INSTRUCTIONS FOR USE

Imegen® P35S Maize Quantification Kit

Ref. IMG-275

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Guide overview

The information in this guide is subject to change without notice.

Health in Code, S.L. guarantees that its products are free from defects, both in used materials as 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 research use only. The user of the product is responsible for validating the usefulness of the protocol proposed by Health in Code, S.L. These protocols are considered a guide only. Health in Code, S.L. does not offer any other warranty, express or implied, which extends beyond the proper functioning of the components of this set. Health in Code S.L., 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. Health in Code, S.L. 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 Health in Code, S.L. are subjected to rigorous quality control (App. A). The **Imegen® P35S Maize 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:

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Instructions for Use (IFU) modifications

V.07	NOV 2024	Content revision in section 2.1
V.06	JUL 2023	Change of kit's name, manufacturer's name and address update. General Master Mix temperature update
V.05	AUG 2022	Contents review

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01

Product information

01.1 General description

Genetically modified organisms (GMOs) are widely distributed, with soy and corn being two of the most extensively cultivated crops worldwide. Indeed, soy, corn and their derivatives (soy protein, corn starch, etc.) are the ingredients of more than the 60% of the food we meet.

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.

01.2 Intended use

The Imegen® P35S Maize Quantification Kit allows the number of copies of the P35 present in the most of the transgenic events to be determined with respect to total Maize in a sample.

This kit uses Real-Time PCR technology and contains all the reagents required to quantify the P35 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 P35 Maize.

The Imegen® P35S Maize Quantification Kit, enables relative quantification of 0.1% of TNOS Maize with respect to the total Maize present in a sample. The limit of absolute quantification (LOQabs), corresponding to the lowest value included in the standard curve, is established to be 20 total copies for each of the quantifiable systems (the endogenous Maize gene, known as Mays Starch Synthase, MSS, and TNOS event).

To perform the reaction to determine the total Maize DNA amount in the sample, this kit includes a master mix with two primers and a hydrolysis probe labelled with the FAM™ fluorophore. This reaction specifically amplifies an endogenous Maize gene known as MSS.

To perform the reaction to determine the P35S promoter DNA amount in the sample, this kit includes a master mix with two primers and a hydrolysis probe labelled with the FAM™ fluorophore. The reaction specifically amplifies P35S promoter.

The kit also includes a plasmid DNA standard containing a copy of each of the targets used during analysis. A comparison of the results obtained with the samples and this standard allows a relative quantification to be made and therefore the percentage of P35S Maize with respect to the total Maize present in the sample to be calculated.

01.3 Content and storage conditions of the kit

Imegen® P35S Maize Quantification Kit contents the necessary reagents to perform 50 reactions:

P35S Master Mix	Master Mix with specific TNOS oligonucleotides, a fluorophore-labelled hydrolysis probe (FAM™) and nuclease-free water.
Maize Master Mix	Master Mix with specific Maize oligonucleotides, fluorophore labelled hydrolysis probe (FAM™) and nuclease-free water.
P35S standard	DNA containing a copy of each of the targets used during analysis.
General Master Mix	Master Mix of PCR with nucleotides, $MgCl_2$, DNA polymerase and buffer to carry out real-time PCR.

Table 1. IMG-275 Imegen® P35S Maize Quantification Kit components and description.

Reagents	Color indicator	Quantity	Conservation
P35S Master Mix*	Blue pad	375 µl	-20 °C
Maize Master Mix*	Red pad	375 µl	-20 °C upon receipt.
General Master Mix*	White pad	2 x 625 µl	-20 °C upon receipt. 2 - 8 °C after initial use. Store protected from light
P35S Standard	Blue cap	4 x 50 µl	-20 °C

(*) See the expiration date on the box and tubes.

