

# A Recommended Procedure for Real-Time Quantitative TaqMan® PCR for YieldGard® Corn Borer Corn, MON 810

## Monsanto Biotechnology Regulatory Sciences

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### Overview

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#### Purpose & Scope

This procedure describes an event-specific real-time quantitative TaqMan® PCR method for determination of the relative content of YieldGard® Corn Borer corn, MON 810 (hereafter referred to as MON 810) DNA to total corn DNA in a sample.

The PCR assay has been optimized for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified.

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#### Summary of the Methodology

For specific detection of MON 810 genomic DNA, a 66-bp fragment of the region that spans the 3' insert-to-plant junction in MON 810 is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-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. The 5'-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence, which is then monitored. For relative quantification of MON 810 DNA, a corn-specific reference system amplifies a 70-bp fragment of *adh1*, a corn endogenous gene, using a pair of *adh1* gene-specific primers and an *adh1* gene-specific probe labeled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of MON 810 DNA in a test sample, MON 810 and *adh1* Ct values are determined for the sample. Standard curves are then used to calculate relative content of MON 810 DNA to total corn DNA.

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#### Important Precautions

- Follow all appropriate safety procedures. Safety glasses should be worn at **all times** in the laboratory. It is recommended to wear protective gloves during the entire procedure to protect the DNA from **contamination** with DNase from the skin.
- It is recommended to use pipettes that are designated for PCR setup to prepare the reactions. Sterile, aerosol resistant pipette tips should be used. Use microtubes or reaction plates appropriate for the instrument sample holder.
- The power supply of the detection system contains electrical circuits operating at **high voltage** that can cause **electrical shock**.

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**Abbreviations**     The following abbreviations are used in this protocol:

<b>Abbreviation</b>	<b>Definition</b>
$\Delta R_n$	Represents the normalized reporter signal minus the baseline signal established in the first few cycles of PCR. $\Delta R_n$ increases during PCR as amplicon copy number increases until the reaction approaches a plateau.
6-FAM	6-carboxyl-fluorescein
<i>adh1</i>	endogenous corn gene encoding alcohol dehydrogenase
bp	base pairs
Ct	threshold cycle
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
RSD	relative standard deviation
TAMRA	tetramethyl-6-carboxyrhodamine
TE	Tris EDTA
Tris	tris(hydroxymethyl)aminomethane

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**Procedure**

**Preparation of Standard DNA Solutions**

Prepare standard solutions of MON 810 DNA by combining purified genomic DNA from known MON 810 with that from known conventional corn. The concentration of MON 810 DNA in the standard solutions should be 50.0, 25.0, 5.0, 2.5, 0.5, 0.25, 0.05, and 0 ng/μl, respectively. The concentration of total corn DNA in all the standard solutions should be 50.0 ng/μl.

Prepare standard solutions of conventional corn DNA by combining purified genomic DNA from known conventional corn with known conventional genomic DNA from another plant species (such as wheat, soybean, or canola). The concentrations of corn DNA in the standard solutions should be 50.0, 25.0, 12.5, 5.0, 2.5, 0.5, and 0 ng/μl, respectively. The concentration of total plant DNA in all the standard solutions should be 50.0 ng/μl.

For consistency of the performance of DNA standards, it is recommended that all DNA stock solutions used in preparation of above-mentioned DNA standard solutions be extracted with the same protocol.

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**Preparing  
the Reaction  
Mixes**

If necessary, thaw all reagents (a 37°C water bath is recommended). It is recommended to store all reagents on ice once thawed. Be sure to thoroughly mix each reagent before use. Two reaction mixes (one for MON 810 PCR and one for *adh1* PCR) must be prepared consisting of all components of the PCR, **except DNA template**, in sufficient quantities for all reactions (including those for standard DNA solutions) to be performed (i.e., prepare each reaction mix for at least one extra reaction). This allows for consistency and minimizes cross-contamination. Note: PCR for MON 810 and *adh1* may be conducted on different days.

It is recommended that all reagents be added in the order listed below for both reaction mixes.

Step	Reagent	Volume (µL)	Final Concentration
1	Nuclease-free water	19	-
2	TaqMan Universal PCR Master Mix (2X)	25	1X
3	Primer 1 (10 µM)	0.75	150 nM
4	Primer 2 (10 µM)	0.75	150 nM
5	Probe* (5 µM)	0.50	50 nM
6	DNA template (standard solutions and test samples, 50 ng/µl)	4.0	200 ng

\*TaqMan probe is labeled with 6-FAM (6-carboxyl-fluorescein, a reporter dye) at its 5'-end and TAMRA (tetramethyl-6-carboxyrhodamine, a quencher dye) at its 3'-end.

**Setup of  
Reactions**

PCR will be conducted in an ABI Prism 7700 sequence detection system following the manufacturer's instructions. It is recommended to print the plate setup before running PCR. Add the appropriate reaction mix to individual wells of a 96-well reaction plate, then add DNA template for each reaction. Firmly seal each well. To quantify the content of MON 810 DNA in test samples, conduct MON 810 PCR on all standard solutions of MON 810 DNA and *adh1* PCR on all standard solutions of conventional corn DNA. Each sample should be analyzed in both PCRs. For each PCR, a known 1% MON 810 control (1% MON 810 DNA in 99% conventional corn DNA), a known conventional corn DNA control, and a no template control must be included. DNA standards and controls must be run in duplicate.

**Running  
the PCR**

Centrifuge the reaction plate to bring the reaction mixture/DNA template to the bottom of the wells (approximately 250 x g for approximately 1 minute at 4°C to room temperature is suggested). Run the PCR with cycling conditions listed below for both MON 810 and *adh1* assays in the ABI Prism 7700 system.

