



PCR Assay for Detection of Maize Transgenic Event Bt10 version 2

Introduction

The following protocol can be used for the detection of the Bt10 transgenic event in corn samples. The silica gel membrane DNA extraction protocol is based upon the respective protocol from the Japanese MHLW guideline for Testing for Foods Produced by Recombinant DNA Techniques. The Polymerase Chain Reaction (PCR) protocol uses target-specific oligonucleotides that have been designed to amplify a 117 bp DNA sequence specific to Bt10 event.

The method was subjected to validation experiments performed by multiple analysts in two different laboratories. The experiments demonstrated adequate robustness, specificity, sensitivity and repeatability. Analysis of a known sample of 0.1 % ground Bt10 in event free ground corn in 50 replicate DNA extractions and 50 respective PCR reactions yielded 50 positive results. Analysis of two different samples of event free corn, each subjected to DNA extraction and PCR analysis in 25 replicates, yielded 50 negative results.

In order to avoid contamination with target DNA at any step of the procedure, observe all applicable precautions, such as use of disposable reaction vial and gloves, disposable pipette tips with aerosol filters, physically separated work areas for sample homogenization, DNA extraction and PCR reaction setup, gel electrophoresis of PCR products. Pay special attention to implementation of rigorous precautions that avoid any carryover of Bt10 specific PCR products from gel electrophoresis to any other work areas.

Sample homogenization

Grind approximately 1 kg of corn until no coarse particles remain. Use equipment that can be rigorously cleaned in between samples, so as to avoid carryover between samples*. Depending on equipment used, it may be required to grind in several portions, which are then combined and blended thoroughly to obtain 1 kg of ground sample. Alternatively, a representative sub-sample from an initial coarse grind of 1 kg corn can be pulverized using a ball mill (e.g. Retsch Mixer Mill MM 200) if available.

**E.g.: Immediately after each use, soak blenders and blades in water with dishwasher detergent. Clean in dishwasher at highest temperature setting and most intense cleaning program available. In doubt, perform plausibility checks for effectiveness of cleaning procedure. E.g., grind a sample that is known to contain substantial amounts a commonly used biotechnology PCR target, such as biotechnology-derived soybeans containing CaMV 35S promoter DNA. Clean grinding equipment and grind a sample of event free corn. Subject the corn sample to DNA extraction and 35S PCR. The sample is expected to test negative for 35S promoter.*

DNA extraction

Reagents, solutions:

DNeasy plant kit (Qiagen, URL: <http://www1.qiagen.com/SelectCountry.aspx>); Note: depending on volumes used for initial lysis, it may be required to purchase additional buffer AP1 and buffer AP2 HPLC H₂O

Procedure:

- Place 10 g of uniformly ground kernel sample in a polypropylene centrifuge tube (50 ml), add 15 ml of AP1 buffer previously warmed at 65 °C and 20 µl of RNase A, mix vigorously to



pulverize lumps of the sample. *Note: for extremely fine grinds, e.g. from a ball mill, use 2 g of the pulverized sample, add 10 ml of AP1 buffer previously warmed at 65 °C and 20 µl of RNase A.* Leave the mixture at 65°C for 15 minutes and shake the sample by overturning the centrifuge tube 2-3 times, or subject them to continuous rotating agitation.

- Add 5000 µl of AP2 buffer (or 3250 µL of AP2 buffer for 2 g pulverized sample in 10 ml AP1), leave the mixture on ice or at 4 °C for 5 minutes, and centrifuge it at 3,000 x g or more for 5 minutes.
- Then, apply 500 µl of the supernatant to a QIAshredder spin column, and perform centrifugation at 10,000 x g or more for 4 minutes and place the eluate in a micro centrifuge tube (15 ml).
- After repeating this procedure, add 1.5 times the eluate volume of AP3 buffer/ethanol mixture*. Apply 500 µl of this solution to a Mini spin column, and perform centrifugation at 10,000 x g or more for 1 minute.
- Apply 500 µl of the remaining solution to the same Mini spin column, perform centrifugation under the same conditions, and dispose of the eluate. Repeat the same procedures until all of the solution is used.
- Apply 500 µl of AW buffer to the column, perform centrifugation at 10,000 x g or more for 1 minute, and dispose of the eluate.
- Apply the AW buffer again and repeat the same procedures.
- After disposing of the eluate, subject the Mini spin column to centrifugation at 10,000 x g or more for 15 minutes to dry it.
- Transfer the Mini spin column to a centrifuge tube of the kit, add 70 µl of previously warmed water, leave the mixture for 5 minutes, and centrifuge it at 10,000 x g or more for 1 minute to elute DNA.
- Add water again and repeat the same procedures, put the obtained eluates together, and use this as the DNA sample stock solution.
- Measure the DNA concentration of the obtained solution by appropriate method, such as comparison to known DNA amount standards using a fluorometer or ethidium bromide stained agarose gel, or UV spectrophotometer absorption at 260/280 nm.

* AP3 buffer/ethanol mixture Mix AP3 buffer and ethanol (96%-100%) at 1:2 and use this as the AP3 buffer/ethanol mixture.