01.4 Equipment, reagents and materials required but not supplied

Equipment

- Real-Time PCR Thermal Cycler with channels for detection of FAM™ (520 nm) and VIC™ (550 nm)
- Micropipettes (10 µl, 20 µl and 200 µl)
- Tabletop centrifuge with adaptors for 96 well PCR plates and/or 0.2 ml tubes
- Vortex

Materials

- Optical 96-well reaction plates or 0.2 ml optical tubes
- Optical adhesive film for 96 well plates or optical caps for 0.2 ml tubes
- Disposable micropipette filter tips (10 µL, 20 µL and 200 µL)
- 1.5 ml sterile tubes
- Powder-free latex gloves

Reagents

- Nuclease-free water

02 Methods

02.1 Preparation of the amplification reactions

Imegen® P35S Maize Quantification Kit is designed to perform two absolute quantifications during the course of the relative quantification of P35S promoter present in a sample.

Sample analysis comprises two real-time PCR simultaneous processes:

- One of them allows the total amount of Maize DNA in the sample.
- The other, allows the amount of P35S promoter DNA present in the sample.

Preparation of the amplification reactions includes:

- **Standard dilutions**
- **Negative PCR and/or extraction controls**
- **Sample analysis in duplicate**

The recommended protocol for preparation of amplification reactions is shown below:

01. Thaw a vial of P35S standard and prepare four 1:10 serial dilutions of this standard. This process results in the quantitative standards with which the samples can be compared.

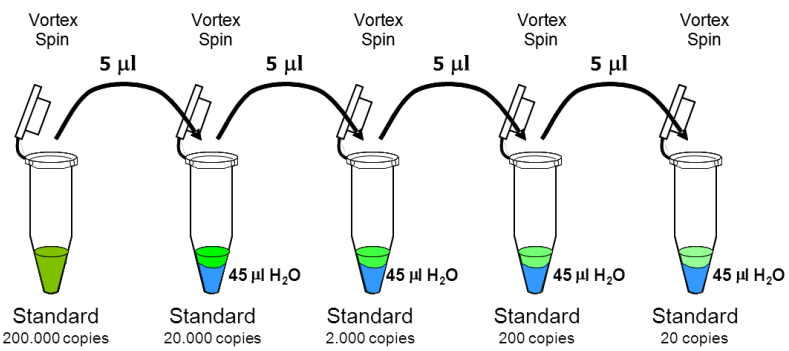

Figure 1. Four serial standard dilutions are made from P35S Standard to perform two standard curves.

02. Thaw the Master Mixes, negative controls and DNA samples (if stored frozen).

03. Shake each of the reagents on the vortex whilst keeping them cold.

04. Add into a 1.5 mL tube (one for each PCR master mix preparation), the following reagents in Table 2. To estimate the amount of necessary reagents, we recommend to make calculations taking into account the number of samples to be simultaneously analyzed, and then considering one more reaction.

Table 2. Reagents and volumes needed to perform PCR reactions.

Reagents	Amount per reaction
Maize Master Mix	7.5 µL
P35S Master Mix	7.5 µL
General Master Mix	12.5 µL

05. Vortex and spin the 1.5 mL tube and dispense 20 µl per well or tube of 0.2 ml.

06. Add 5 µl of each sample DNA (10-25 ng/µL) to the corresponding wells:
    - Total Maize reactions
    - P35S amplification reactions

07. Add 5 µl of each standard dilution to the corresponding wells:
    - Total Maize reactions
    - P35S amplification reactions

08. Add 5 µl of each control (negative control and DNA extraction control) to the corresponding wells:
    - Total Maize reactions
    - P35S amplification reactions

09. Seal the plate with optical film and spin.

10. Load the plate into a thermal cycler and then perform a run using the conditions showed in the next section.

(*) 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.

02.2 Settings for the Real-Time PCR program

This kit is compatible with the Real-Time PCR platforms 7500 FAST, StepOne Real-Time PCR System (Thermo Fisher Scientific) and QuantStudio™ 5 Real-Time PCR system.

