Primerdesign™ Ltd

GMO event quantification

Maize MON810

Detection and quantification of
GMO integration events by real-time PCR

100 tests

GENESIG

Kits by Primerdesign

Kit contents

- **Maize-WT DNA primer/probe mix (**100 reactions **BROWN)**
- **MON810 (GMO) DNA primer/probe mix (**100 reactions **BROWN)**
- **Positive control template (RED)**
- **GMO reference control sample (BLUE)**
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control template

Reagents and equipment to be supplied by user

- **Real-time PCR Instrument**
- **oasig™ Lyophilised or Precision®PLUS 2X qPCR Master Mix**
  This kit is intended for use with oasig or PrecisionPLUS 2X qPCR Master Mix.
- **Pipettes and Tips**
- **Vortex and centrifuge**
- **Thin walled 0.2 ml PCR reaction tubes**

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from date of resuspension under these circumstances.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration and DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the test sample DNA with RNase/DNase free water.

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Introduction

The Maize line MON810 was produced by biolistic transformation of maize genotype Hi-II with a mixture of plasmid DNAs, PV-ZMBK07 and PV-ZMGT10. The PV-ZMBK07 plasmid contained the cry1Ab gene and PV-ZMGT10 plasmid contained the CP4 EPSPS and gox genes. Both plasmids contained the nptII gene under the control of a bacterial promoter required for selection of bacteria containing either plasmid, and an origin of replication from a pUC plasmid (ori-pUC) required for replication of the plasmids in bacteria.

Maize line MON810 (trade name YieldGard) was developed through a specific genetic modification to be resistant to attack by European corn borer (ECB; *Ostrinia nubilalis*), a major insect pest of maize in agriculture. The novel variety produces a truncated version of the insecticidal protein, Cry1Ab, derived from *Bacillus thuringiensis*. Delta-endotoxins, such as the Cry1Ab protein expressed in MON810, act by selectively binding to specific sites localized on the brush border midgut epithelium of susceptible insect species. Following binding, cation-specific pores are formed that disrupt midgut ion flow and thereby cause paralysis and death. Cry1Ab is insecticidal only to lepidopteran insects, and its specificity of action is directly attributable to the presence of specific binding sites in the target insects. There are no binding sites for delta-endotoxins of *B. thuringiensis* on the surface of mammalian intestinal cells, therefore, livestock animals and humans are not susceptible to these proteins.

Principles of the test

This kit provides a method for detecting gene insertion events by real-time PCR. The kit is based on the PCR amplification and detection across the integration site of the inserted genetic marker. This DNA sequence is artificial and therefore only detectable in a genetically modified organism (GMO) containing that specific modification.

Real-time PCR

The primer and probe mixes provided exploit the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the sample DNA. Fluorogenic probes are included in the reaction mixtures which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be used to detect genetically modified DNA present in the sample.

As well as primers and probe that detect the insertion event, the kit also contains primers and probe to detect the wild-type sequence. Also included is a control DNA containing GM DNA at a ratio of 1 part in 100 (1% GMO). This enables the proportion of GM DNA present in any given sample to be calculated.

GMO Event specific primer and probes

The kit provides “event specific” primers and probe that detect a GM insertion event. The primers and probe are read through the FAM channel and detect the presence of the precise DNA modification event under test and no others.

Wild-type control primers and probe

The kit also provides primers and probe to detect the endogenous wild-type DNA of the host plant. These primers and probe are also detected through the FAM channel and are tested in a separate well. Detection of the wild-type DNA indicates that the extraction process has been successful. With detection levels of the wild-type sequence enabling calculation of the proportion of modified DNA included in the sample. This is critical for determining the % of GM DNA in mixed populations of plants.

Positive control for MON810 and Maize sequences

The kit provides a positive control template for both primer and probes sets. The positive control enables the user to obtain positive traces thereby proving that the PCR reactions have been set up and run correctly. A positive control for each primer set should be included each time a run is performed. The PCR amplicons for the two primer sets have been cloned into the same vector so that the same positive control template can be used for both primer sets.

GMO reference control sample (crushed seeds)

The kit contains crushed wild-type seeds which have been spiked with GMO seeds at a level of 1%. The DNA extracted from this sample is a control for the extraction process to show that DNA can be successfully extracted using your extraction protocol. Data from this control sample for the wild-type primer set and event specific primer set can be used to calculate the percentage of GM DNA present in test samples.

Resuspension protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening**
   This will ensure the lyophilised primer/probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component – Resuspend in water	Volume
Pre-PCR pack	
Maize primer/probe mix (**BROWN**)	110 µl
MON810 primer/probe mix (**BROWN**)	110 µl

3. **Resuspend the positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – Resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
Positive control template (**RED**) *	500 µl

* This component contains high copy number template DNA and is a significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

Perform DNA extraction on test samples and crushed seeds according to the DNA extraction kit protocol.

