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GMO*Quant* (LR)
35S Screen Soy

Test kit for quantification of the CaMV 35S promoter in soy DNA

Cat. No. 5125207201, -10

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Test kits, their components and instructions for use are subject to alterations. They are intended for research purposes only.

GMO*Quant*
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Kit for the quantification of the CaMV 35S promoter in soy samples

1. INTRODUCTION AND TEST PRINCIPLE

The GMO*Quant* 35S Screen Soy kit provides materials for the quantitative measurement of genetically modified soy DNA in DNA extracted from food, feed, seeds and environmental samples.

The 35S MasterMix contains all components for the specific detection of the cauliflower mosaic virus (CaMV) 35S promoter element sequence present in several transgenic soy lines, e.g. RoundupReady™ soy and Dupont GM soy.
The soy reference MasterMix contains all reagents necessary to detect DNA from both conventional and transgenic soy. The two detection systems use FAM™-labeled probes.
The GMO*Quant* 35S Screen Soy kit is a highly economical tool for a semi-quantitative GMO screening

in food and feed. The 35S promoter is an element of the genetic modification in different GMO varieties of soy and also other species.
It is important to make sure that the sample does only contain soy as GMO source (and no other species as botanical impurities), because in this case, the 35S result will be biased (if i.e. GM corn contributes to the 35S quantification result).
However, even when the sample contains only soy as possible GMO source, there are still a number of different GM soy varieties in question, which may contain different copy numbers of the 35S promoter.

For this reason it is very important to keep in mind that the results achieved with this kit cannot be more than a rough estimation of the GMO content.

The kit is calibrated with RoundupReady™ soy and thus based on a 1:1 target ratio. A 1% GMO:non-GMO mixture on a weight/weight basis of homozygous material of a GMO as e.g. RR soy containing only one integrated 35s copy per transgenic genome should give a result of 1 %. For a GMO with 4 copies per genome (e.g. A2704-12) the expected measured value is approximately 4 % for a homozygous 1% w/w sample and the w/w percentage will thus be over-estimated.

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GMO	OECD identifier	Copy no. of the p35S per haploid genome
A2704-12 Soy	ACS-GMØØ5-3	2 *(GM Crop database)* or 4 *(Biosafty scanner, GMDD)*
GU262 Soybean	ACS-GMØØ3-1	2
A2704-21 /	ACS-GMØØ4-2,	5
A5547-127 Soy	ACS-GMØØ6-4	1
DuPont Soy	DD-Ø26ØØ5-3	1
Roundup Ready Soy	MON-Ø4Ø32-6	1
Optimum GAT Soy	DP-356Ø43-5	1
Event W89 Liberty Link	ACS-GMØØ2-9,	12 or more
Event W62 Liberty Link	ACS-GMØØ1-8	2 or more
MON89788 Soy	MON-89788-1	0
MON87705 Soy	MON-877Ø5-6	0

Table 1: CaMV 35S promoter copies in different GM soy lines. (source: Biosafty Scanner, GM Crop Database, GMO Detection method Database (GMDD) - no responsibility is taken for this information.)

2. METHOD

DNA amplification and detection methods take advantage of the relative stability of the DNA, which resists the processing steps which are used in food and feed production. DNA can be detected and quantified with high sensitivity and precision. The procedure follows the basic principles of the real-time PCR amplification method and of relative quantification using the standard curve method.
The GMO*Quant* 35S Screen Soy amplifies a DNA fragment which is present in a large number of different genetically modified (GM) plant varieties.

For relative quantification of the detected amounts of GM soy DNA, a soy reference gene sequence is amplified from the same sample DNA.

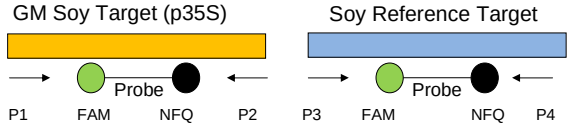

Figure 1: FAM is the fluorescent reporter dye attached to the probes for the 35S promoter and the soy reference (le1). The quencher is a non-fluorescent quencher.

