Event 5307 Maize:

Real-time, Event-specific Polymerase Chain Reaction Method

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES STANDARDS

The provisions of Good Laboratory Practices Standards (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act do not apply to this report because it summarizes an analytical method. Relevant study records (including raw data) have been retained.

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LIST OF ACRONYMS AND ABBREVIATIONS

3′	three prime
5′	five prime
adh1	alcohol dehydrogenase 1 gene
bp	base pair
CT	cycle threshold
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
FAM	6-carboxy-fluorescein
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HPLC	high performance liquid chromatography
M	molar
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram
nM	nanomolar
PCR	polymerase chain reaction
pg	picogram
TAMRA	6-carboxytetramethylrhodamine, succinimidyl ester
TE	Tris (2-amino-2(hydroxymethyl)-propane-1,3-diol)/EDTA (ethylenediaminetetraacetic acid)
Tris	2-amino-2(hydroxymethyl)-propane-1,3-diol
US EPA	United States Environmental Protection Agency
X	times (as in 2 X or 100 X)
®	registered trademark
™	trademark
µl	microliter
µM	micromolar
Δ CT	delta CT (difference in CT values)

SUMMARY

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays)* to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species.

A real-time, event-specific polymerase chain reaction (PCR) method was developed to detect and quantify Event 5307 deoxyribonucleic acid (DNA) extracted from grain and seed samples. The method consists of a maize-specific PCR method as a reference, and an event-specific PCR method for detection and quantification of Event 5307 maize DNA.

INTRODUCTION

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays)* to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species.

A real-time, event-specific polymerase chain reaction (PCR) method was developed to detect and quantify Event 5307 deoxyribonucleic acid (DNA) extracted from grain and seed samples. The method consists of a maize-specific PCR method as a reference and an event-specific PCR method for the detection and quantification of Event 5307 maize DNA. This method determines the relative content of Event 5307 maize DNA in proportion to total maize DNA in samples. The method should be used in conjunction with a DNA extraction method which yields DNA of sufficient purity and quantity. The following report describes the real-time, event-specific PCR method.

METHOD

Principle of the Event 5307 Real-time, Event-specific Method

The Event 5307 real-time, event-specific PCR method is optimized for use on an Applied Biosystems PRISM® 7900HT Sequence Detection System. The amount of PCR product is determined during each cycle (real-time) by measuring the fluorescence produced by a target-specific oligonucleotide probe labeled with two fluorescent dyes.

- Event 5307-specific PCR method: oligonucleotide probe with 6-carboxy-fluorescein (FAM™) as a reporter dye at its 5′ end and 6-carboxytetramethylrhodamine, succinimidyl ester (TAMRA™) as a quencher dye at its 3′ end

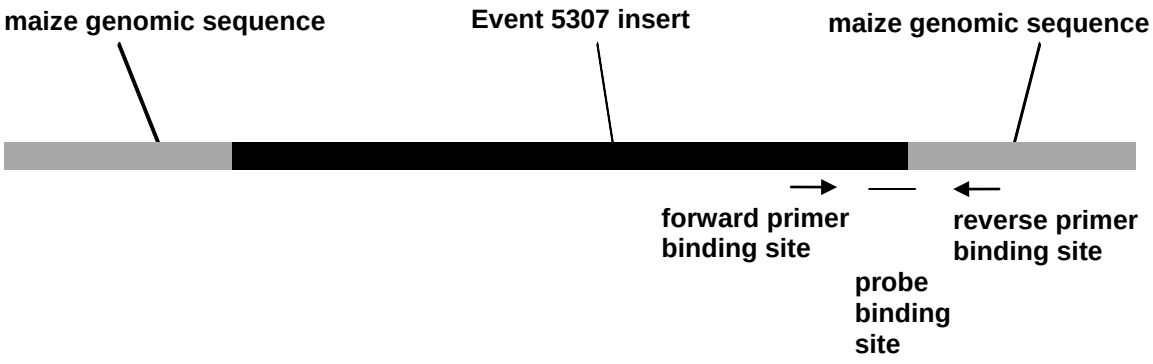
- Maize-specific PCR method: oligonucleotide probe with VIC® as a reporter dye at its 5′ end and TAMRA™ as a quencher dye at its 3′ end

The 5′-nuclease activity of *Taq* DNA polymerase cleaves the probe and liberates the fluorescent moiety from proximity to the quenching moiety during the amplification process. The resulting increase in fluorescence during amplification is monitored and recorded.