Table 3. IMG-275 Imegen® P35S Maize Quantification Kit probes and specifications.

Target	Receptor	Quencher
MSS DNA	FAM™	MGB
P35S DNA	FAM™	MGB

The following instructions should be taken into account in order to setup the amplification program:

- **Reaction volume:** 25 µL.
- **Targets:** FAM™.
- In case the quencher has to be defined, select MGB for all probes. If the real time PCR system does not take into account the quenchers, select only the receptors (FAM™).
- If the Real-Time PCR system is a 7500 Fast, a StepOne Real-Time PCR system (Thermo Fisher Scientific) or a QuantStudio™ 5 Real-Time PCR system, select Quantitation - Standard curve as a type of experiment and include ROX™ as a reference.
- **Ramp rate:** standard
- **Optimal program:**

Table 4. Optimal PCR program.

Fields	Step 1 Enzyme activation	Step 2 PCR	
No. of cycles	1 initial cycle	50 cycles	
		Denaturation	Primers binding/extention
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	1 minute *

(*) Fluorescence detection.

02.3 Analysis of results

To analyze the results, it is recommended to establish the Threshold at 0.1 and to keep the default Baseline value to minimize the residual signal in the detection channels.

Ct settings	Threshold	0.1
	Baseline	AUTO

Before analyzing the samples results, it should be checked if obtained results in controls are as expected:

- **Negative control:** No amplification should be detected in either the reaction corresponding to Maize or that corresponding to P35S promoter. Amplification in a negative control indicates the presence of contamination and therefore that the assay should be repeated.
- **P35S standard:** Amplification should be detected for the five points corresponding to the Maize standard and the five points corresponding to the P35S promoter standard.

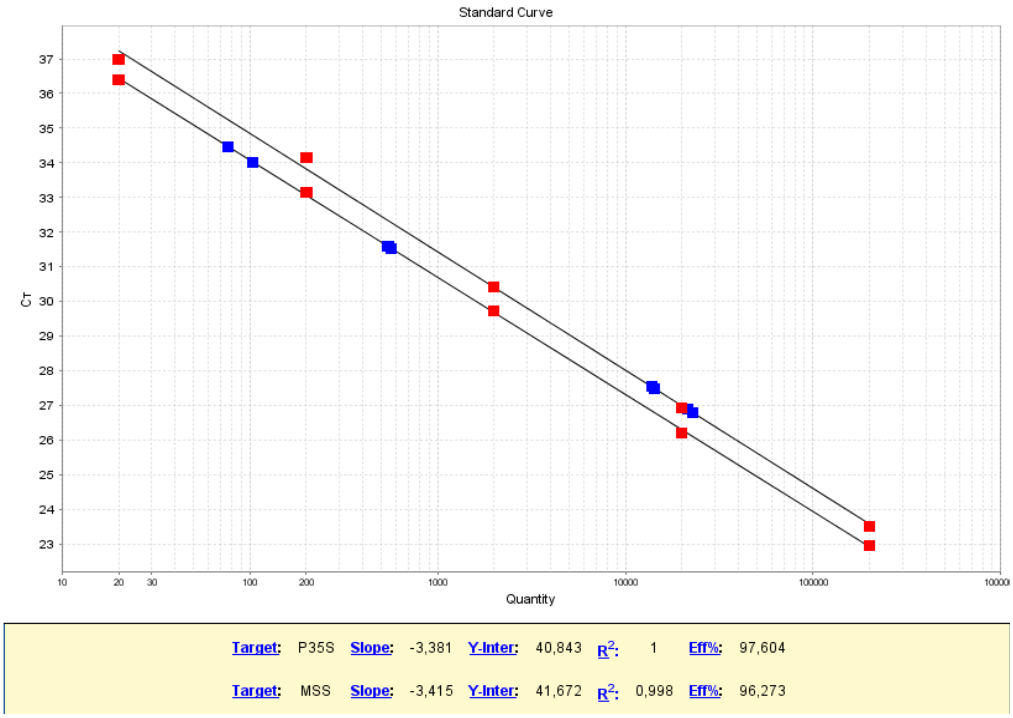

Figure 2. Standard curves for total Maize and P35S targets. Red dots represent the dilutions of the standard.