The crushed seeds should be extracted in parallel and using the same protocol as for all of the samples under test.

N.B. the kit contains the correct amount of seeds required for 1 extraction only.

qPCR detection protocol

1. **Prepare complete reaction mixes for each target primer/probe mix according to the table below:**
   Include sufficient reactions for positive and negative controls as well as each sample and extracted crushed seeds.

Component	1 reaction
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Maize or **MON810** primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final volume	**15 µl**

2. **Pipette 15µl of each mix into individual wells according to your qPCR experimental plate set up.**

3. **Prepare sample DNA templates for each of your samples.**

4. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

5. **Pipette 5µl of positive control template into the positive control wells.**
   The final volume in each well is 20µl

qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR Master Mix

	Step	Time	Temp
Cycling x50	Enzyme activation	2 min	95ºC
	Denaturation	10 s	95ºC
	DATA COLLECTION*	60 s	60ºC

*Fluorogenic data should be collected during this step through the FAM channel.

Interpretation of results

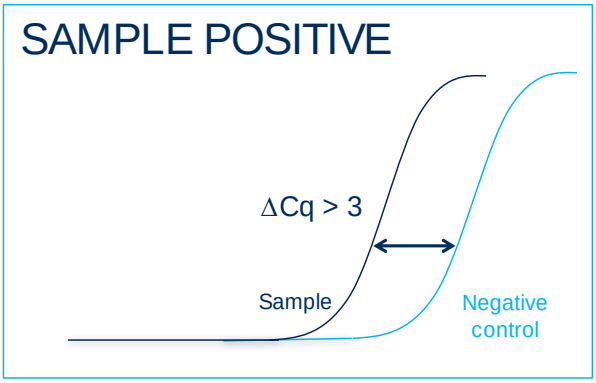
Results from both the test sample and the reference control can be analysed using the table below to determine whether a result can be called as positive or negative.

GMO Target (FAM)	WT Maize (FAM)	Positive control	Negative control	Interpretation
+	+	+	-	**POSITIVE QUANTITATIVE RESULT** Calculate GMO %
+	-	+	-	**POSITIVE QUALITATIVE RESULT** Do not attempt to calculate GMO %
-	+	+	-	**NEGATIVE RESULT**

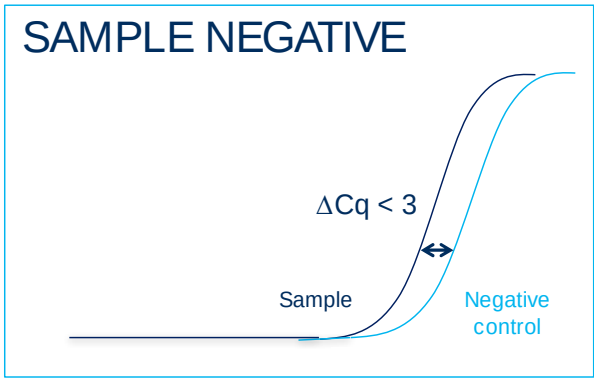
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** Due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	**INAPPROPRIATE SAMPLE TYPE / PREPARATION FAILURE**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 3 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 3 Cq earlier than the negative control then the positive sample result is invalidated and a negative call is the correct result.

Calculating GMO %

If both the test sample and reference control produce positive quantitative results, the presence of the GMO sequence within a sample can be calculated in percentage terms using the formulae below:

Step 1: Calculate the relative WT Maize amounts

$$\text{WT Maize Relative Amount} = 2^{-(\text{WT Maize Sample Cq} - \text{WT Maize Reference Control Cq})} \times 99$$

Step 2: Calculate the relative GM MON810 amounts

$$\text{GM Relative Amount} = 2^{-(\text{GM MON810 Sample Cq} - \text{GM MON810 Reference Control Cq})}$$

Step 3: Calculate GM percentage

$$\text{GM Percentage} = \frac{\text{GM MON810 Relative Amount}}{\text{WT Maize Relative Amount} + \text{GM MON810 Relative Amount}} \times 100$$

Worked example

Test sample
WT event target Cq = **25**
GMO event target Cq = **29**

1% GMO Reference control (supplied)
WT event target Cq = **26.5**
GMO event target Cq = **32**

Step 1: $2^{-(25-26.5)} \times 99 = 280.01$
Step 2: $2^{-(29-32)} = 8$
Step 3: $[8 \div (280.01 + 8)] \times 100 =$ **2.77%**

The test sample percentage = 2.77%