GMO*Quant* (HR)
Roundup Ready™ Soy

Test kit for quantification of MON4032-6 soy DNA

Cat. No. 5125200401

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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*
Roundup Ready™ Soy Kit

Kit for the quantitation of DNA from Roundup Ready™ soy in food and feed samples

1 INTRODUCTION

The quantitative Roundup Ready™ Soy DNA detection kit provides materials for the quantitative measurement of Roundup Ready™ soy (OECD unique identifier: MON-Ø4Ø32-6) DNA in DNA extracted e.g. from food, feed, seed and environmental samples.

DNA detection methods take advantage of the relative stability of DNA, which resists the processing steps used in food and feed production better than e.g. proteins. DNA can be detected and quantified with high sensitivity and accuracy with RT-PCR (real-time polymerase chain reaction).

2 PRINCIPLE OF THE TEST

The GMO*Quant* Roundup Ready™ Soy test kit employs a quantitative real-time PCR system for ABI-PRISM® 7900 and equivalent real-time PCR cyclers with two single systems for the detection of soy (species/soy reference) and Roundup Ready™ soy (GMO).
DNA segments are amplified with two specific primers. PCR products are measured real-time with a target-specific oligonucleotide probe labelled with fluorescent dyes:

- Probe 5´: FAM
  (Reporter detection systems RRS and soy reference)
- Probe 3´: TAMRA (Quencher)
- ROX (passive reference)

The kit contains ROX as normalizing dye in a concentration apt for ABI PRISM® 7700, 7900 and similar machines.[1]
The GMO system is modification specific and detects the transition of CaMV 35S promoter and petunia transit peptide sequences.
The reference system is species specific and detects a segment of the lectin (le1) housekeeping gene of soy.

[1] for ABI 7500, Stratagene MX and similar machines please preferentially use our special low-ROX kits.

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Two standard curves are generated, one each with the GMO system and the species reference system.
For each standard curve four calibration points are determined with the calibration DNA standards included in the kit. Subsequently the percentage of GMO DNA in the unknown DNA sample is calculated by relative quantification.

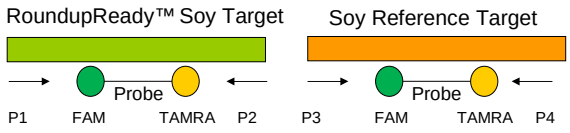

Figure 1: FAM and TAMRA are fluorescent dyes (reporter and quencher) attached to the 5' and 3' ends of the probes for RoundupReady™ Soy and the soy reference (le1).

3 MATERIALS

The GMO*Quant* RoundupReady™ Soy kit contains sufficient reagents for 2 x 48 reactions:

1. MasterMix (HR) GS-P-07.042 • RRS-HT-P/C (RED CAPS) PCR reaction mix incl. primers, probe, dNTPs, hot-start Taq DNA polymerase, buffer; 1 x 1000 µL
2. MasterMix (HR) Soy lectin1 (GREEN CAPS) PCR reaction mix incl. primers, probe, dNTPs, hot-start Taq DNA polymerase, buffer; 1 x 1000 µL
3. Calibration Standards 1-4 (STRIPED VIOLET CAPS) 4 x 65 µL of calibration DNA, made from CRM ERM-BF410gk soymeal containing 10% RRS (w/w) (see also table 1)
4. 1% RR Soy DNA (CLEAR CAP) 65 µL quantitation control, made from CRM ERM-BF410dk soymeal containing 1% (w/w) RRS

Store all reagents light protected at –20°C.

MATERIAL	Copy Numbers Soy Ref/Rxn	Copy Numbers RRS/Rxn
Standard 1	86,400	8,640
Standard 2	14,400	1,440
Standard 3	2,400	240
Standard 4	400	40

Table 1: Shown are the assigned target copy numbers and DNA amounts per 5 µL of the calibration standards.

Material required but not supplied

- Optical tubes or plates and optical caps or seals
- Accuracy pipette to deliver 5 µL and 20 µL
- Vortex mixer
- Centrifuge (1500 x g, preferably refrigerated)
- Centrifuge for micro titer plates (preferably refrigerated)
- ABI PRISM® 7700, 7900 SDS 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 calibrated regularly.

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5 PROCEDURAL NOTES

A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained when precise laboratory techniques are employed and the package insert is accurately followed.

The standard curve must be included in each quantification run.

Do not mix kit components of various kit lots within one 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 volumes needed for the run. 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 accuracy of the analysis. Furthermore, different ways to check sample DNAs for inhibitory effects may be applied – e.g. analysing 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:

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.

6.1.1 Calculation and Setup of Reactions

Calculate the number of reactions and amount of equivalent MasterMix volume before thawing the reagents and starting the practical work. The calibration standard and positive control materials are supplied with the kit.
The MasterMix solutions supplied are pre-made reagents ready for direct use. Quantitative analyses require at least the following reactions:

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

Table 2: Number of reactions needed for 1 or 9 samples.

6.1.2 Setup and Programming of Plate Documents

Before starting the practical work, program the plate document.