Two standard curves are generated, one each with the 35S system and with the soy reference system. For each standard curve four calibration points are determined with the calibration DNA standards included in the kit. The sample DNA is compared to the calibration curves and "virtual" copy-numbers are deducted. Subsequently the concentration of the unknown DNA sample is calculated by relative quantification (35S/soy ref).

The kit uses

- FAM (reporter dye for both, 35S and soy reference)
- NFQ (Non Fluorescent Quencher)
- ROX (Normalizer)

The ROX concentration of this kit is laid out for PCR real-time machines requiring a low ROX level, like e.g. the ABI 7500 from Applied Biosystems.
The kit can also be used with machines not requiring ROX normalisation like e.g. the Agilent MX3005P.

3. REAGENTS

The GMO*Quant* 35S Screen Soy kit contains sufficient reagents for 2 x 48 reactions or 10 x 2 x 48 reactions for cat# -10, respectively.

1. MasterMix (LR) GS-P-07.060 • p35S (RED CAPS) ready-to-use PCR reaction mix;
   1 x 1000 µL, for # -10: 10 x 1000 µL
2. MasterMix (LR) Soy lectin1 (GREEN CAPS) ready-to-use PCR reaction mix;
   1 x 1000 µL, for # -10: 10 x 1000 µL
3. Calibration Standards 1-4 (STRIPED VIOLET CAPS): 4 x 65 µL of calibration DNA,
   for #-10: 10 x 4 x 65 µL (see also table below)
4. 1.0% RR Soy DNA (CLEAR CAP)
   65 µL; for #-10: 10 x 65 µL
   quantitation control, made from CRM ERM-BF410dk soy meal containing 1% RRS (w/w).

Store all reagents light protected at –20°C.

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MATERIAL	Soy Reference Copy Nos/Rxn	35S Copy Nos/Rxn
Standard 1	86,400	8,640
Standard 2	14,400	1,440
Standard 3	2,400	240
Standard 4	400	40

Shown are the assigned target copy numbers and DNA amounts per 5 µL of the calibration standards.

3.1 Materials Required But Not Supplied

- Optical plates and optical seals
- Precision pipette to deliver 5µL and 20 µL
- Vortex mixer
- Centrifuge (1500 x g, preferably refrigerated)
- Centrifuge for micro titer plates (preferably refrigerated)
- ABI 7500 SDS, Agilent MX3005P or equivalent RT-PCR instrument

4 RECOMMENDATIONS AND WORKING PRECAUTIONS

For DNA extraction use only molecular-biology grade reagents and sterile-filtrated, deionised water (DNA-free) and 0,1x TE buffer for DNA dilutions (Tris-HCl pH=8,0 c=1 mmol/L; EDTA pH=8,0 c=0,1mmol/L):

Maintain separate working areas for DNA preparation, reaction setup, amplifications and reaction product analyses. Never transfer amplified products into the reaction setup, or extraction area. Use filter-plugged pipette tips in order to avoid possible cross-contamination. Use only powder-free gloves and change them frequently during the process. Clean lab-benches and equipment periodically with 10% bleach solution. To obtain reliable quantification results all pipettes have to be checked regularly for precision and if necessary have to be calibrated.

5 PROCEDURAL NOTES

A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert. The standard curve must be included in each quantification run.
Do not mix various lots of any kit component within an individual run. Do not use any component beyond the expiration date shown on its label.
After removing reagents from the refrigerator, allow them to thaw slowly and mix them by vortexing and briefly centrifuge before use. Keep the MasterMix cooled during the working steps. Thaw only the MasterMix amounts needed for the run. If smaller volumes are needed, aliquot reagents at the first use.
Unused reagents should be stored at –20°C. Standards and positive controls should be mixed by vortexing and briefly centrifuged before use.

