Primerdesign™ Ltd

GMO event quantification

CaMV promoter & NOS terminator detection

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

100 tests

GENESIG

Kits by Primerdesign

Kit contents

- **CaMV-GM primer/probe mix (**100 reactions **BROWN)**
- **CaMV-WT primer/probe mix (**100 reactions **BROWN)**
- **NOS-GM primer/probe mix (**100 reactions **BROWN)**
- **NOS-WT primer/probe mix (**100 reactions **BROWN)**
- **Soya-WT endogenous control primer/probe mix (**100 reactions **BROWN)**
- **Positive control (RED)**
- **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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Principles of the test

The kit provides a method for detecting gene insertion events by real-time PCR. The kit is based on the PCR amplification and detection of two genetic sequences that are commonly used in the genetic modification of plants. The Cauliflower mosaic virus (CaMV) promoter is preferred above other potential promoters because it is highly transcriptionally active and is not greatly influenced by environmental conditions or tissue types. The second sequence, NOS terminator, is derived from *Agrobacterium tumefaciens* and is used as a transcriptional terminator.

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.

Control primers and probes

Since both CaMV and *A. tumefaciens* are naturally found in the soil, it is necessary to exclude false positive results by proving that these wild type organisms have not infected or contaminated a non GM sample. This kit provides control primers and probe that detect both of these organisms in regions of their genome that are not used in the genetic modification of plants.

Endogenous wild-type control primers and probe

The kit provides primers and probe to detect the endogenous wild-type DNA of the soya host plant. These primers and probe are detected through the FAM channel and are tested in a separate well. Detection of the wild-type sequence indicates that the extraction process has been successful.

Positive control

The kit provides a positive control for all primer and probe sets in the kit. This is a vector that contains the cloned amplicon for each primer and probe set. The one positive control is therefore used for all primer and probe sets in the kit.

Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings, a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

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	
CaMV-GM primer/probe mix (**BROWN**)	110 µl
CaMV-WT primer/probe mix (**BROWN**)	110 µl
NOS-GM primer/probe mix (**BROWN**)	110 µl
NOS-WT primer/probe mix (**BROWN**)	110 µl
Soya-WT endogenous control 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 a high copy number template and is a VERY 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 according to the DNA extraction kit protocol.

qPCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below for each of the 5 primer/probe sets:**
   Include sufficient reactions for positive and negative controls as well as each sample.

Component	1 reaction
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
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

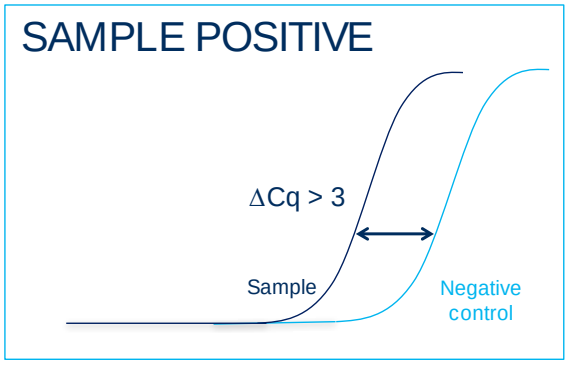
Results from each GM and WT target can be analysed using the table below to determine whether a result can be called as positive or negative.

Target (FAM)	Endog. control (FAM)	Positive control	Negative control	Interpretation
≤ 30	**+ / -**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**+**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**-**	**+**	**-**	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample preparation
-	**+**	**+**	**-**	**NEGATIVE RESULT**

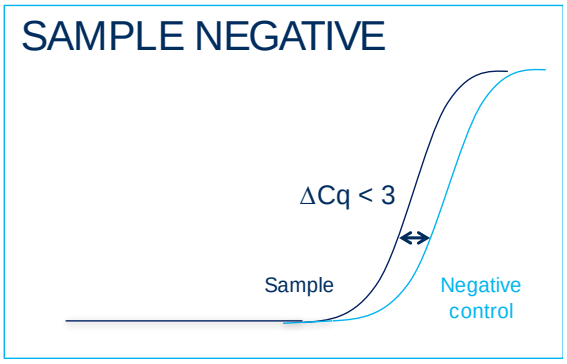
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED** due to test contamination
+ / -	**+ / -**	**+**	**> 35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

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:



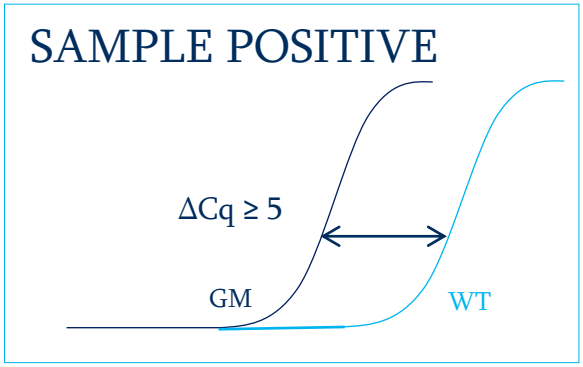
If the sample amplifies > 3 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



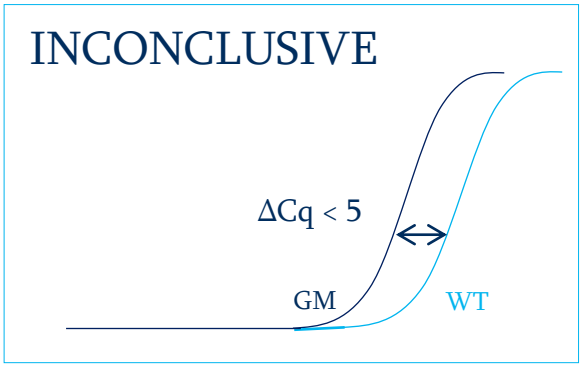
If the sample amplifies < 3 Cq earlier than the negative control then the positive sample result is invalidated and a negative call is the correct result.

Once results for both the GM and WT target of the CaMV or NOS have been analysed individually using the table on the previous page, these results may be used to determine the presence of a genetically modified sample using the following table:

GM Result	WT Result	Decision
POSITIVE RESULT	**NEGATIVE RESULT**	**Significant presence of genetically modified material in tested sample**
NEGATIVE RESULT	**POSITIVE RESULT**	**No significant presence of genetically modified material in the tested sample**
NEGATIVE RESULT FOR BOTH TARGETS		**No significant presence of genetically modified material in the tested sample**
POSITIVE RESULT FOR BOTH TARGETS		Where a positive signal is obtained with both the GM and the WT primer/probe sets, this indicates that both naturally occurring and GM material is present in the samples. The data must be carefully considered by looking at the relative signal strength of the two results to determine if the GM material is present at a significant level.



If the GM primer/probe amplifies ≥ 5Cq values earlier than the WT control primer/probe, then the test confirms the presence of both the CaMV or *A. tumefaciens* organism and GM plant material.



If the traces for the WT and GM are closer together with a < 5Cq difference, then the test is indeterminate since the presence of WT CaMV or *A. tumefaciens* prevents the analysis of the GM content of the sample.