A maize-specific PCR method (Hernandez *et al.* 2004), which amplifies a 135 base pair (bp) fragment of the alcohol dehydrogenase 1 gene (*adh1*) of maize (Entrez® Database Accession No. AY691949 [NCBI 2010]), is used as a reference method.

For specific detection of Event 5307 genomic DNA, a 107 bp fragment of the region that spans the 3′ insert to plant genome junction in Event 5307 maize is amplified using two specific primers. The forward primer binding site is located in the Event 5307 insert, the reverse primer binding site is located in the maize genomic sequence, and the probe binding site spans the junction between the Event 5307 insert and the adjacent maize genomic DNA (Figure 1).

Figure 1. Location of the Event 5307 real-time, event-specific PCR primer and probe binding sites



Reagents and Equipment

All materials (*e.g.*, vials, containers, pipette tips, *etc.*) should be suitable for PCR and molecular biology applications (Table 1). Materials should be deoxyribonuclease (DNase)-free, DNA-free, sterile, and unable to absorb protein or DNA. Table 2 contains a list of reagents, buffers, and solutions needed to perform the PCR method.

Table 1. Equipment and materials

Equipment and materials	Specification
Applied Biosystems PRISM® 7900HT Sequence Detection System	Applied Biosystems™ Part Number 4329003
Vortex	NeoLab Vortex VM-300 or equivalent
Thermo-Fast® 96 PCR Detection Plate MKII	Abgene® Catalog Number AB-1400 or equivalent
Clear Seal Diamond Heat Sealing Foil	Abgene® Catalog Number AB-0-812 or equivalent
Thermo-Sealer	Abgene® Catalog Number AB-0384/240 or equivalent
Pipettes with adjustable volume	Eppendorf® Research, 2 – 20 µl, 20 – 200 µl, 100 – 1000 µl or equivalent
Aerosol resistent tips	Molecular BioProducts Catalog Number 2149P
Microcentrifuge tubes 1.5 ml	Roth Catalog Number 4182.1 or equivalent
Microcentrifuge tubes 1.5 ml, screw lids	Sarstedt Part Number 72.692.005 or equivalent

Table 2. Reagents, buffers, and solutions

Reagents, buffers, and solutions	Specification
JumpStart™ *Taq* ReadyMix™ (requires supplement see below)	Sigma-Aldrich® Catalog Number P2893
1X TE buffer pH 8.0	Applichem Part Number A2575, 1000
Water HPLC Gradient Grade	Rotisolv®HPLC Gradient Grade Product Number A511.1
1 M $MgCl_2$	Sigma-Aldrich® Catalog Number M1028
sulforhodamine 101	Sigma-Aldrich® Catalog Number S7635

TE = Tris (2-amino-2(hydroxymethyl)-1,3-propanediol)/EDTA (ethylenediaminetetraacetic acid)
HPLC = high performance liquid chromatography

10,000 X sulforhodamine 101 stock

For 10,000 X stock: Resuspend 227.5 mg of sulforhodamine 101 in 250 ml nuclease-free water to make a 1.5 mM stock solution

Solution should be vortexed and stored at -20°C

Supplemented 2 X JumpStart™ *Taq* ReadyMix™

For 50 ml: To 2 X JumpStart™ *Taq* ReadyMix™, add:
550 µl of 1 M $MgCl_2$
20 µl 10,000 X sulforhodamine 101 stock

Solution should be vortexed and stored at 4°C for up to one year.

The handling of all reagents and controls should be carried out under sterile conditions and in a manner that precludes contamination of reagents or controls with exogenous DNA or undesired enzymatic activities (*e.g.*, DNase).

DNA Concentration of Samples

For the unknown samples, the use of 250 ng of template DNA per reaction is recommended. This corresponds to approximately 100,000 haploid copies of the maize genome, assuming a genome weight of 2.5 pg (Arumuganathan and Earle 1991).

