imegenagro
Food Safety

Instructions for Use

imegen™ MON89788 Quantification Kit

Quantification of MON89788 soy
by real-time PCR

REF **IMG-256**

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imegen guarantees that its products are free from defects, both in the used materials and in its manufacturing process. This warranty is extended to the expiration date, as long as the storage conditions specified in this manual are met.

Our products are designed for use in research. *The user of the product is responsible for validating the usefulness of the protocol proposed by imegen. These protocols are considered as a guide only. imegen does not offer any other warranty, express or implied, which extend beyond the proper functioning of the components of this set. imegen sole obligation in respect of the preceding guarantees, will be to replace the product or return the purchase price thereof, as desired by the customer, as long as the existence of a defect in the materials test, or in the manufacture of its products. Imegen will not be responsible for any damage, direct or indirect, resulting in economic losses or damages resulting from the use of this product by the purchaser or user.*

All products sold by the imegen are subjected to rigorous quality control. The ***imegen® MON89788 Quantification kit*** *has passed all internal validation tests, ensuring the reliability and reproducibility of each assay.*

For any questions about the applications of this product or its protocols, please contact our Technical Department:

Phone number: *963 212 340*
e-Mail: *tech.support@imegen.es*

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1. General Information

Genetically modified organisms (GMOs) are widely distributed, with soy and corn being two of the most extensively cultivated crops worldwide. Indeed, Roundup Ready transgenic soy accounts for some 77% of the soy cultivated worldwide, thus making it the most widespread transgenic event and therefore the one that generates the most problems. Developments in biotechnology and molecular-assisted breeding have enabled Monsanto to generate a second-generation glyphosate-tolerant soybean product, Roundup Ready2Yield™ or MON89788.

Overall, MON89788 accounts for over 7% yield advantage compared to Roundup Ready soybean 40-3-2.

The European Union has established a legal framework to regulate the use, release into the environment and, above all, labelling of foodstuffs containing genetically modified organisms.

The ***imegen-MON89788 Quantification Kit*** *allows the percentage of Roundup Ready 2 or MON89788 soy in a sample to be determined with respect to total soy.*

This kit uses Real-Time PCR technology and contains all the reagents required to quantify the MON89788 soy in DNA obtained from any food or feed. Furthermore, the kit contains the plasmid DNA used as a standard with which the samples can be compared to determine the percentage of MON89788 soy.

2. Intended Use

imegen® MON89788 Quantification kit *enables the user to quantify the relative amount of MON89788 in a soysample.*

For this purpose, it is necessary to extract the total DNA from the test sample to later use it a real-time PCR assay which will enable to the relative quantification of MON89788 soy. The assay is composed of two specific PCR systems containing oligonucleotides and hydrolysis probes labelled with the FAM™ fluorophore that will enable to amplify the transgenic event, MON89788, as well as the amplification of a soy endogenous gene, Lectin. The MON89788 PCR system specifically enables the amplification of a fragment in the integration region where the construct was inserted into the plant genome (5' insert-to-plant junction). This fragment is event-specific, in other words it amplifies the binding region between the soy genome and the construct introduced in MON89788 soy.

The inclusion of a plasmid system containing each of the targets, MON89788 and lectin in an equimolar proportion enables the construction of a standard curve of known copy number to compute the proportion of each one of the transgenic events.

3. Technical Characteristics

imegen® MON89788 Quantification kit *has been validated using GM and non-GM soy and other crop samples, as well as animal samples.*

The repeatability and the reproducibility of the assay has been proven using dilutions of the DNA of interest. The limit of relative quantification (LOQrel) has been established in 0.1%, whereas the limit of absolute quantification (LOQabs), corresponding with the lowest value included in the standard curve, is established to be 20 total copies for each of the quantifiable systems (soy lectin and MON89788).

The theoretical sensibility and specificity have been verified by in silico comparisons with the sequences retrieved from public databases. In addition, the analytical specificity has been experimentally tested using DNA samples from and GM crops including soy [1], maize [2], cotton [3] and rapeseed [4], as well as non-GM crops and farmed animals.