Controls:

Negative control: blank extraction without sample, processed in duplicate and in parallel with unknown samples throughout the entire protocol starting with buffer AP1

Or

DNA extraction from a known event free sample of ground corn in duplicate, extracted in parallel with unknown samples

Positive control: DNA extraction from a known sample of 0.1 % ground Bt10 in event free ground corn in duplicate, extracted in parallel with unknown samples



PCR

Reagents, solutions:

AmpliTaq Gold, Applied Biosystems (5 U / μ l; URL: <http://www.appliedbiosystems.com>)
10 x Applied Biosystems PCR buffer II without $MgCl_2$
 $MgCl_2$ (25 mM)
dNTP Mix (25 mM each)
primer JSF5 (100 μ M) 5' CAC ACA GGA GAT TAT TAT AGG GTT ACT CA
primer JSR5 (100 μ M) 5' ACA CGG AAA TGT TGA ATA CTC ATA CTC T
HPLC H_2O
Event free DNA solution (10 ng / μ l)
0.1 % Bt10 DNA in event free corn DNA solution (10 ng / μ l)
Bt10 DNA solution (0.25 ng / μ l)

Procedure:

Adjust the concentration of sample DNA solutions to approx 10 ng / μ l.

Note: When using certain DNA extraction protocols, e.g. protocols that involve a phenol / chloroform extraction step, concentration of the obtained DNA solutions may be considerably overestimated when measured by UV spectrophotometer absorption. It is advisable to perform plausibility checks by comparison with other DNA quantification methods.

Combine the following components:

component	Concentration	final concentration	volume per reaction* (μ l)	example 100 reactions*
HPLC H_2O			15.38	1538
$MgCl_2$	25 mM	1.5 mM	1.5	150
PCR buffer	10 x	1 x	2.5	250
dNTPs	25 mM each	160 μ M	0.16	16
forward primer	100 μ M	0.6 μ M	0.15	15
reverse primer	100 μ M	0.6 μ M	0.15	15
Taq polymerase	5 U / μ l	0.032 U / μ l	0.16	16
subtotal			20	2000
DNA	Approx 10 ng / μ l	50 ng / reaction	5	
total			25 μ l	

*for inhibition control reactions, replace 0.2 μ l H_2O / reaction with 0.2 μ l Bt10 DNA solution (0.25 ng / μ l) / reaction

Dispense 20 μ l of the reaction mixture into reaction tubes / wells.

Samples: Add 5 μ l of a 10 ng / μ l DNA solution.

Controls:

Negative controls for PCR setup:

- add 5 μ l of HPLC H_2O , duplicate reactions and / or
- add 5 μ l of event free corn DNA solution (10 ng / μ l, duplicate reactions)



Negative controls for DNA extraction / PCR setup:

- add 5 µl of buffer blank extraction solution, extracted in parallel with unknown samples, duplicate reactions

and / or

- add 5 µl of DNA solution from a known event free sample of ground corn, extracted in parallel with unknown samples, duplicate reactions

Positive controls:

- add 5 µl 0.1 % Bt10 DNA in event free corn DNA solution (10 ng / µl), duplicate reactions

and / or

- add 5 µl of DNA solution from a known sample of 0.1 % ground Bt10 in event free ground corn, extracted in parallel with unknown samples, duplicate reactions
- To monitor potential PCR inhibition, prepare spiked PCR reaction counterparts for all DNA solutions from unknown samples. This is especially helpful for PCR analysis of DNA solutions from sample matrices other than ground corn and PCR analysis of DNA solutions from any DNA extraction protocol other than recommended above. The spiked reactions contain 0.05 ng Bt10 DNA each, in addition to all other components. When combined with 50 ng of DNA from an unknown corn sample, this translates to at least 0.1 % Bt10 DNA. Failure of a spiked reaction to generate the expected PCR product indicates inhibitory effects from the particular sample DNA solution that was added to the reaction.

PCR profile*

Step 1	Step 2			Step 3	Step 4
10 min	25 sec	30 sec	45 sec	Cycles	7 min
94°C	94°C	62°C	72°C	40	∞
					4°C

**Note: Method development and validation were carried out with Applied Biosystems GeneAmp9700 instruments. The ramp speed was set to 'GeneAmp9600'. Validation data indicate that the PCR test is not susceptible to annealing temperatures deliberately changed to 60 °C or 64 °C instead of 62 °C. Nevertheless, it is recommended to verify reliable detection of Bt10 in replicate DNA solutions extracted from a known sample of 0.1 % ground Bt10 in event free ground corn when using different types of PCR instruments*

Gel electrophoresis:

Run approx 12 µl of the PCR reactions on a 1 x TAE or TBE, 2.5 - 3 % agarose gel with ethidium bromide staining¹⁾. The PCR products specific to event Bt10 are 117 bp in length. Run each spiked reaction side by side with its respective counterpart without spike DNA. This enables the analyst to compare the size of a PCR product in reactions without spike directly to the size of the PCR product in the respective positive control reaction next to it. Only PCR products that migrated exactly the same distance as PCR products from spiked reactions or any other positive control reaction as judged by side by side comparison can be considered the expected 117 bp PCR product. Use an appropriate length standard, e.g. pUC19 digested with HpaI.

If a 117 bp PCR product is obtained from an unknown sample, add 10 units of SspI to the retained portion of the PCR reaction and to the retained portion of the corresponding spiked control reaction. Mix well, incubate for 1 h at 37 °C²⁾, and then subject to electrophoresis on a 3 % agarose gel¹⁾. 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 40 bp fragment. Only if the product from the putative positive reaction has been cleaved as expected, it can be considered confirmed as Bt10 positive³⁾.



Notes:

- 1) 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 polyacrylamide gels at room temperature.
- 2) 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.
- 3) 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 117 bp that migrated exactly the same distance as PCR products from spiked reactions or other positive control reactions (see above)
- the 117 bp PCR products were successfully cleaved with SspI, so as to obtain same pattern of fragments like a positive control reaction cleaved with SspI
- and all negative control reactions do not show 117 bp PCR products

The result for an unknown sample is *negative* if

- all replicate PCR reactions for the sample do not show 117 bp products
- the respective inhibition control reactions (if performed) for the sample show discernable PCR products of 117 bp
- all positive control reactions show discernable PCR products of 117 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 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*.