
A Recommended Procedure for Real-Time Quantitative TaqMan[®] PCR for Bollgard[®] Cotton 531 Monsanto Biotechnology Regulatory Sciences

Overview

Purpose & Scope

This procedure describes an event-specific real-time quantitative TaqMan[®] PCR method for determination of the relative content of Bollgard[®] cotton 531 (hereafter referred to as 531) DNA to total cotton 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.

Monsanto has optimized and performed internal validation on these test methods using the protocols, procedures, conditions, equipment, reagents, test matrix, and DNA isolation method described in the method protocols. While this method protocol has been verified to a high standard in Monsanto's laboratories, variations in laboratory conditions and capabilities require that this protocol must be considered only as a guideline for other users of this method. As with all PCR-based methods, each laboratory and user must validate each method protocol in their individual applications and in their laboratory, and conduct appropriate proficiency testing to establish the reliability, accuracy and reproducibility of the method for that use in that laboratory.

Summary of the Methodology

For specific detection of 531 cotton genomic DNA, a 72-bp fragment of the region that spans the 5' insert-to-plant junction in cotton 531 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 531 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acp1*, an endogenous cotton gene encoding a cotton fiber-specific acyl carrier protein, using a pair of *acp1* gene-specific primers and an *acp1* 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 531 DNA in a test sample, 531 and *acp1* Ct values are determined for the sample. Standard curves are then used to calculate relative content of 531 DNA to total cotton 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**.

Abbreviations

The following abbreviations are used in this protocol:

Abbreviation	Definition
ΔR_n	Represents the normalized reporter signal minus the baseline signal established in the first few cycles of PCR. ΔR_n increases during PCR as amplicon copy number increases until the reaction approaches a plateau.
6-FAM	6-carboxyl-fluorescein
<i>acp1</i>	endogenous cotton gene encoding a cotton fiber-specific acyl carrier protein
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 531 DNA by combining purified genomic DNA from known 531 cotton with that from known non-531 cotton. The concentration of 531 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 cotton DNA in all the standard solutions should be 50.0 ng/μl.

Prepare standard solutions of conventional cotton DNA by combining purified genomic DNA from known conventional cotton with known conventional genomic DNA from another plant species (such as soybean, corn, or canola). The concentrations of cotton 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.

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 531 PCR and one for *acp1* 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 531 and *acp1* 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

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*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 531 DNA in test samples, conduct 531 PCR on all standard solutions of 531 DNA and *acp1* PCR on all standard solutions of non-conventional cotton DNA. Each sample should be analyzed in both PCRs. For each PCR, a known 1% 531 control (1% 531 DNA in 99% conventional cotton DNA), a known conventional cotton 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 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 531 assay in the ABI Prism 7700 system:

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 55°C 1 minute	no yes

For *acp1* assay in the ABI Prism 7700 system:

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 ΔR_n vs. Ct), the standard curve, and the experimental report for each PCR.

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Note: Perform quantitative data analysis for 531 PCR separately from that of *acp1* 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/μl) of 531 DNA and total cotton DNA will be calculated for all samples using the standard curve for 531 PCR and that for *acp1* PCR, respectively, and are then used to calculate the relative content (%) of 531 DNA to total cotton DNA by the following formula:

$$\text{relative content (\% of 531 DNA to total cotton DNA)} = \frac{[(\text{concentration of 531 DNA}) / (\text{concentration of cotton DNA})] \times 100.}$$

To accept a run, the following criteria must be met: (1) the absolute value of standard curve correlation coefficients must be ≥ 0.95 ; (2) the result with the known 531-positive containing 1% 531 DNA must be within a range of 0.4 to 1.6%; (3) the known conventional cotton DNA must have a Ct value of ≥ 40 in the 531 PCR and a Ct value of ≤ 40 in the *acp1* PCR; and (4) the no template control must have a Ct value of ≥ 40 in both 531 and *acp1* PCRs.

To accept the result of a test sample, the relative standard deviation of the sample must be $\leq 50\%$ if the relative content of 531 DNA to total cotton DNA is $\leq 5\%$, and the RSD must be $\leq 30\%$ if the relative content of 531 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.

Raw Data

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

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Attachments

Materials

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

Equipment	Number/Specification
ABI Prism [®] 7700 sequence detection system	Applied Biosystems Part No. 7700-01-200/208
Software: Sequence Detection System version 1.7	Applied Biosystems Part No. 4311876
ABI Prism [®] 7900HT sequence detection system	Applied Biosystems Part No. 4329002 or 4329004
MicroAmp [®] optical 96-well reaction plates	Applied Biosystems Part No. N801-0560
MicroAmp [®] optical tubes	Applied Biosystems Part No. N801-0933
MicroAmp [®] optical caps (8 caps/strip)	Applied Biosystems Part No. N801-0935
ABI PRISM [™] optical adhesive covers	Applied Biosystems Part No. 4311971
ABI PRISM [™] optical adhesive cover starter kit	Applied Biosystems Part No. 4313663
ABI PRISM [™] optical cover compression pads	Applied Biosystems Part No. 4312639
MicroAmp [®] cap-installing tool	Applied Biosystems Part No. N801-0438

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

Reagent	Number/Specification
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 and fluorescent oligonucleotide probes	Synthesized by Applied Biosystems, Operon Technologies, Inc., or Integrated DNA Technologies, Inc.
TaqMan [®] 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

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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.

**Primers &
Probes**

	Sequence (5' to 3')
531 primer 1	TCCCATTCGAGTTTCTCACGT
531 primer 2	AACCAATGCCACCCCACTGA
531 probe	6-FAM-TTGTCCCTCCACTTCTTCTC-TAMRA
<i>acp1</i> primer 1	ATTGTGATGGGACTTGAGGAAGA
<i>acp1</i> primer 2	CTTGAACAGTTGTGATGGATTGTG
<i>acp1</i> probe	6-FAM-ATTGTCTCTTCCACCGTGATTCCGAA-TAMRA

Buffers and Solutions

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.

**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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