Table 1. *Animal and plant species analised during the specificity assays*

MON89788 Quantification Assay			
GM-Crops	Result	GM-Crops	Result
MON89788 [1]	D	MON88017 [2]	ND
RR [1]	D	DAS40278 [2]	ND
MON87769 [1]	ND	Bt11 [2]	ND
MON87705[1]	ND	MON863 [2]	ND
A2704-12[1]	ND	DAS01507 [2]	ND
A5547-127 [1]	ND	*DAS21023* [3]	ND
DP305423 [1]	ND	*LLCotton25* [3]	ND
CV127 [1]	ND	*MON1445* [3]	ND
MON87708 [1]	ND	*MON15985* [3]	ND
DAS68416 [1]	ND	*T45* [4]	ND
GA21 [2]	ND	*Ms8* [4]	ND
MON87460 [2]	ND	*GT73* [4]	ND
NK603 [2]	ND	*Ms1* [4]	ND

MON89788 Quantification Assay			
Non-GM Samples	Result	Non-GM Samples	Result
Maize	ND	*Rapeseed*	ND
Carrot	ND	*Cow*	ND
Lentil	ND	*Sheep*	ND
Sesame	ND	*Buffalo*	ND
Oats	ND	*Atlantic cod*	ND
Wheat	ND	*Common dab*	ND
Cotton	ND	*Pork*	ND

D: Detected
ND: Not Detected

** The analytical method is detailed in section 8. Results analysis (Figure 7).*

This product meets the quality requirements for the ISO 9001, not only the materials used in its manufacturing process but the final marketed product as well. It is also analytically and functionally stable during the optimum time of use, provided that the instructions for use and storage, specified in Section 5 of this manual (Contents and storage conditions), are followed.

4. Warnings and precautionary statements

1. *Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.*
2. *Do not pipette by mouth.*
3. *Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.*
4. *You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.*
5. *Send down the drain only those materials found on the safe list. Compounds not listed are not suitable for drain disposal. Use waste containers according to the local legislation and manage their treatment through an authorised waste manager.*
6. *In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.*
7. *The materials safety data sheets of all hazardous components contained in this kit are available on request to imegen.*
8. *This product could require the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.*
9. *Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.*
10. *This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.*

5. Contents and Storage Conditions

imegen® MON89788 Quantification kit *includes all the reagents needed to quantify the amount of MON89788 soy in a DNA sample extracted from a pure soy sample, foodstuff or animal feed. In addition, a plasmid containing equimolar quantities of the targets is included in order to construct a quantification standard curve for each one of the PCR systems (Endogenous gene, Lectin; transgenic event, MON89788).*

The kit enables to perform a total of 48 determinations per system, and includes the following reagents:

- ***MON89788 Master Mix.*** *Contains specific oligonucleotides and one hydrolysis probe labelled with a FAM™ fluorophore to amplify the MON89788 transgenic event.*
- ***Soy Master Mix.*** *Contains specific oligonucleotides and one hydrolysis probe labelled with a FAM™ fluorophore to amplify the endogenous gene, Lectin.*
- ***General Master Mix.*** *Contains the DNA polymerase, dNTPs and amplification buffer.*
- ***MON89788 Standard.*** *Plasmid containing equimolar quantities of the targets (Lectin:MON89788 in a 1:1 ratio).*

Table 2. *Kit contents and storage conditions.*

Reagents	Colour	Volume	Storage
MON89788 Master Mix	Blue pad	360 µL	-20ºC
Soy Master Mix	Purple pad	360 µL	-20ºC
General Master Mix	White pad	2 x 600 µL	4ºC
MON89788 Standard	Brown cap	6 x 50 µL	-20ºC

6. Equipment and materials required but not supplied

6.1 Equipment and material required

The following table includes the equipment and materials not supplied but needed to use ***imegen® MON89788 Quantification kit*** *:*

Equipment	
1	Real-time thermal cycler (FAM channel)
2	Micropipettes (10 µL, 20 µL and 200 µL)
3	Vortex
4	Top bench centrifuge for 96-well plates and/or 8-well strips