For setup and evaluation information for your individual cycler, please send an email to kits@eurofins.com or see our website www.eurofins.de/kits.

Program the ‘Thermocycler Conditions‘ as given in below.

Thermal Cycler Times and Temperatures		
HOLD	CYCLE (45 repeats)	
10 min at 95°C	15 sec at 95°C	60 sec at 60°C

Save the created file document in the normal file format with the ‘**Save‘** function in the file menu.

6.1.3 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 100 ng DNA of the species of interest per reaction. The concentration of DNA should be adjusted accordingly prior to the setup 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 matrices known with regard to extraction yield and purity, DNA measurement may be omitted.

6.1.4 Plate Setup "Quantitative Run"

The following plate document shows the distribution of reactions used for quantitative analysis of 9 species 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 ref. MasterMix, orange: GM MasterMix; NTC = no template control, St = Standard (row A: species ref., row B: GM); a and b = duplicates of sample; D = Dilution of sample

In this example, 48 reactions are needed for each system (GM = Roundup Ready™ soy MasterMix; Species Ref. = Soy 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).

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6.1.5 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, and start the run.

7 RESULTS

The four calibration standards analysed in both systems (GMO and Species-Ref.) permit the calculation of the number of copies for these DNA targets in every sample. The number of copies of GM-target divided by the amount of species DNA in each sample gives the GMO percentage for the respective sample. Then, the ratios of GMO/species ref. can be compared among all samples (e.g. replicates, dilutions) analysed. A sample of known GMO percentage is included in the analysis, this sample serves as a control of the calibration standards and the quantification process.

Quantification can be accomplished either within the cycler´s software or on a local spreadsheet (e.g. Microsoft Excel™), with standard curve calculations made by the user or an evaluation (Excel™) sheet can be requested at kits@eurofins.com. Slightly different results may be obtained.
The following section gives a short protocol for data analysis of quantitative runs.

The actual unit of measurement is “percent of haploid genome equivalents (HGE) GMO” or “percent (g/g) GMO”, because the calibration standards are made from homozygous Roundup Ready™ Soy.

Sample Name	RRS Quantity	Soy Ref. Quantity	Relative RRS DNA Content	Mean RRS DNA Content
#1/A dil.1	340	35,325	0.96%	1.02%
#1/B dil.1	322	27,622	1.17%	
#1/A dil.2	68	7,958	0.85%	
#1/B dil.2	78	7,211	1.08%	

Example for an evaluation of a quantitative result.

This example above shows a possible relative Roundup Ready™ Soy DNA quantification result, calculated with the procedure described above. Two DNA extracts were prepared in parallel from the material and two dilutions of these DNA extracts were analysed. The mean Roundup Ready™ Soy DNA content as well as the corresponding standard deviation is calculated from the four individual results obtained with the DNA sample dilutions.

7.1 Interpretation of Results

Depending on the assay format chosen, the interpretation of the results may differ. Here it is described for the recommended format as described above. The following parameters should be judged:

7.1.1 Parameters and Acceptance criteria

These criteria have to be fulfilled:

- Correlation coefficient of regression line (R^2) ≥ 0.98.
- The average value of the slope of the standard curves should be approximately in the range of -3.1 ≥ slope ≥ -3.6, corresponding to amplification efficiencies of 90% to 110%.
  (*Efficiency [%] = [$10^{(-1/slope)}$] -1) x 100*).
- Deviation of quantification result of quantification control (prepared from CRM) from expected result +/- 30%.
- No template controls (NTC) show no amplification.

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7.1.2 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 quantitation results) or to non-uniformities in the DNA-extraction efficiency (if differences in the determined copy numbers).
If the two quantitation results and/or DNA copy numbers for the species reference system show significant differences, repeat DNA extraction and homogenise sample more thoroughly.

7.1.3 Inhibition Control with Sample Dilution

At least two dilutions of the DNA test sample should be used to detect inhibitory effects.
As inhibition control the experimentally determined dilution factors in the species specific as well as in the GMO specific systems are calculated using the following equation:

$$Dilution\ factor = \frac{Copy\ number_{undiluted\ sample}}{Copy\ number_{diluted\ sample}}$$

The calculated dilution factor is than compared with the dilution of the sample.
Deviation of +/- 20% can be accepted.
Deviation from calculated dilution factor to actual dilution factor:

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

If deviations occur, samples can be accepted after case to case evaluation. But deviation of quantification of

duplicates should not be greater than -50% to +100%. For greater deviations, the analysis has to be repeated.

8 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 than 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 fit-ness for a particular purpose. GeneScan's sole obli-gation with the respect to the foregoing warranties shall be, at its option, to either replace or to refund the pur-chase 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.
GeneScan shall not be liable for any direct, indirect or consequential damages resulting from economic loss or property damages sustained by buyer or any customer from the use of the product(s). A copy of Eurofins GeneScan Technologies GmbH terms and conditions can be obtained on request, and is also provided in our price lists.

9 PRODUCT USE LIMITATIONS

This 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.

10 TECHNICAL SUPPORT

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 GeneScan.

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 call Eurofins GeneScan Technologies or your local distributor.