INSTRUCTIONS FOR USE

Imegen® Roundup Ready Alfalfa Quantification Kit

Ref. IMG-315

Manufactured by HEALTH IN CODE, S.L.
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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® Roundup Ready Alfalfa 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.03	OCT 2023	Contents review. Change of the manufacturer's identification, going from Imegen to Health in Code, and update of the front page (logo ISO 9001 removed). Modification of the storage temperature of the General Master Mix
V.02	SEP 2018	Contents review

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01

Product information

01.1 General description

Genetically modified organisms (GMO) are widely distributed around the globe as GM varieties can improve crop yields, nutritional value, and reduce environmental risks.

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. Whereas alfalfa (Medicago sativa) is widely distributed, genetically engineered alfalfa is restricted to very few countries and small geographical areas.

One of the most constant challenges alfalfa growers face is effective weed control. The dairy and horse industries require high quality alfalfa, free of weeds, for their livestock. Most weeds are low quality in comparison to alfalfa; they have little nutritional value, are unpalatable, and are sometimes noxious or poisonous to animals. Farmers control weeds with traditional herbicides, but often have to use several different herbicides in order to retain effective weed control for their fields.

Glyphosate-resistant crops, also known as Roundup Ready (RR), represent a significant proportion within transgenics. RR technology provides genetic resistance to glyphosate in vegetables by inserting a bacterial gene that modifies an essential enzyme for the plant growth. In the particular case of alfalfa, two transgenic events, J101 (MON-ØØ1Ø1-8) and J163 (MON-ØØ163-7) confer resistance to glyphosate, thus making the use of that herbicide possible and, consequently, the control of a wide range of weeds that commonly grow in the crops of this species.

References:

Undersander, Dan & Martin, Neal & Hall, Marvin & Mueller, Shannon. (2009). Review of Roundup Ready Alfalfa. Forage and Grazinglands. 10.1094/FG-2009-1019-01-RV.

01.2 Intended use

Imegen® Roundup Ready Alfalfa Quantification Kit enables the user to quantify the relative amount of Roundup Ready alfalfa in an alfalfa sample.

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 Roundup Ready alfalfa. The assay is composed of three specific PCR systems containing oligonucleotides and hydrolysis probes labelled with the FAM™ fluorophore, that will enable to amplify each one of the transgenic events, J101 (MON- ØØ1Ø1-8) and J163 (MON-ØØ163-7), as well as the amplification of an alfalfa endogenous gene, Acetyl CoA-carboxylase, known as Acc.

The inclusion of a plasmid system containing each of the targets, Acc, J101 and J163 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.

01.3 Content and storage conditions of the kit

Then kit contents the necessary reagents to perform 48 reactions:

Alfalfa Master Mix	Master Mix with two specific oligonucleotides and a hydrolysis probe labelled with the FAM™ fluorophore to amplify the endogenous gene, Acc.
J101 Master Mix	Master Mix with two specific oligonucleotides and a hydrolysis probe labelled with the FAM™ fluorophore to amplify the J101 transgenic event.
J103 Master Mix	Master Mix with two specific oligonucleotides and a hydrolysis probe labelled with the FAM™ fluorophore to amplify the J163 transgenic event.
General Master Mix	Master Mix of PCR with nucleotides, MgCl2, DNA polymerase and buffer needed to carry out RT- PCR.
Alfalfa Standard*	Includes a plasmid DNA standard containing equimolar quantities of the targets (Acc:J101:J163 in a 1:1:1 ratio).

Table 1. IMG-315 Imegen® Roundup Ready Alfalfa Quantification Kit components and description.

Reagents	Color indicator	Quantity	Conservation
Alfalfa Master Mix*	Green disc	360 µl	-20 °C
J101 Master Mix*	Yellow disc	360 µl	-20 °C
J163 Master Mix*	Blue disc	360 µl	-20 °C
General Master Mix*	White disc	3 x 600 µl	-20 °C upon receipt. 2 - 8 °C after initial use. Store protected from light
Alfalfa standard*	Black lid	120 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

Related kits

- Imegen® Food Extraction Kit (IMG-262)
- Imegen® GMO Screening Kit (IMG-273)
- Imegen® Roundup Ready Soya Quantification Kit (IMG-274)
- Imegen® P35S Maize Quantification Kit

02 Methods

02.1 Preparation of the amplification reactions

Imegen® Roundup Ready Alfalfa Quantification Kit includes all the reagents needed to quantify the amount of Roundup Ready Alfalfa in a DNA sample extracted from a pure alfalfa 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, Acc; transgenic events, J101 and J163).