Materials	
1	Optical PCR tubes 0.1 mL
2	Optical lids for the PCR tubes
3	Filter tips (10 µL, 20 µL and 200 µL)
4	Sterile tubes 1.5 mL
5	Dust-free gloves

6.2 Related kits

Imegen provides a DNA extraction kit suitable for GMOs, food and feedstufss:
4466336 GMO Extraction Kit

In addition, additional kits for the detection or quantification of GM-crops:
4466334 TaqMan GMO Screening Kit
4466335 TaqMan Roundup Ready Soya Quantification Kit
4481972 TaqMan GMO Maize Quantification Kit

7. Assay Protocol

7.1 Preparation of the PCR assay

The protocol for preparation of amplification reactions is shown below:

1. Thaw all the reagents needed for the analysis including,
   a. Specific master mixes:
      1. MON89788 Master Mix
      2. Soy Master Mix
   b. MON89788 Standard (Positive control)
   c. DNA samples (10-25 ng/µl)
   d. Nuclease-free water for the negative controls (PCR and Extraction controls)
   e. General Master Mix
2. Vortex and spin each reagent to mix thoroughly and keep on ice.
3. Using the MON897888 Standard, prepare serial dilutions, 1/10, to construct the standard curves that will enable the calculation of copy numbers for each one of the targets.

   As represented in Figure 1, four serial dilutions should be prepared starting from the undiluted MON89788 Standard containing $2x10^5$ copies. For this, add 5 µL of the MON89788 standard and 45 µL of nuclease-free water, until the preparation of the lowest concentration containing 20 total copies.

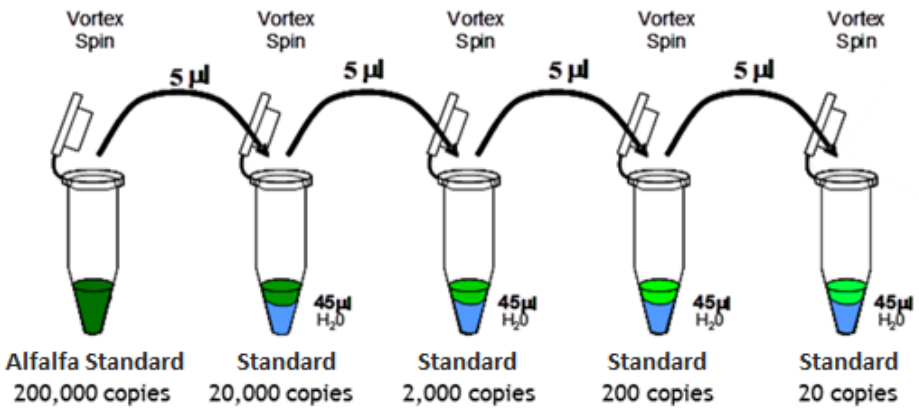


Figure 1. *Four serial dilutions are prepared from the MON89788 standard to construct three standard curves.*

Vortex and spin each dilution mix before starting to prepare the next dilution.

4. *Prepare the PCR master mixes in 1.5 mL tubes for each one of the target genes. For this, independent master mixes have to be setup accordingly:*

MON89788 Master Mix (Transgenic event)

Reagents	Volume per reaction
MON89788 Master Mix	7.5 µL
General Master Mix	12.5 µL

Soy Master Mix (endogenous gene)

Reagents	Volume per reaction
Soy Master Mix	7.5 µL
General Master Mix	12.5 µL

The volumes required of each mix have to be scaled up based on the number of samples that will be analysed, the 5 dilutions required to build the standard curve and the negative controls, including PCR controls and extractions controls.