Furthermore, the curves obtained using the standard points should meet the following requirements:

- The efficiency of the curve should be between 80% and 110%.
- The slope of the curve should be between -3.1 and -3.9.
- The correlation coefficient (R2) should be greater than 0.98.

Once the controls have been verified, the results obtained with the samples can be analyzed. If duplicated have been performed, the results for both replicates should be similar.

Three results are possible for each amplification reaction of both Maize and P35S promoter:

- **Not detected:** No amplification in the sample. The amplification curve is flat.
- **Not quantifiable:** Amplification is detected in the sample but to an extent lower than the last point on the curve. When the Ct for the sample is greater than the Ct for the 20-copy standard, it can be concluded that the analyte is present in the sample but is not quantifiable.

- **Quantifiable:** Amplification is detected in the sample to an extent greater than the last point on the 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 calculate the percentage of P35S promoter.

The following formula should be used to calculate the percentage of P35S promoter with respect to total Maize present in the sample:

$$\%\ P35S = \frac{Nº\ of\ copies\ of\ P35S\ x\ 100}{Nº\ of\ Maize\ copies}$$

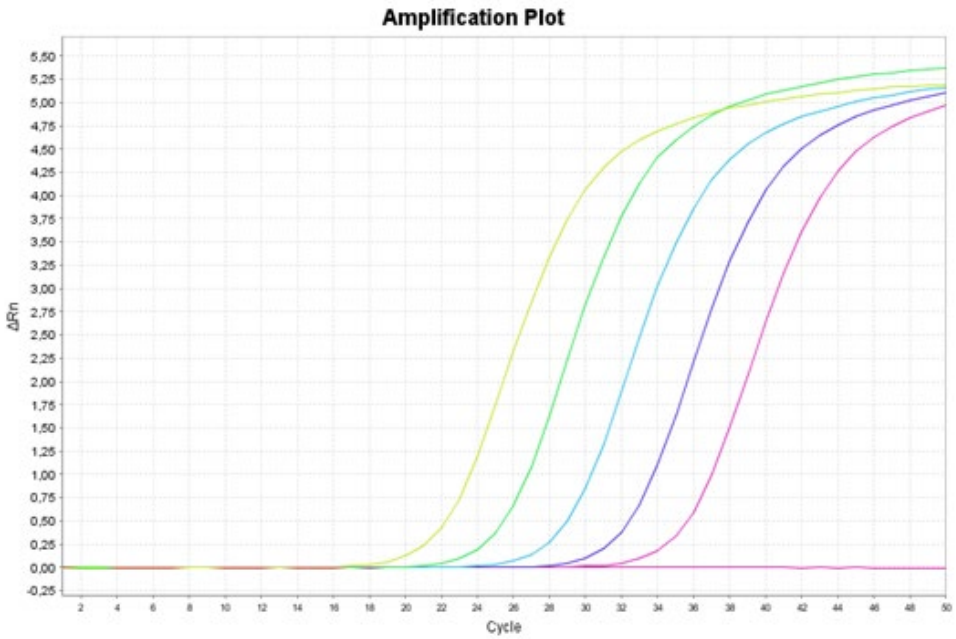

Figure 3. Amplification curves for each of the dilutions of the P35S Standard using P35S master mix.

03 Troubleshooting

The following table shows graphically the results that may be obtained from the analysis of different assay controls, as well as the interpretation that should be done from the obtained result:

Table 5. Possible results and their interpretation.

