GA21	Maize	F64	Maize
[1]MON863	Maize		
T25	Maize		
TC1507	Maize		
[1]NK603	Maize		
STARLINK	Maize		
H7-1	Sugar beet		
[1]Roundup Ready	Soy		
No Template Control			

[1]Certified Reference Material (JRC, IRMM, Belgium)

2.2.1 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_2O			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

2.2.2 Primer sequence

Forward primer (JSF5) 5’- CAC ACA GGA GAT TAT TAT AGG GTT ACT CA -3’

Reverse primer (JSR5) 5’- ACA CGG AAA TGT TGA ATA CTC ATA CTC T -3’

Primers have been synthesized at Microsynth (CH).

2.2.3 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

2.2.4 Amplicon length in event Bt10

When amplifying Bt10 gDNA with the primer pair JSF5/JSR5 at the conditions detailed in Table 3, the expected amplicon length is 117 bp.

2.2.5 Results on agarose gel electrophoresis

Fifty nanograms of gDNA from different non-GM lines of maize (W85, B14, CM7, EM, IA153, W117, F64) and GM lines of maize (Bt11 maize certified reference material, Bt11 used in method validation at the Community Reference Laboratory, MON 810, DAS-59122-7, MIR 604, Bt 176, GA 21, MON 863, T 25, TC 1507, NK 603, Starlink), in addition to GM-sugarbeet (H7-1) and GM-soy (Roundup Ready) and to 50 ng of Bt10 and wild-type isogenic Bt10 gDNA were amplified in duplicate with event Bt10 specific primers and analyzed on 2.7% agarose gel electrophoresis. An example of amplification results is provided in Figure 1. The molecular weight of the resulting amplicons was compared to a ladder marker of molecular weight (M).

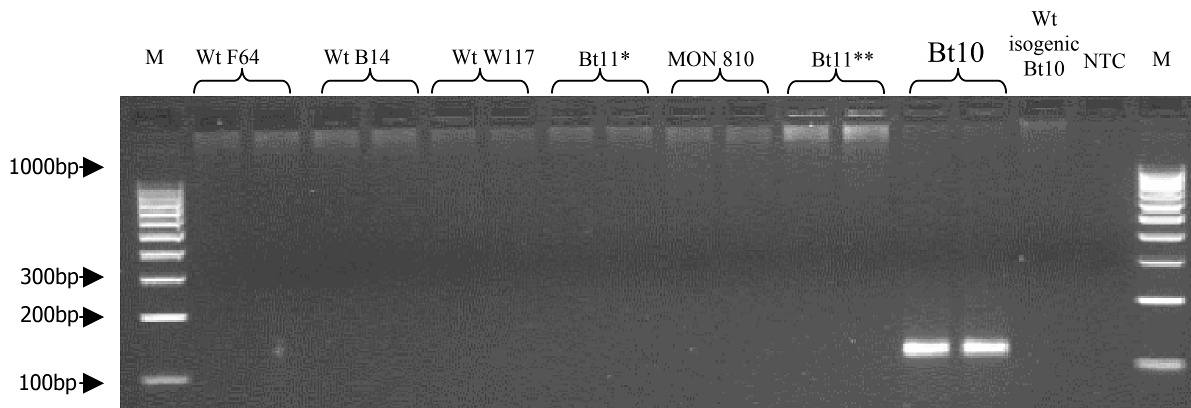

Fig. 1 Specificity test.

As shown in Figure 1, a 117 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.

The results of the specificity assay are presented in Table 4. Presence of amplicon at 117-bp was classified as positive (+) and absence as negative (-) response. The test was carried out according to the conditions outlined in Tables 2 and 3, using the primer pair JSF5/JSR5, in duplicate reactions in GeneAmp PCR system 9700, Applied Biosystems.