Note: *To estimate the amount of necessary reagents, we recommend to make the calculations taking into account the volume of reagents needed to analyse all the samples to be included in the same PCR analysis and add 10% extra of each reagent.*

5. *Vortex and spin the tubes containing the PCR master mixes and dispense 20 µL in each well.*
6. *Once the master mixes have been dispensed, add the following volumes into the corresponding wells:*
   - *5 µL of DNA (10-25 ng/µl) in duplicate (R1 and R2 for replicates in Figure 2)*
   - *5 µL of each dilution of the MON89788 Standard*
   - *5 µL of nuclease-free water (PCR control, NTC; Extraction Control)*
7. *Seal the plate with optical film and spin down the plate.*
8. *Load the plate into a thermal cycler and then perform a run using the conditions showed in the section.*

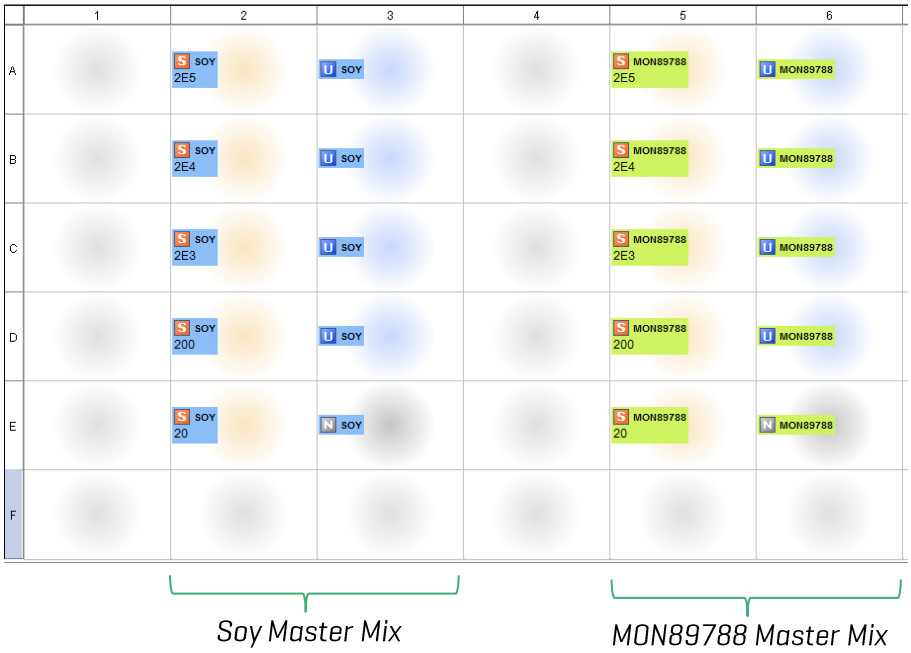


Figure 2: Proposed design analysis for 2 samples obtained from the same DNA extraction round.

*NOTE: We strongly recommend using an **extraction negative control** for each run of extractions carried out. This control consists in one tube to which no sample is added and which is summited to the same extraction process as the other samples. Likewise, we recommended using a **PCR negative control** for each PCR run; this tube contains no DNA but all PCR reagents.*

7.2 Setup of the real-time PCR program

The following instructions must be followed in order to setup the amplification program:

7500 Fast or StepOne Real-Time PCR system (ThermoFisher Scientific)

- *Experiment: Quantitation- Standard curve*
- *Ramp rate: standard*
- *Reaction volume: 25 µL*
- *Reference ROX™: Include*
- *TaqMan® probes fluorophores:*

Table 3. Hydrolysis probe information.

Hydrolysis probe	Reporter Dye	Quencher
MON89788-P	FAM™	MGB
Soy-P	FAM™	MGB

** In the StepOne PCR System (ThermoFisher Scientific) this field should be filled as "None"*

- *Optimal PCR programme:*

Table 4. Optimal PCR program for 7500 FAST or StepOne PCR Systems

Fields	Step 1 Enzymatic activation	Step 2 PCR	
Cycles	1 initial cycle	50 cycles	
		Denaturation	Annealing / Extension
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	1 minute*