The recommended protocol for preparation of reactions is showed below:

01. Thaw all the reagents needed for the analysis including,
    - **Specific master mixes:**
      - Alfalfa Master Mix
      - J101 Master Mix
      - J163 Master Mix
    - **Alfalfa Standard (Positive control):**
      - DNA samples (10-25 ng/µl)
      - Nuclease-free water for the negative controls (PCR and Extraction controls)
      - General Master Mix
02. Shake each of the reagents on the vortex whilst keeping them cold.
03. Using the Alfalfa 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.

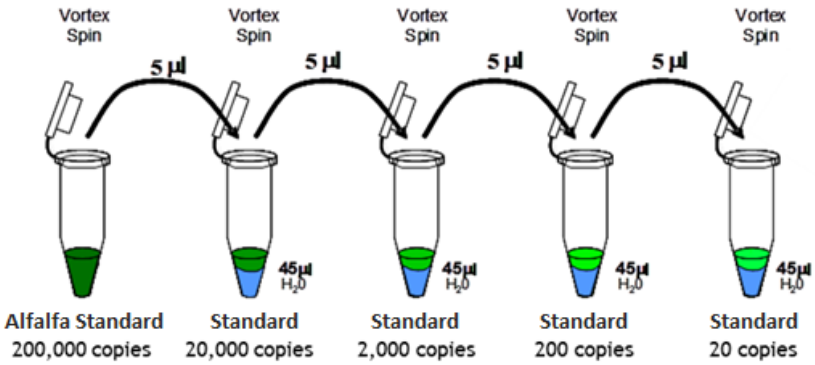

Figure 1. Four serial standard dilutions are made from RR Soy Standard to perform two standard curves.

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

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

06. Add into a 1.2 mL tube, the following reagents:

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

Reagents	Amount per reaction
Specific Master Mix (Alfalfa/J101/J1163)	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.

07. Vortex and spin the tubes and dispense 20 µL per well or tube of 0.2 ml

08. 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.
- 5 µL of each dilution of the Alfalfa Standard.
- 5 µL of nuclease-free water (PCR control, NTC; Extraction Control).

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-315 Imegen® Roundup Ready Alfalfa Quantification Kit probes and specifications.

Target	Receptor	Quencher
Alfalfa	FAM™	MGB
J101	FAM™	MGB
J163™	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™ and VIC™).
- 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	36 cycles	
		Denaturation	Primers binding/extension
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 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 Alfalfa Standard (plasmid) produce suitable standard curves for all the targets when a linear regression is fitted to logarithmic copy numbers:
  - The efficiency of the curve should be between 90% and 110%
  - The slope of the curve should be between -3.1 and -3.7
  - The correlation coefficient (R2) should be greater than 0.98

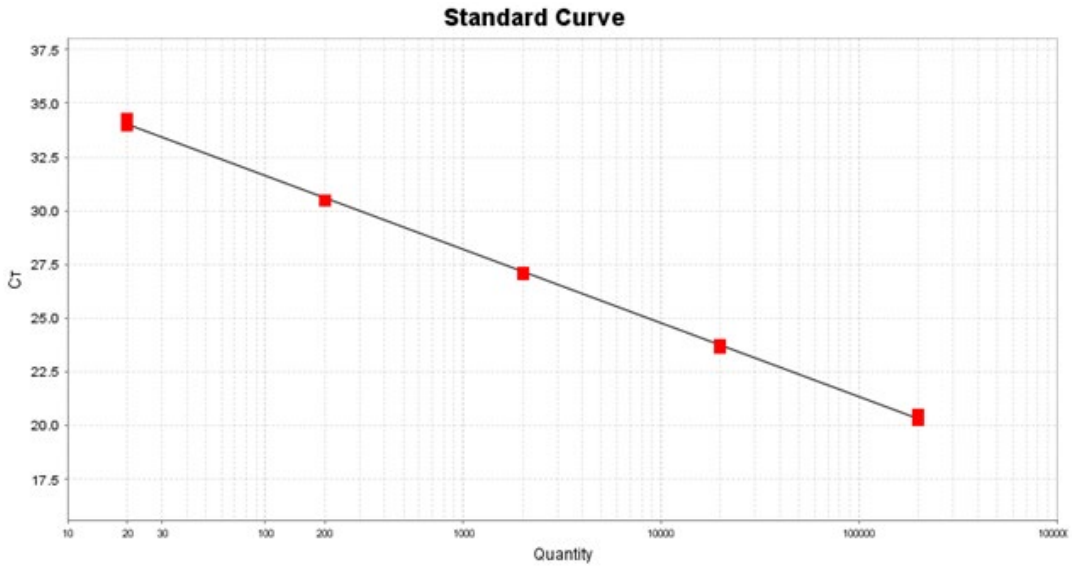

Figure 2: Standard curves for total Alfalfa and RR Soy targets. Red dots represent the dilutions of the standard.