Table 4. Results of specificity test

GM EVENTS		NON GM EVENTS	
Sample DNA	Plant species	Sample DNA	Plant species
Bt10	+	Wt isogenic Bt 10 (Syngenta)	-
[1]Bt11	-	W85	-
Bt11	-	B14	-
[1]MON810	-	CM7	-
DAS-59122-7	-	EM	-
MIR 604	-	IA153	-
Bt176	-	W117	-
[1]GA21	-	F64	-
[1]MON863	-		
T25	-		
TC1507	-		
[1]NK603	-		
STARLINK	-		
H7-1	-		
[1]Roundup Ready			
No Template Control			

[1]Certified Reference Material (JRC, IRMM, Belgium)

Only Bt10 gDNA provided a positive response and no other amplicons were visible in the other GM-lines of maize, GM-soybean and GM-suga rbeet as well as in wild-type maize DNA.

2.3 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 times, ensuring a ≤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 JSF5/JSR5). Results are shown in Table 5.

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	20	1
0	21	0	21

The absolute LOD of the event Bt10 specific method is at least 4 copies. This corresponds to 0.022% in relative terms when 50 ng are analysed in PCR.

2.4 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 JSF5/JSR5). The amplification reactions were run on a 2.7% agarose gel. Results are reported in Table 6. Figure 2 shows selective amplification of Bt10 maize at 0.1% and 1% concentrations.

Table 6. Selectivity test results

B10 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.

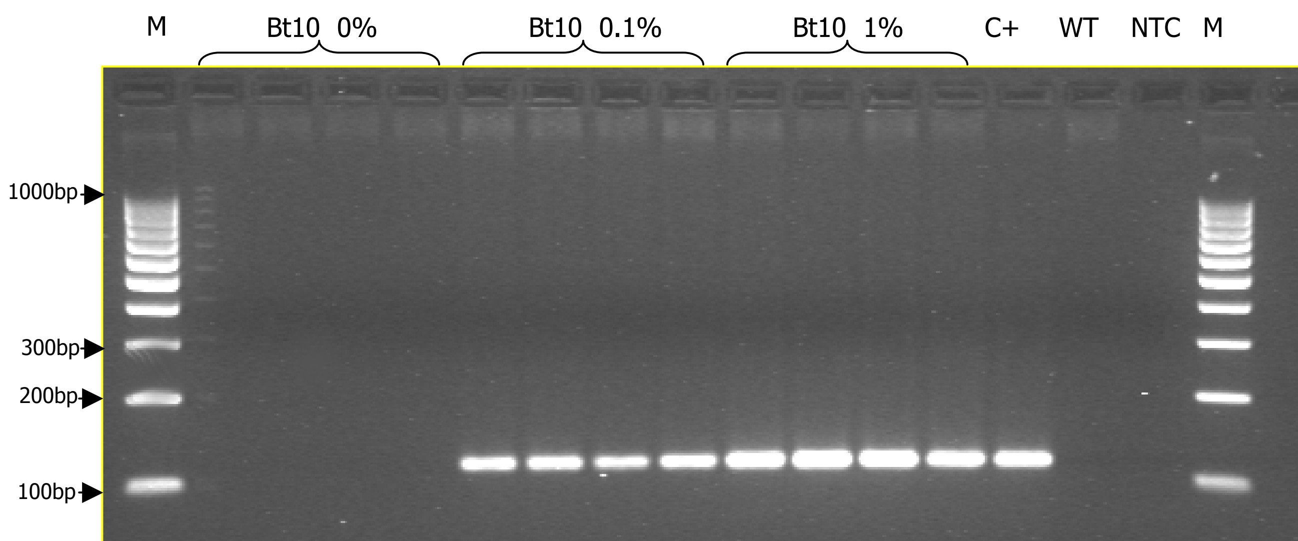

Fig.2. Selective amplification of Bt10 at 0.1% and 1% concentrations.
C+: positive control Bt10 100%

2.5 Restriction Analysis

The 117-bp amplicon, amplified according to the present PCR-based qualitative method of detection, has been digested with SspI. The retained portion of the PCR reaction was incubated with 10 units of the restriction enzyme SspI at 37ºC for 1 hour, as described in the protocol (available at http://gmo-crl.jrc.it/statusofdoss.htm).