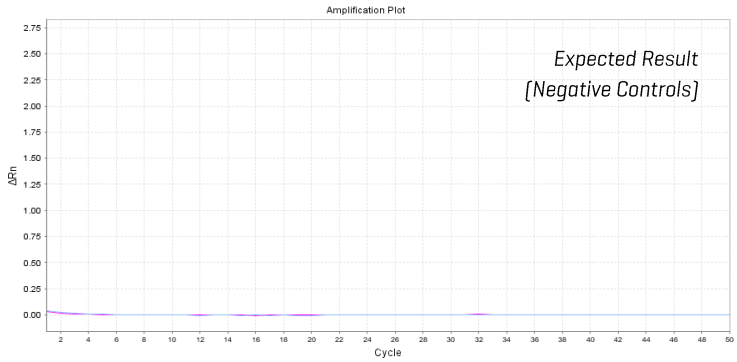
** Fluorescence detection*

8. Results analysis

For the correct interpretation of the results, the following recommendations are given:

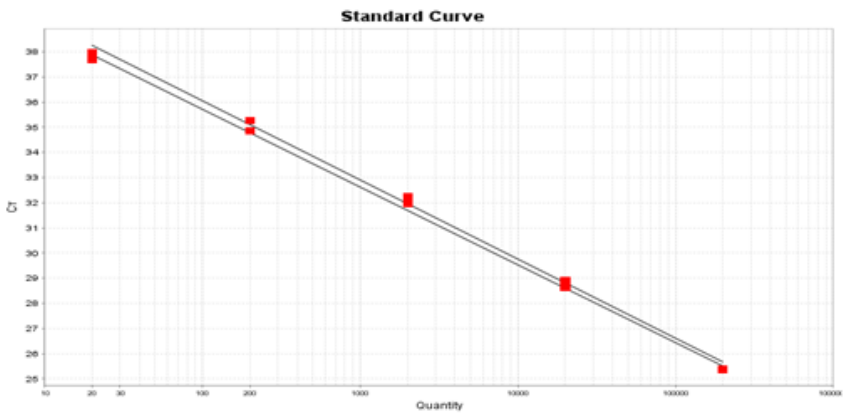
NEGATIVE CONTROLS

- *Confirm that there is no amplification in the negative controls (NTC). The presence of an amplification curve is indicative of accidental contamination and the assay should be repeated.*

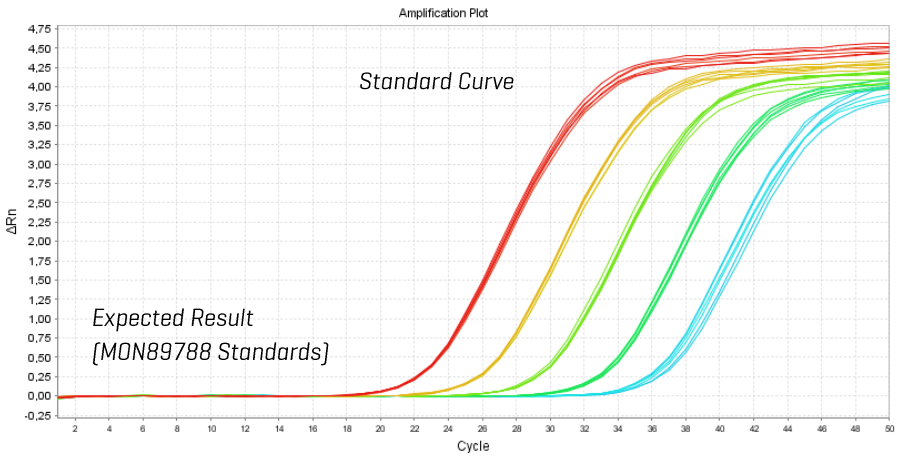


STANDARD CURVE

- *Confirm that the serial dilutions prepared using the MON89788 Standard (plasmid) produce suitable standard curves for all the targets when a linear regression is fitted to logarithmic copy numbers:*
  - *Slope: Ranged between -3.1 and -3.7*
  - *Coefficient of determination: R^2 > 0.998*
  - *PCR Efficiency between 90% and 110%*



- *If no amplification is detected in the MON89788 Standards, see section 9 (Troubleshooting). The highest concentration of the MON89788 Standard corresponds to 200,000 total copies.*



DNA SAMPLES

Once the controls have been verified, the results obtained with the samples can be analysed. If duplicates have been performed, the average should be estimated.