Controls	P35S	Interpretation
Quantifiable	Not detected	No P35S promoter detected in the sample
Quantifiable	Not quantifiable	The amount of P35S promoter detected in the sample is lower than the limit of quantification
Quantifiable	Quantifiable	The amount of P35S promoter with respect to total Maize in the sample is X%
Not quantifiable	Not detected	No P35S promoter detected in the sample, the amount of Maize present in the sample is lower than the limit of quantification
Not quantifiable	Not quantifiable	The amounts of Maize or P35S promoter detected in the sample are lower than the limit of quantification
Not detected	Not detected	No Maize or P35S promoter detected in the sample *

(*) It is possible that the inability to detect Maize 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 Maize 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.

04 Limitations

04.1 Equipment

Imegen® P35S Maize Quantification Kit has been validated using the following Real-Time PCR systems:

- 7500 FAST Real-Time PCR System (Thermo Fisher Scientific)
- StepOne™ Plus Real-Time PCR System (Thermo Fisher Scientific)
- QuantStudio5™ Real-Time PCR System (Thermo Fisher Scientific)

Technically, this kit is compatible with any Real-Time PCR systems that enable the detection of the fluorescence emitted by FAM™ and VIC™ fluorophores.

If a PCR system different from the systems described in this section is going to be used, it is possible that the PCR program might need to be readjusted. In this case, please contact our Technical Support Team for more details.

04.2 Reagents

Imegen® P35S Maize Quantification Kit has been validated using the reagents included in the kit and the DNA polymerase recommended by the supplier of the Real-Time PCR systems used in the validation as follows:

- TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific)

If a PCR master mix (DNA polymerase) different from the DNA polymerase used in the validation is going to be used to perform the analysis, a validation with the new reagents is recommended beforehand. Please, contact our Technical Support Team if you request any further information.

04.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied as specified on Section 01.3 (Contents and Storage Conditions) from the reception of the kit until the expiry date assigned to each production batch.

Appendix A

Supplemental information

AA.1 Sensitivity and specificity

The specificity of the kit was tested through comparison with the NCBI sequence database and was also experimentally tested with success on a collection of reference DNAs.

AA.2 Detection limit

Detection and quantification limits of the Imegen® P35S Maize Quantification Kit:

- **Limit of quantification:** 20 copies of DNA
- **Detection limit of the PCR technique (Maize system):** 3 copies of DNA
- **Detection limit of the PCR technique (P35S Maize system):** 3 copies of DNA

This Kit allows relative quantifications of up to 0.1% of P35S Maize to be determined with respect to total Maize in a sample. The relative limit of quantification varies depending on the sample analyzed.

AA.3 Quality certifications

- Health in Code, S.L. is certified against the standard UNE-EN ISO 9001:2015 "Quality management systems" for the design, development, manufacture, and commercialization of kits for genetic analysis.
- Health in Code, S.L. is certified against the standard UNE-EN ISO 14001:2015 "Environmental Management Systems" for the design, development, manufacture, and commercialization of kits for genetic analysis.

Appendix B

Safety warnings and precautions

	Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.
	Do not pipette by mouth.
	Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
	You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.
	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.
	In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.
	The materials safety data sheets of all hazardous components contained in this kit are available on request to Health in Code, S.L.
	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.
	Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.

	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.
	The manufacturer is not responsible for the malfunction of the assay when one or more reagents included in the kit are replaced by other reagents not supplied by Health in Code, S.L.
	The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Health in Code, S.L., considering them equivalent to those provided in the kit.

Appendix C

Documentation and support

AC.1 Food safety support

Please, visit our website for the latest services, orders and support information:

imegenagro.es

Health in Code certificates of analysis and other product documentation:

portal.imegen.es/en/certificate-of-analysis/

AC.2 Customer and technical support

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

+34 963 212 340

tech.support@healthincode.com

NOTE: For SDSs for reagents and chemicals from other manufacturers, please contact the appropriate manufacturer.