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

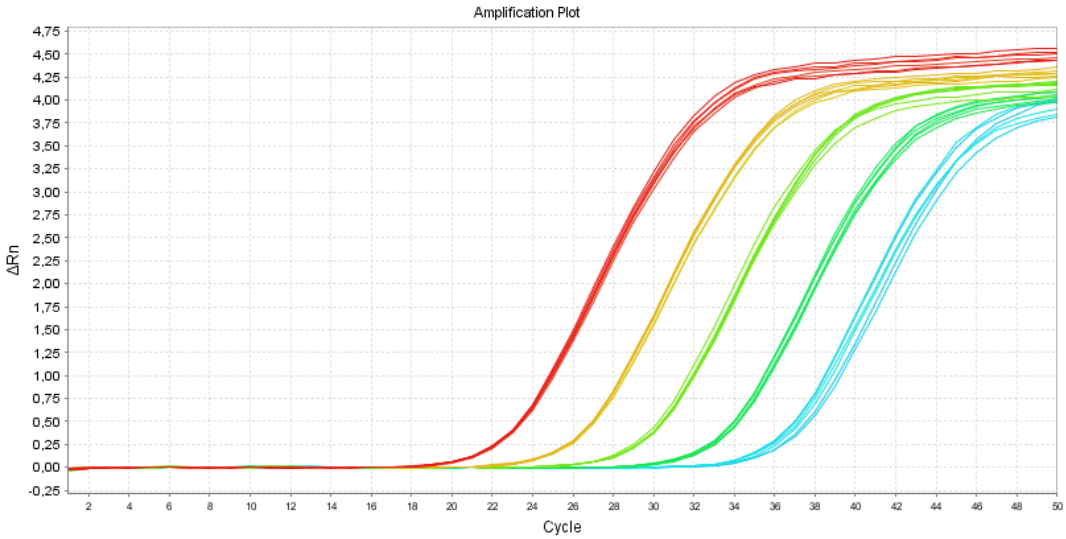

Figure 3: Standard curves for total Alfalfa and RR Soy targets. Red dots represent the dilutions of the standard.

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

Three results are possible for each amplification reaction of both Alfalfa and Roundup Ready Alfalfa:

- **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 Roundup Ready Alfalfa.

The following formula should be used to calculate the percentage of Roundup Ready Alfalfa with respect to total Alfalfa present in the sample:

$$\%\,J101 = \frac{Nº\ of\ copies\ of\ J101\ x\ 100}{Nº\ of\ Alfalfa\ copies}$$

$$\%\,J163 = \frac{Nº\ of\ copies\ of\ J163\ x\ 100}{Nº\ of\ Alfalfa\ copies}$$

The following table shows graphically the results that may be obtained from one sample analysis, as well as the interpretation that should be done from the obtained result:

Table 5. Possible results and their interpretation.

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

*It is possible that the inability to detect Alfalfa 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 Alfalfa 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.

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

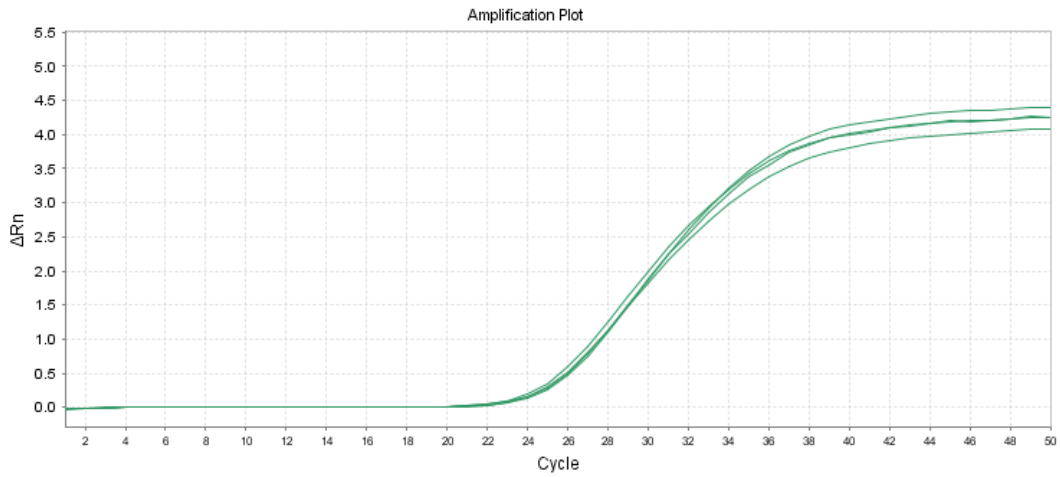

Figure 4: Reference gen amplification.

