EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION

COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Detection Method for Event Bt 10 using a qualitative PCR assay

Protocol for verification of positive results by restriction analysis

Method development:
Syngenta Crop Protection AG

In-house validation and reporting:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Document Approval		
Name / *Function*	**Date**	**Signature**
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Procedure:

If a 130 bp PCR product is obtained from an unknown sample using the method described at http://gmo-crl.jrc.it/detectionmethods/Bt10%20Detection%20Protocol.pdf, add 10 units of SspI to the retained portion of the PCR reaction and to the retained portion of the corresponding positive control reaction. Mix well, incubate for 1 h at 37 °C**, and then subject to electrophoresis on a 3 % agarose gel*. Load the cleaved putative positive reaction side by side with the corresponding cleaved control reaction. Cleavage of the PCR product specific to event Bt10 is expected to result in a 77 bp and a 53 bp fragment. Only if the product from the putative positive reaction has been cleaved as expected, it can be considered confirmed as Bt10 positive***.

**Note: While polyacrylamide gel electrophoresis is suitable, it may not have a considerable advantage over agarose gels since the AT-rich amplified DNA region may exhibit anomalous migration in polya crylamide gels at room temperature. **Note: Using 1* µl *of SspI (Invitrogen Cat. No. 1545801,10 units /* µl), *these conditions allowed for complete digest in 1 x Applied Biosystems PCR buffer II with 1.5 mM MgCl2. If activity and specificity of SspI varies in between vendors and star activity or partial digest should be observed, optimize the digest accordingly. ***Note: While comparison to a low molecular weight DNA ladder in agarose gel electrophoresis may not allow for a sufficiently exact assessment of the size of the cleavage products, the described side-by-side comparison will still unambiguously demonstrate whether cleavage products from the putative positive reaction and cleavage products from the authentic positive control match.*

The result for an unknown sample is *positive* if*:*

- all replicate PCR reactions for the sample show discernable PCR products of 130 bp that migrated exactly the same distance as PCR products from spiked reactions or other positive control reactions (see above)
- the 130 bp PCR products were successfully cleaved with SspI, so as to obtain same pattern of fragments like a positive control reaction cleaved with Sspl
- and all negative control reactions do not show 130 bp PCR products

The result for an unknown sample is *negative* if:

- all replicate PCR reactions for the sample do not show 130 bp products
- the respective inhibition control reactions (if performed) for the sample show discernable PCR products of 130 bp
- all positive control reactions show discernable PCR products of 130 bp

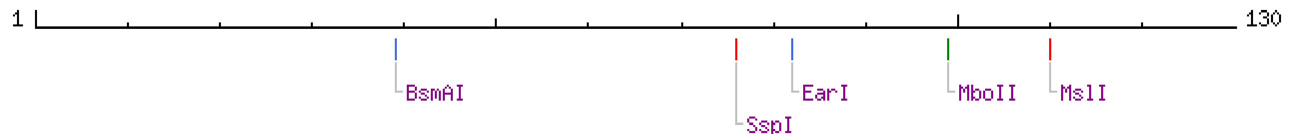
In case of conflicting results for PCR reactions performed with replicate DNA extracts from the same sample (e.g. one replicate positive, one replicate negative) repeat the PCR reactions. If replicate results from this second PCR setup concur (i.e., all replicates negative or all replicates positive), the overall conclusion is *negative* or *positive* respectively. If replicate results from this second PCR remain ambiguous (positive and negative replicates) repeat the DNA extraction and perform a further PCR setup.
If replicate results from this third PCR setup concur (i.e., all replicates negative or 1 all replicates positive), the overall conclusion is *negative* or *positive* respectively. If replicate results from the third PCR setup again do not concur (positive and negative replicates), the DNA target is not present in the sample in amounts that can be detected reproducibly, and the result is *negative.*

Restriction map of the 130 bp amplification product:

Cut position	MS Enzyme	Recognition sequence
40	BsmAI	GTCTCN^NNNN_
77	SspI	AAT|ATT
83	EarI	CTCTTCN^NNN_
100	MboII	GAAGA(N)$_{7}$_N^
111	MslI	CAYNN|NNRTG

Cleavage code
- blunt end cut
- 5′ extension
- 3′ extension
- cuts 1 strand

Enzyme name code
- Available from NEB
- Has other supplier
- Not commercially available
- *: cleavage affected by CpG meth.
- #: cleavage affected by other meth.
- (enz.name): ambiguous site



Sequence of the 130 bp amplification product:

1 CACACAGGAG ATTATTATAG GGTTACTCAC ATTTCCCCGA ATGTTGAGAC AATAACCCTG
61 ATAAATGCTT CAAT**AATATT** GAAAAAGGAA GAGTATGAGT ATTCAACATT TCCGTGTCGC
121 CCTTATTCCC

In bold font, the SspI restriction site.