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Stage	Cycle No.	Settings	Data Collection
1	1	50°C 2 minutes	no
2	1	95°C 10 minutes	no
3	45	95°C 15 seconds 60°C 1 minute	no yes

**Analyzing  
the Data**

Once the PCR is completed, analyze the data and print, sign/initial, and date the amplification plot(s) (as  $\Delta R_n$  vs. Ct), the standard curve, and the experimental report for each PCR

Note: Perform quantitative data analysis for MON 810 PCR separately from that of *adh1* PCR since only one standard curve can be programmed with the current version (1.7) of software for the ABI Prism 7700 system. Be sure to use identical baseline range and threshold value settings in data analysis for both PCRs.

**Acceptance  
Criteria &  
Interpretation  
of Results**

The concentrations (pg/ $\mu$ l) of MON 810 DNA and those of total corn DNA will be calculated for all samples using the standard curve for MON 810 PCR and that for *adh1* PCR, respectively, and are then used to calculate the relative content (%) of MON 810 DNA to total corn DNA by the following formula:

$$\text{relative content (\% of MON 810 DNA to total corn DNA)} = \frac{\text{[concentration of MON 810 DNA]}}{\text{[concentration of corn DNA]}} \times 100.$$

To accept a run, the following criteria must met: (1) the absolute value of standard curve correlation coefficients must be  $\geq 0.95$ ; (2) the result with the known MON 810-positive containing 1% MON 810 DNA must be within a range of 0.5 to 1.5%; (3) the known non-transgenic corn DNA must have a Ct value of  $\geq 40$  in the MON 810 PCR and a Ct value of  $\leq 40$  in the *adh1* PCR; and (4) the no template control must have a Ct value of  $\geq 40$  in both MON 810 and *adh1* PCRs.

To accept the result of a test sample, the relative standard deviation (RSD) of the sample must be  $\leq 50\%$  if the relative content of MON 810 DNA to total corn DNA is  $\leq 5\%$ , and the RSD must be  $\leq 30\%$  if the relative content of MON 810 DNA is  $> 5\%$ .

If a run or sample does not meet these criteria, it must be rejected. The reason for rejection must be documented in the raw data.

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**Raw Data**            The raw data in the form of printed, uniquely identified amplification plots, standard curves, and experimental reports, as well as completed worksheets and the printed plate setup, should be retained.

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**Attachments  
Materials**

**Equipment** The following equipment may be used in this procedure (**equivalents may be substituted**):

<b>Equipment</b>	<b>Number/Specification</b>
ABI Prism <sup>®</sup> 7700 sequence detection system	Applied Biosystems Part No. 7700-01-200/208
Software: Sequence Detection System version 1.7	Applied Biosystems Part No. 4311876
MicroAmp <sup>®</sup> optical 96-well reaction plates	Applied Biosystems Part No. N801-0560
MicroAmp <sup>®</sup> optical tubes	Applied Biosystems Part No. N801-0933
MicroAmp <sup>®</sup> optical caps (8 caps/strip)	Applied Biosystems Part No. N801-0935
MicroAmp <sup>®</sup> cap-installing tool	Applied Biosystems Part No. N801-0438

**Reagents** The following reagents are used in this procedure (**equivalents may be substituted**):

<b>Reagent</b>	<b>Number/Specification</b>
0.5 M EDTA	Sigma Cat. No. E-7889
HCl, 36.5-38.0%	J.T. Baker Cat. No. 7647-01-0
Nuclease-free water	Sigma Cat. No. W-4502
PCR primers (10 µM) and fluorescent oligonucleotide probes (5 µM)	Synthesized by Applied Biosystems, Operon Technologies, Inc., or Integrated DNA Technologies, Inc.
TaqMan <sup>®</sup> universal PCR master mix (2X)	Applied Biosystems Part No. 4304437
1 M Tris-HCl, pH 8.0	Sigma Cat. No. T-3038

**Note:** Fluorescent oligonucleotide probes should be kept in the dark during storage as light can slowly degrade the fluorescent moieties. It is recommended to resuspend the oligonucleotide probes in a sterile buffered solution (e.g., TE buffer, pH 7.0). For optimal long term storage, probe solutions should be aliquoted, lyophilized, and stored in a -20°C freezer.

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<b>Primers &amp; Probes</b>	<b>Sequence (5' to 3')</b>
MON 810 primer 1	AGCCACCACTTCTCCTTGGA
MON 810 primer 2	AGGCTACCGAAAGTCCTCGTT
MON 810 probe	6-FAM-ATCGATGTGGGCTGCACCGACCT-TAMRA
<i>adh1</i> primer 1	CCAGCCTCATGGCCAAAG
<i>adh1</i> primer 2	CCTTCTTGGCGGCTTATCTG
<i>adh1</i> probe	6-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT-TAMRA

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**Buffers and Solutions**

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**Overview**            The following describes the preparation, storage, and stability of the buffer used in this procedure. **Note:** Volume may be scaled as needed. Equivalent reagent may be substituted. To filter sterilize, vacuum filter through a maximum of 0.45 µm filter to a sterile receptacle.

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**TE Buffer, pH 7.0**            **10 mM Tris, 1 mM EDTA, pH 7.0** - For 250 ml:

Mix 100 ml of nuclease-free water, 2.5 ml of 1 M Tris, pH 8.0, and 0.5 ml of 0.5 M EDTA.

Adjust pH to 7.0 with HCl.

Adjust final volume to 250 ml with nuclease-free water.

Filter sterilize.

Store at room temperature for up to 5 years.

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