Primerdesign™ Ltd

GMO event quantification

CaMV 35S promoter

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

100 tests

GENESIG

Kits by Primerdesign

Kit contents

- **CaMV-P35S-GM primer/probe mix (**100 reactions **BROWN)**
- **CaMV-WT primer/probe mix (**100 reactions **BROWN)**
- **Internal extraction control primer/probe mix (**150 reactions **BROWN)**
- **Internal extraction control template (**100 reactions **BLUE)**
- **Positive control (RED)**
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**
  for resuspension of internal control template and 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 the Cauliflower mosaic virus (CaMV) promoter. This promoter is preferred above other potential promoters because it is highly transcriptionally active and is not greatly influenced by environmental conditions or tissue types.

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 CaMV is naturally found in the soil, it is necessary to exclude false positive results by proving that the wild type virus has not infected or contaminated a non GM sample. This kit provides control primers and probe that detect a region of the CaMV genome that is not used in the genetic modification of plants.

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 CaMV 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.

Internal extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR amplification of the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the target DNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a Cq value of 28+/-3.

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-P35S-GM DNA primer/probe mix (**BROWN**)	110 µl
CaMV-WT DNA primer/probe mix (**BROWN**)	110 µl
Internal control primer/probe mix (**BROWN**)	165 µl

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

Component – Resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control template (**BLUE**)	600 µl
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

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this can lead to degradation and a loss in signal.

1. **Add 4µl of Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer.**

2. **Complete DNA extraction according to the manufacturer's protocols.**

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.

Component	1 reaction
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
WT or GM primer/probe mix (**BROWN**)	1 µl
Internal extraction control primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	3 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 and VIC channels.

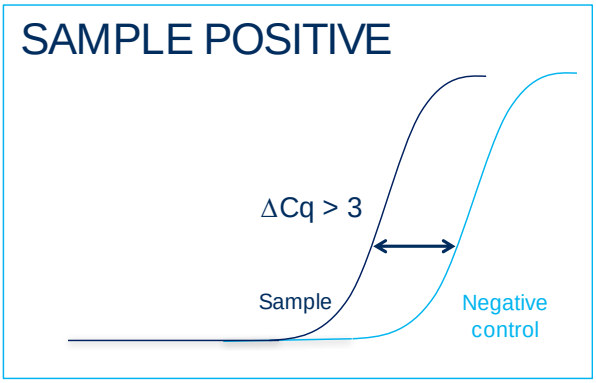
Interpretation of results

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

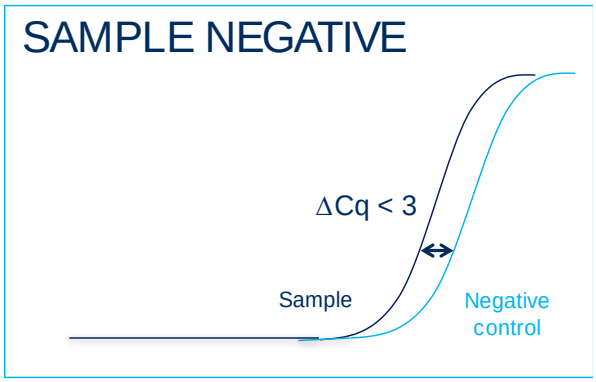
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	**+ / -**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**+**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**-**	**+**	**-**	**POSITIVE QUALITATIVE RESULT** do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



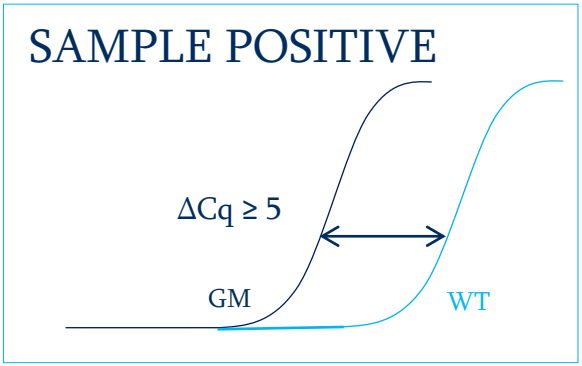
If the sample amplifies > 3 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



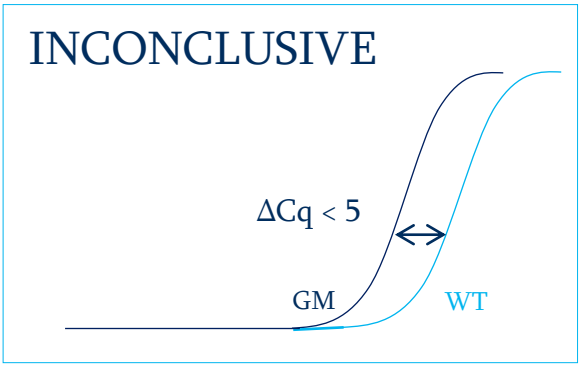
If the sample amplifies < 3 Cq earlier than the negative control then the positive sample result is invalidated and a negative call is the correct result.

Once results for both the GM and WT target 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 CaMV-WT primer/probe sets, this indicates that both naturally occurring and GM CaMV 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 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 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 prevents the analysis of the GM content of the sample.