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# A Recommended Procedure for Real-Time Quantitative TaqMan<sup>®</sup> PCR for Roundup Ready<sup>®</sup> Canola RT73 Monsanto Biotechnology Regulatory Sciences

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## Overview

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### Purpose & Scope

This procedure describes an event-specific real-time TaqMan<sup>®</sup> PCR method for determination of the relative content of Roundup Ready<sup>®</sup> canola RT73 (hereafter referred to as RT73) DNA to total canola DNA in a sample. The PCR assay has been optimized for use in an ABI Prism<sup>®</sup> 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.

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### Summary of the Methodology

For specific detection of RT73 canola genomic DNA, a 108-bp fragment of the region that spans the 3' insert-to-plant junction in RT73 canola 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 RT73 DNA, a canola-specific reference system amplifies a 76-bp fragment of *FatA*, a canola endogenous gene, using a pair of *FatA* gene-specific primers and a *FatA* 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 RT73 DNA in a test sample, RT73 and *FatA* Ct values are determined for the sample. Standard curves are then used to calculate relative content of RT73 DNA to total canola 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
$\Delta R_n$	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
bp	base pairs
Ct	threshold cycle
EDTA	ethylenediaminetetraacetic acid
<i>FatA</i>	endogenous canola gene encoding acyl-acyl carrier protein thioesterase
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 RT73 DNA by combining purified genomic DNA from known RT73 canola with that from known non-RT73 canola. The concentration of RT73 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 canola DNA in all the standard solutions should be 50.0 ng/μl.

Prepare standard solutions of conventional canola DNA by combining purified genomic DNA from known conventional canola with known conventional genomic DNA from another plant species (such as corn, wheat, or soybean). The concentrations of canola 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 RT73 PCR and one for *FatA* 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 RT73 and *FatA* 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 RT73 DNA in test samples, conduct RT73 PCR on all standard solutions of RT73 DNA and *FatA* PCR on all standard solutions of conventional canola DNA. Each sample should be analyzed in both PCRs. For each PCR, a known 1% RT73 control (1% RT73 DNA in 99% conventional canola DNA), a known conventional canola 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 both RT73 and *FatA* assays 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  $\Delta R_n$  vs. Ct), the standard curve, and the experimental report for each PCR.

Note: Perform quantitative data analysis for RT73 PCR separately from that of *FatA* 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. In the case that the Ct value produced for the 50.0 ng/ $\mu$ l standard solution is greater than the Ct value produced for the 25.0 ng/ $\mu$ l standard solution, omit the Ct value for the 50.0 ng/ $\mu$ l standard solution from the standard curve calculation.

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**Acceptance  
Criteria &  
Interpretation  
of Results**

The concentrations (pg/μl) of RT73 DNA and total canola DNA will be calculated for all samples using the standard curve for RT73 PCR and that for *FatA* PCR, respectively, and are then used to calculate the relative content (%) of RT73 DNA to total canola DNA by the following formula:

$$\text{relative content (\% of RT73 DNA to total canola DNA) =} \\ \frac{\text{[concentration of RT73 DNA]}}{\text{[concentration of canola DNA]}} \times 100.$$

To accept a run, the following criteria must be met: (1) the absolute value of standard curve correlation coefficients must be  $\geq 0.95$ ; (2) the result with the known RT73-positive containing 1% RT73 DNA must be within a range of 0.4 to 1.6%; (3) the known conventional canola DNA must have a Ct value of  $\geq 40$  in the RT73 PCR and a Ct value of  $\leq 40$  in the *FatA* PCR; and (4) the no template control must have a Ct value of  $\geq 40$  in both RT73 and *FatA* 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 RT73 DNA to total canola DNA is  $\leq 5\%$ , and the RSD must be  $\leq 30\%$  if the relative content of RT73 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 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 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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**Primers &  
Probes**

	Sequence (5' to 3')
RT73 primer 1	CCATATTGACCATCATACTCATTGCT
RT73 primer 2	GCTTATACGAAGGCAAGAAAAGGA
RT73 probe	6-FAM-TTCCCGGACATGAAGATCATCCTCCTT-TAMRA
<i>FatA</i> primer 1	GGTCTCTCAGCAAGTGGGTGAT
<i>FatA</i> primer 2	TCG TCCCGAAGTTCATCTGTAA
<i>FatA</i> probe	6-FAM-ATGAACCAAGACACAAGGCGGCTTCA-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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