The specific Bt10 amplicon is cleaved in two bands of respectively 40-bp and 77-bp as shown in Figure 3.

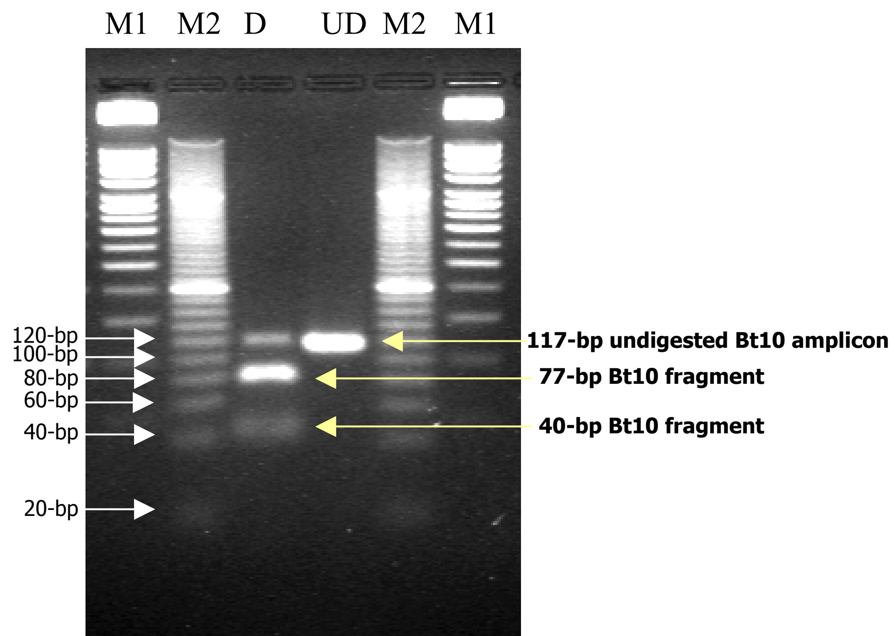

Fig.3. A two-band pattern is obtained following SspI enzymatic digestion of Bt10 amplicon and electrophoresis run at 50 V for 2.5 hours in 2.8% agarose gel.
M1: 50-bp ladder; M2: 20-bp ladder; D: 117-bp amplicon digested with SspI; UD: undigested amplicon 117-bp

Due to the low molecular weight of the resulting fragments, a side-by-side comparison with SspI-digested positive control will unequivocally confirm the size of the bands.

Therefore, whenever a 117-bp amplicon is obtained from an unknown sample while applying the present method**, the two-band pattern of 40-bp and 77-bp will confirm the presence of Bt10 DNA**.

3. Conclusion

A 117-bp amplicon is detected in event Bt10 maize DNA following the procedure performed according to the third version of the PCR-assay method provided by Genescan and in-house validated according to the present Report. The same amplicon band is not detected when analyzing either event Bt11 maize DNA or a wide range of GM and non-GM maize lines, in addition to GM soybean and GM sugarbeet. At the same time the new method has high specificity for the Bt10 event and no other amplicon can be detected in conventional maize or in the tested maize GM-events or GM-soybean and GM-sugarbeet.
Finally, a specific cleavage site for SspI exists in the Bt10 amplicon. The presence of two bands at 40 and 77 base pairs following SspI digestion of the 117-bp band, amplified from an unknown sample according to the conditions herewith described, will confirm the presence of Bt10 DNA in that sample.

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

The absolute LOD of the event Bt10 specific method is 4 copies. This corresponds to 0.022% 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 and can be used for compliance with the Commission Decision of 18 April 2005 (317/2005/EC) "*on emergency measures regarding the non-authorised genetically modified organism Bt10 in maize products*".

4. Literature

Mazzara, M., Maretti, M., Foti, N., Price, S., Paoletti, C., Savini, C., Van den Eede, G. "Report on the In-House Validation of a Detection Method for Event Bt10 Maize using a Qualitative PCR Assay", 22 April 2005 (http://gmo-crl.jrc.it/statusofdoss.htm)

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