6 TEST PROCEDURE

6.1 General Description

Different assay formats are possible: For one laboratory sample either one DNA extraction with two or more PCR-replicates or two parallel (“A” and “B”) DNA-extractions (e.g. as recommended by certain national and international standards) may be analysed. Different numbers of replicates analysed per DNA sample (e.g. triplicates in case of only one DNA-sample) may be chosen. The mean value of several replicates will yield increased precision of the analysis. Furthermore, different ways to check sample DNAs for inhibitory effects may be applied – e.g. with analysis of the DNA in two defined dilutions. This can either be done in a preceding monitor run or, according to the user´s preference, it can also be done in the quantitation run without prior monitor run.

The proceedings described are made for the following format and assumption.

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DNA- Test-Samples
Two independent DNA extracts yielding an “A” and a “B” sample DNA are taken. Each DNA is analysed in two different defined concentrations (dilution 1 (dil.1) and dilution 2 (dil.2)). For example, each DNA could be analysed undiluted (dil. 1) and a known dilution like e.g. a fivefold dilution with water or TE (dil. 2). Each DNA dilution is analysed by a single determination (no replicates).

Calibration curves
Four concentrations per target are analysed in duplicate each.

6.2 Calculation of Reactions Needed for Analysis

Calculate the number of reactions and amount of equivalent MasterMix volume before thawing the reagents and starting the practical work. Quantitative analyses require at least the following reactions:

Table 3: Number of reactions needed when analysing 1 or 9 samples.

Reaction Type		Sample Number per run			
		1		9	
		35S Rxns	Soy Ref. Rxns	35S Rxns	Soy Ref. Rxns
Sample DNA Two Extracts in 2 Dilutions	DNA #1A (dil.1)	1	1	9	9
	DNA #1A (dil.2)	1	1	9	9
	DNA #1B (dil.1)	1	1	9	9
	DNA #1B (dil.2)	1	1	9	9
DNA Standards 4 Calibration Points in Duplicates	35S STND	8	-	8	-
	Soy Ref. STND	-	8	-	8
Controls	No Template Control (NTC)	2	2	2	2
	Quantification Control	2	2	2	2
	Number of Rxns	16	16	48	48

6.3 Setup and Programming of Plate Documents

Before starting the practical work, program the plate document. For general and more detailed instructions please refer to the user guide of the instrument and respective software version and to our application notes for use with your cycler model (see www.eurofins.com/kits).

Program the 'Thermocycler Conditions' as given below. Before amplification cycling the polymerase activity must be activated by thermal incubation at 95°C for 10 min.

The PCR temperature profile is:

Temperature	Time	
95°C	10 min	
95°C	15 sec	45 cycles
60°C	90 sec	

For other thermocyclers, it may be necessary to optimise the PCR parameters.

6.4 Sample DNA

Absolute DNA amount and purity (meaning: free of PCR inhibiting substances) affect overall sensitivity as well as accuracy of the analysis. Low DNA amount subjected to analysis results in poor LOD and LOQ with regard to the specific test sample. Insufficiently pure DNA displaying inhibitory effects increase inaccuracy or even lead to totally biased results.

It is recommended to use 200 ng DNA of the species of interest per reaction. The concentration of DNA should be adjusted accordingly prior to the set-up of the reactions. DNA amount can be determined prior to analysis by one of the following methods:
- Real Time PCR monitor run (preferred method)
- Fluorimetric or spectrophotometric or other physical measurements, e.g. gel electrophoresis may be applied.

Especially the spectrophotometric methods can suffer significant errors due to interfering DNA impurities or partial DNA-denaturation/degradation. When analysing samples for which there is a lot of experience in the extraction yield and purity, DNA measurement may be omitted.