Three results are possible for each amplification reaction of both soy and MON89788 soy:

- ***Not detected:** No amplification is detected in the sample. The amplification curve is flat.*
- ***Not quantifiable:** Presence of the transgenic event is detected in the sample but it cannot be reliably quantified as the value falls outside the coverage of the standard curve.*
- ***Quantifiable:** Amplification is detected in the sample and it can be quantified as the value falls within the coverage of the standard curve. When the amplification Ct for the sample is interpolated between the values for the standard points, the quantitative result can be considered to be reliable and can be used to compute the percentage of MON89788 soy.*

Quantification using Copy Numbers

To calculate the relative amount of transgenic soy (%) the copy number of both the reference gene (Lectin) and the transgenic event shall be computed. Lectin gene is an endogenous gene exists in natural soy. The copy number of Lectin represents the total amount of soy in a test sample.

For the quantifiable sample, the target copy number will be interpolated from the standard curve by the software. The equation used to interpolate the copy number will be:

Y = aX + b, where:

Y = Ct value,

a = slope,

X = log (copy number),

b = Y-intercept.

Both **a** *and* **b** *are shown underneath the standard curve in the software.*

For an unknown sample, the Ct value will be plugged into above equation and a copy number will be interpolated by the software. The copy number will be reported in the results table.

The percentage is obtained using the following formula:

$$\text{MON89788 \%} = \frac{\text{MON89788 copy number}}{\text{Soy copy number}} \times 100$$

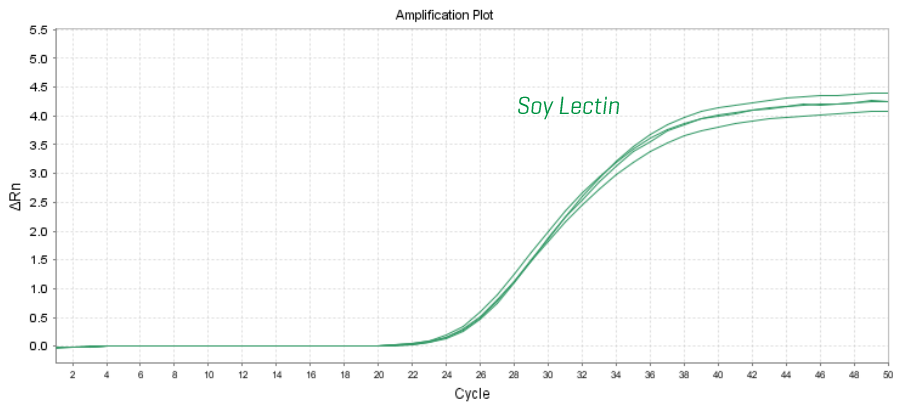
The following table shows the interpretation that should be done from the obtained result:

***Table 5**. Possible results and their interpretation.*

Soy	MON89788	Interpretation
Quantifiable	Not detected	No MON89788 soy is detected in the sample
Quantifiable	Not quantifiable	The amount of MON89788 soy detected in the sample is lower than the limit of quantification
Quantifiable	Quantifiable	The amount of MON89788 soy with respect to total soy can be reliably quantified
Not quantifiable	Not detected	No MON89788 soy is detected in the sample, the amount of soy present in the sample is lower than the limit of quantification
Not quantifiable	Not quantifiable	The amounts of wild-type soy and MON89788 soy detected in the sample are lower than the limit of quantification
Not detected	Not detected	No soy or MON89788 soy is detected in the sample

Master Mix Soy

- *Confirm that the reference gen (reactions prepared with **Soy Master Mix**) is detected in all the DNA samples. Lectin is a reference gene constitutively expressed, thus this reaction informs the user of the good quality and integrity of the soy DNA sample.*