Calibration Standards

The method format uses five calibration standards containing different amounts of Event 5307 DNA (Standard 1 to Standard 5); each calibration standard is analyzed in both the Event 5307-specific and *adh1*-specific PCR methods. The calibration standards are produced by preparing solutions of 50 ng/µl (250 ng/reaction) of total genomic DNA with 10%, 5%, 1%, 0.5%, and 0.1% Event 5307 DNA in DNA extracted from a nontransgenic maize background. Table 3 lists the total DNA content, as well as the total Event 5307 DNA content in the PCR reaction, for each calibration standard.

Table 3. Dilution scheme of the calibration standards

Sample	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5

Total DNA content in PCR	250 ng	250 ng	250 ng	250 ng	250 ng
Total Event 5307 DNA content in PCR	25 ng	12.5 ng	2.5 ng	1.25 ng	0.25 ng

The *adh1*-specific PCR method

The *adh1*-specific PCR method serves as a reference method. Primers and probe sequences are described in (Hernandez *et al*. 2004). Reaction conditions are described in this report. For specific detection of *adh1*, two specific primers (Table 4) are used to amplify a 135 bp DNA fragment of the region of the gene *adh1* (Figure 2).

Table 4. The *adh1*-specific PCR primers and probe sequences

Primer/probe name	Primer sequence 5' to 3'
Zm *adh1* primer F	CGTCGTTTCCCATCTCTTCCTCC
Zm *adh1* primer R	CCACTCCGAGACCCTCAGTC
Zm *adh1* probe	VIC®-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA™

Figure 2. Sequence of the 135 bp amplicon generated by PCR amplification with *adh1*-specific PCR method

Zm *adh1* primer F — Zm *adh1* probe

CGTCGTTTCCCATCTCTTCCTCCTTTAGAGCTACCACTATATAAATCAGGGCTCATTTTCTCGCTCCTCAC

AGGCTCATCTCGCTTTGGATCGATTGGTTTCGTAAGTGGTGAGGGACTGAGGGTCTCGGAGTGG

Zm *adh1* primer R

Primer and probe binding sites are underlined.

All reagents should be thawed, as necessary, and thoroughly mixed before each use. The master mix for the *adh1*-specific PCR method that contains all components of the PCR method except template DNA (Table 5) can be prepared in sufficient quantities before reactions are performed.

Table 5. Master mix for the *adh1*-specific PCR method

Components	Concentration	Final concentration*	µl/reaction	µl/50 reactions
JumpStart™ *Taq* ReadyMix™ (supplemented with 600 nM sulforhodamine 101 and 11 mM $MgCl_2$)	2 X	1 X	12.50	**625.0**
Zm *adh1* primer F	10 µM	300 nM	0.75	**37.5**
Zm *adh1* primer R	10 µM	300 nM	0.75	**37.5**
Zm *adh1* probe	10 µM	200 nM	0.50	**25.0**
Nuclease-free water	N/A	N/A	5.50	**275.0**
Total volume	N/A	N/A	**20.00**	**1000.0**

*Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template)
N/A = Not applicable

Event-specific PCR Method for the Detection and Quantification of Event 5307 DNA

For specific detection of Event 5307 maize genomic DNA, two specific primers (Table 6) are used to amplify a 107 bp fragment of the region that spans the 3′ insert to plant genome junction (Figure 3). The amount of PCR products is determined during each cycle (real-time) by measuring the fluorescence produced by an Event 5307-specific oligonucleotide probe labeled with two fluorescent dyes: FAM™ as a reporter dye at its 5′ end and TAMRA™ as a quencher dye at its 3′ end.

Table 6. Event 5307 real-time, event-specific PCR primer and probe sequences

Primer/probe name	Primer sequence 5' to 3'
5307i3' forward primer	CATGGCCGTATCCGCAATGTG
5307i3' reverse primer	TGCACCCTTTGCCAGTGG
5307i3'-S2 probe	6FAM™–ACCACAATATACCCTCTTCCCTGGGCCAG-TAMRA™

Figure 3. Sequence of the 107 bp amplicon generated by PCR amplification with Event 5307 real-time, event-specific PCR method

5307i3′ forward primer 5307i3′-S2 probe
CATGGCCGTATCCGCAATGTGTTATTAAGTTGTCTAAGCGTCAATTTGTTTACACCACAATATACCCTCTT

CCCTGGGCCAGGCTGGGCCCACTGGCAAAGGGTGCA
5307i3′ reverse primer

Primer and probe binding sites are underlined.