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6.5 Plate Setup

The following plate document (table 4) shows the distribution of reactions for quantitative analysis of 9 samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St1	St1	St2	St2	St3	St3	St4	St4	NTC	NTC	NTC	NTC
B	St 1	St1	St2	St2	St3	St3	St4	St4	1%	1%	1%	1%
C	1a	1b	1a	1b	1a D	1b D	1a D	1b D	2a	2b	2a	2b
D	2a D	2b D	2a D	2b D	3a	3b	3a	3b	3a D	3b D	3a D	3b D
E	4a	4b	4a	4b	4a D	4b D	4a D	4b D	5a	5b	5a	5b
F	5a D	5b D	5a D	5b D	6a	6b	6a	6b	6a D	6b D	6a D	6b D
G	7a	7b	7a	7b	7a D	7b D	7a D	7b D	8a	8b	8a	8b
H	8a D	8b D	8a D	8b D	9a	9b	9a	9b	9a D	9b D	9a D	9b D

Table 4: Plate layout for 9 samples:
green: Species MasterMix,
orange: GM MasterMix; NTC= no template control,
St = Standard (row A: species ref. standards, row B: GM standards);
a and b = Duplicates of Sample; D = Dilution of Sample

In this example, 48 reactions are needed for each system (GM = 35S MasterMix; SpeciesRef.= species reference MasterMix).

Thirty-six of these reactions in each system will analyse 9 different test samples (#01 to #09), where DNA-extracts a and b are tested at two dilutions (e.g. undil. and dil. fivefold). The remaining 12 reactions in each system will analyse the 4 calibration standards (STND1 to STND4) in duplicate, two no template controls in duplicate and two quantitation controls (1% RRS).

7 PROCEDURE

Before starting the practical working steps switch on the instrument and make sure the plate document is properly set and programmed.
Allow the MasterMix reagents to thaw and mix them by vortexing. Centrifuge carefully before use.

1. Add 20 µL of the MasterMix into appropriate tubes or wells of the plate according to the programmed plate document.
2. Add 5 µL of DNA solution (Standards, Controls or Unknowns) to the inner side of the tube or well.
3. Carefully close all tubes/plates with optical caps/seals.
4. Centrifuge tubes or plate carefully at low speed.
5. Place tubes or plate into the thermal block of the instrument, close lid and start the run.

8 RESULTS

The four calibration standards analysed in both systems (GM soy and soy reference) allow for the calculation of the copy numbers for both targets in every sample. The number of copies of GM soy is then divided by the number of copies of soy reference in the same volume, of the same dilution, of the same sample in order to obtain the relative GM ratio (%). Then, the ratios of GM soy/soy reference can be compared among all replicates of a sample. A sample of known GMO concentration (DNA from 1.0% RRS certified reference material) is included in the analysis, this sample serves as a control for the calibration and the quantification process and should not show a deviation of more than 30% from the expected result.

Our recommendation is to export the Ct values from the cycler´s software to a local spreadsheet (e.g. Microsoft Excel™) with subsequent standard curve calculation, because this saves individual programming of plates. (Slightly different results may be obtained compared to evaluation in the cycler software due to rounding errors.) The following section describes a short protocol for data analysis of quantitative runs.

8.1 Evaluation

Refer to your cycler´s manual for details and to our application notes for use with your cycler model (see www.eurofins.com/kits). An evaluation (Excel™) sheet can be requested at kits@eurofins.com.

Threshold: The threshold should be placed in the region of exponential amplification across all of the amplification plots. This region is depicted in the log view of the amplification plots as the portion of the plot, which is linear. The threshold line should neither be placed in the plateau phase nor in the initial linear phase of amplification. Thresholds are typically in the range 0.03 – 0.1.

Baseline:
ABI 7500/7500 Fast: Set manually, 3-15
Stratagene Mx: Adaptive

The percentage of 35S containing DNA in each sample is calculated as the ratio of the 35S DNA quantity divided by the soy reference DNA quantity (in copy numbers). The spreadsheet table can have implemented functions to calculate the 35S/GM soy DNA content in percent.