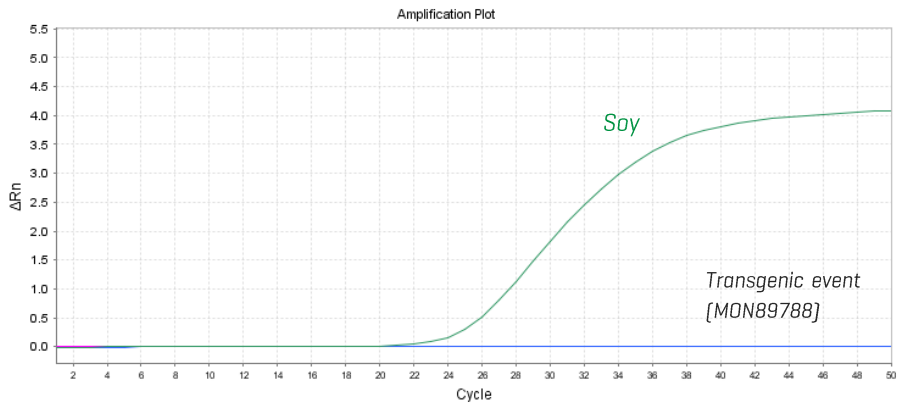


Note: *If a sample different from soy is being analysed, food or feedstuff free from any soy traces, no amplification will be expected on this screen.*

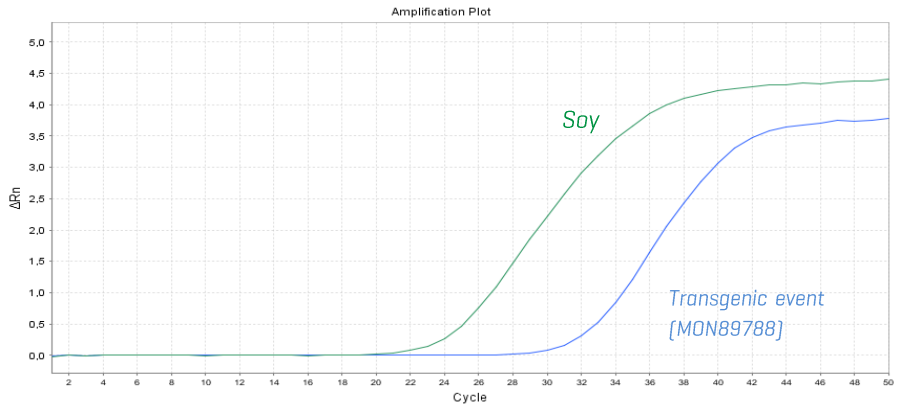
MON89788 Master Mix

- *After verifying all the controls included in the analysis are correct, the DNA samples are analysed. The sample analysed contains transgenic MON89788 if amplification is detected with* ***MON89788 Master Mix*** *as indicated below.*

Negative sample



Positive sample



9. Troubleshooting

The table below represents the results that could be obtained using the positive and negative controls and the DNA samples. In case an unexpected result is obtained, the interpretation of the result and the cause most likely reason for such result is given in the table below.

***Table 6.** Interpretation of the possible results obtained using imegen MON89788 Quantification kit*

Control	Soy	MON89788	Result / Interpretation
Positive Control	+	+	*Expected result*
	-	-	*Fail in the PCR setup* [1]
DNA sample	+	-	*Expected result*
	+	+	
	-	-	*Fail to amplify the DNA sample* [2]
Negative Control (NTC)	-	-	*Expected Result*
	+	+	*Contamination with soy DNA or with the positive control* [3]

[1] ***Fail in the PCR setup**: An error in the amplification might be due to a technical issue during the configuration of the PCR setup. Check the amplification program and the setup of the fluorescence detection.*

[2] ***Fail to amplify the DNA sample:** An error to amplify the reference gene in the DNA sample might suggest the quantity or the quality of the DNA sample is compromised. In this situation, a second analysis would be recommended before an interpretation of the results is made.*