All reagents should be thawed, as necessary, and thoroughly mixed before each use. The master mix for the Event 5307-specific PCR method that contains all components of the PCR method except template DNA (Table 7) can be prepared in sufficient quantities before reactions are performed.

Table 7. Master mix for the Event 5307-specific PCR method

Components	Concentration	Final concentration	µl/reaction	µl/50 reactions
JumpStart™ *Taq* ReadyMix™ (supplemented with 600 nM sulforhodamine 101 and 11 mM $MgCl_2$)	2 X	1 X	12.500	**625.00**
5307i3' forward primer	10 µM	350 nM	0.875	**43.75**
5307i3' reverse primer	10 µM	350 nM	0.875	**43.75**
5307i3'-s2 probe	10 µM	125 nM	0.313	**15.65**
Nuclease-free water	N/A	N/A	5.437	**271.85**
Total volume*	N/A	N/A	**20.000**	**1000.00**

*Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template)
N/A = not applicable

Cycling Parameters

An Applied Biosystems PRISM® 7900HT Sequence Detection System using the 9600 emulation mode in the instrument set-up is used with the Event 5307 real-time, event-specific PCR method and the *adh1*-specific PCR method. Table 8 shows the PCR cycling conditions.

Table 8. Cycling parameters

Cycle	Step	Temperature (°C)	Time (seconds)	Number of cycles
A	1	95	600	1
B	1	95	15	40
	2	60	60	

PCR Plate Layout

The calibration of the Event 5307 real-time, event-specific method includes five calibration standards, each containing different percentages of Event 5307 maize DNA, with a total amount of 250 ng maize DNA. The Event 5307 DNA content of the standard samples ranges from 0.1% to 10%. Each calibration standard is analyzed in duplicate in the Event 5307-specific PCR method, as well as in the *adh1*-specific PCR method. Duplicate negative control samples containing no template DNA verify the purity of the reagents. Unknown samples are analyzed in triplicate for both the 5307-specific and *adh1*-specific PCR methods. Figure 4 summarizes the PCR plate layout.

Figure 4. PCR plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1		Standard 1		Standard 2		Standard 2		Standard 3		Standard 3	
B	Standard 4		Standard 4		Standard 5		Standard 5		Negative control		Negative control	
C	Unknown 1			Unknown 1			Unknown 2			Unknown 2		
D	Unknown 3			Unknown 3			Unknown 4			Unknown 4		
E	Unknown 5			Unknown 5			Unknown 6			Unknown 6		
F	Unknown 7			Unknown 7			Unknown 8			Unknown 8		
G	Unknown 9			Unknown 9			Unknown 10			Unknown 10		
H	Unknown 11			Unknown 11			Unknown 12			Unknown 12		

Standard 1 to 5: calibration standards
Unknown 1 to 12: unknown samples
Negative control: samples containing no template DNA
White: *adh1*-specific PCR method
Gray: Event 5307-specific PCR method

Data Analysis and Evaluation of Results

Analyze the results according to the following procedure:

a) Place the threshold in the region of exponential amplification across all of the amplification plots. This region is recognized in the logarithmic view of the amplification plots as the portion of the plot that is linear. Do not place the threshold line in the plateau phase or in the initial linear phase of amplification.

Place the threshold line clearly above the background fluorescence and above the level where splitting or fork effects between replicates can be observed.

b) Determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (*e.g.*, if the earliest cycle threshold (CT) = 24, set the baseline crossing at 21, or CT of 24 - 3).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other method.

e) Save the settings and export all the data into Microsoft Excel® 2007 file for further calculations.

The option "Use spectral compensation for real-time" should be deactivated in the menu "Instrument\Diagnostics\Advanced Options" when using an ABI PRISM® 7700 Sequence Detection System.

A calibration curve can be produced by plotting mean delta CT (Δ CT) values of calibration samples against the logarithm of the respective percent Event 5307 DNA contents; the slope (a) and the intercept (b) of the calibration curve ($y = ax + b$) can then be used to calculate the percent Event 5307 DNA content of the reference samples based on their normalized mean Δ CT values.

CONCLUSION

A real-time, event-specific PCR method was developed to detect and quantify Event 5307 DNA extracted from grain and seed samples.

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