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8.2 Interpretation of Results

Depending on the assay format chosen, the interpretation of the results may differ. Here it is described for the recommended format.

Parameters and Acceptance criteria
These criteria have to be fulfilled:

- Correlation coefficient of regression line (R^2) ≥ 0.995
- Slope of regression line between -1.3 and -1.5
- PCR Efficiency ($[10^{(-1/slope)}]$-1) from 90% to 110%
- Deviation of quantification result of quantification control from expected result +/- 30%
- No template controls (NTC) show no amplification

Table 5: Example for the quantification of the 1% control.

Sample Name	35S Quantity	Soy Ref. Quantity	Relative 35S DNA Content	Mean 35S DNA Content
#1/A dil.1	680	70,652	0.96 %	1.02 %
#1/B dil.1	644	55,241	1.17 %	
#1/A dil.2	136	15,902	0.86 %	
#1/B dil.2	156	14,420	1.08 %	

This example (table 5) shows a possible relative GM Soy DNA content for the quantification control, calculated using the procedure above. Two DNA extracts were prepared in parallel from the material and two dilutions of these DNA extracts were analysed. The mean 35S DNA content as well as the corresponding standard deviation is calculated from the four individual results obtained with the DNA sample dilutions.

The unit of measurement is "% GM DNA", because the calibration standards are made from genomic homozygous DNA. For copy number deviations of individual GMOs, please also see chapter 1.

Homogeneity / DNA Extraction
If independently extracted DNAs show significant deviations between "A" and "B", this may be due to sample material non-homogeneity (if differences in the quantification results) or to non-uniformities in the DNA-extraction efficiency (if differences in the determined copy numbers). If the two quantification results and/or DNA copy numbers for the species reference system show significant differences, repeat DNA extraction and homogenise sample more thoroughly.

Inhibition
At least two dilutions of the DNA test sample should be used to detect inhibitory effects. If the delta Ct does not reflect the dilution (approximation: dilution factor of 4 must yield a delta Ct of 2) or if GMO quantities determined on the two dilution levels differ significantly (>30%), inhibitory effects can be suspected and thus the analysis needs to be repeated with either more purified DNA or with more diluted DNA (please be aware that dilution will affect your sample/practical LOQ).

Dilution of sample	Acceptable result factor
1:5	1:4 to 1:6
1:10	1:8 to 1:12
1:20	1:16 to 1:24

9 PRODUCT WARRANTIES AND SATISFACTION GUARANTEE

Eurofins GeneScan Technologies GmbH ("GeneScan") warrants the products manufactured by it will be free of defects in materials and workmanship when used in accordance with the applicable instructions for a period equal to or shorter of one year from the date of shipment of the product(s) or the expiration date marked on the product packaging under the storage conditions, recommended in the instructions and/or on the package. Application protocols published by GeneScan are intended to be only guidelines for the buyers of the products. Each buyer is expected to validate the applicability of each application protocol to his individual application. GeneScan makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. GeneScan's sole obligation with the respect to the foregoing warranties shall be, at its option, to either replace or to refund the purchase price of the product(s) or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies GeneScan promptly of any such defect.

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10 PRODUCT USE LIMITATIONS

This Eurofins GeneScan Technologies GMO*Quant* Kit is developed, designed, and sold for research purposes only. It is not to be used for diagnostic purposes or analysis of food and feed unless expressly cleared for that purpose by the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

11 TECHNICAL SERVICE

If you have any questions or experience any difficulties regarding this kit or Eurofins GeneScan Technologies products in general, please do not hesitate to contact us. Our customers are a major source of information regarding advanced or specialised uses of our products. This information is helpful to other scientists as well as to the researchers at Eurofins GeneScan Technologies.

We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques. For technical assistance and more information please contact the Eurofins GeneScan Technologies Technical Service Department or your local distributor.