[3] ***Contamination with soy DNA or with the positive control:** PCR contamination might be caused by an inappropriate handling of the sample, the use of contaminated reagents or caused by an environmental contamination. To solve this issue, a thorough cleanse of the laboratory where the PCRs are prepared, including the equipment and material used is recommended. If necessary, use fresh aliquots of the PCR reagents and prepare last, the PCR reactions containing the positive controls in order to avoid any cross contamination.*

10. Limitations

10.1 Equipment

imegen™ MON89788 Quantification kit *has been validated using the following real-time PCR systems:*

- *StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific)*
- *7500 FAST Real-Time PCR System (ThermoFisher Scientific)*

Technically, this kit is compatible with any real-time PCR systems that enable the detection of the fluorescence emitted by FAM™ fluorophore.

If a real-time PCR thermal cycler different from the systems described in this section is going to be used for the quantification of transgenic MON89788, it is possible that the PCR program might need to be readjusted. In this case, please contact our Technical Support Team for more details.

10.2 Reagents

Imegen™ MON89788 Quantification kit *has been validated using the reagents included in the kit.*

If a thermal cycler different from the thermal cyclers used in the validation is to be used, consult our Technical Support Team to confirm the suitability of the DNA polymerase. Similarly, if a DNA polymerase different from the DNA polymerase provided in the kit is to be used, a validation with the new reagents is recommended beforehand. Please, contact our Technical Support Team if you request any further information.

10.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied as specified on Section 5 (Contents and Storage Conditions) from the reception of the kit until the expiry date assigned to each production batch.

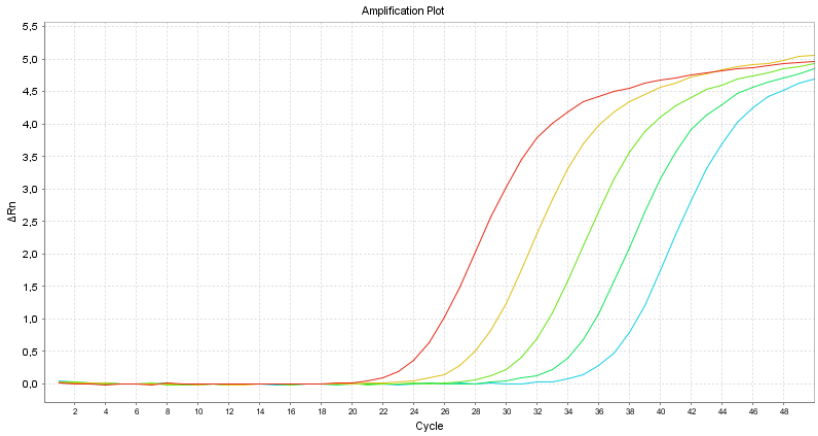


Figure 4: Amplification curves for each of the dilutions of the MON89788 soy Standard using MON89788 master mix

The following table shows the interpretation that should be done from the obtained result:

Soy	MON89788	Interpretation
Quantifiable	Not detected	No MON89788 soy detected in the sample
Quantifiable	Not quantifiable	The amount of MON89788 soy detected in the sample is lower than the limit of quantification
Quantifiable	Quantifiable	The amount of MON89788 soy with respect to total soy in the sample is X%
Not quantifiable	Not detected	No MON89788 soy detected in the sample, the amount of soy present in the sample is lower than the limit of quantification
Not quantifiable	Not quantifiable	The amounts of soy and MON89788 soy detected in the sample are lower than the limit of quantification
Not detected	Not detected	No soy or MON89788 soy detected in the sample*

Table 8. Possible results and their interpretation

** It is possible that the inability to detect soy DNA in a sample is due to the presence of inhibitors in the DNA used. To check for the absence of inhibitors in the sample, we recommend that you use an inhibition control consisting of amplification with Soy Master Mix in a well containing test sample DNA together with 1 µL of the inhibition control corresponding to the dilution containing 20,000 copies of the standard. Another well containing 5 µL of water and 1 µL of the same inhibition control should be amplified in parallel. If the amplification of both reactions is similar, it can be concluded that the sample is not inhibited.*