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

- After verifying all the controls included in the analysis are correct, the DNA samples are analysed. The sample analysed presents contains a transgenic event if amplification is detected with either one of the J101 Master Mix or J163 Master Mix as indicated below.

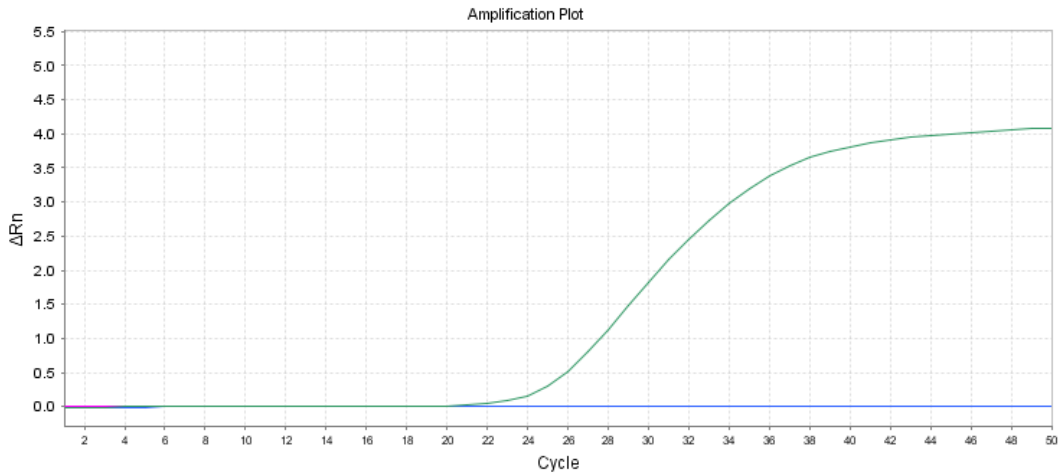

Figure 5. Negative sample.

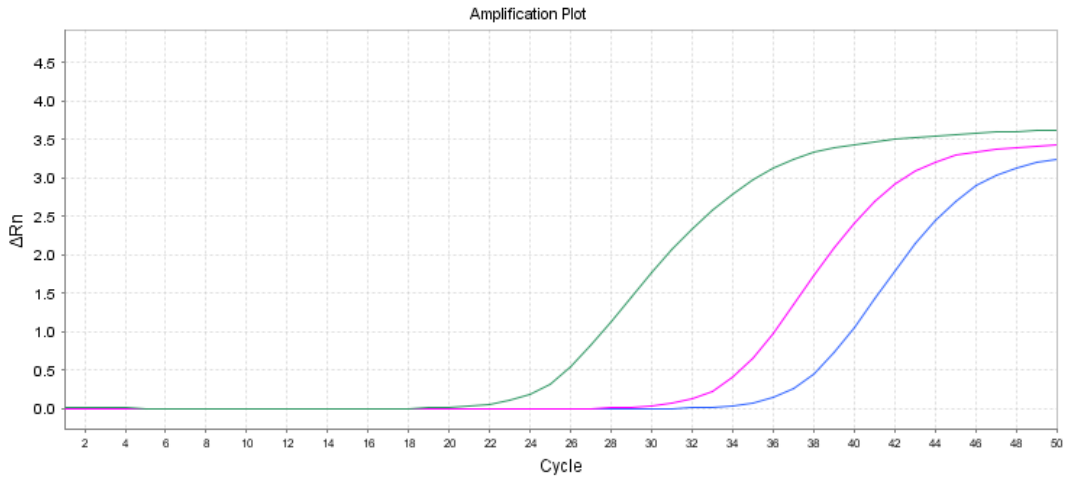

Figure 6. Positive sample.

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.

Control	Alfalfa	J101	J163	Result/Interpretation
Positive control	+	+	+	Expected result
	-	-	-	[1]Fail in the PCR setup
DNA sample	+	-	-	Expected result
	+	+	+	
	+	-	+	
	+	+	-	
	-	-	-	[2]Fall to amplify the DNA sample
Negative Control (NTC)	-	-	-	Expected result
	+	+	+	[3]Contamination with Alfalfa DNA or with the positive control

(1) PCR Amplification Failure: check amplification program and configuration of fluorescence capture. Amplification failure may be due to a setup technical problem or PCR inhibition.

(2) Contamination in the DNA extraction procedure: contamination may be due to some error made in the process of sample handling, reagents contamination, or environmental contamination. Check DNA extraction protocol, wipe the laboratory where DNA extraction process was performed and take care to avoid any contamination during sample homogenization. If necessary, use new aliquots of the reagents used in DNA extraction.

(3) PCR contaminations with Alfalfa DNA: contamination of PCR reactions may be due to an error made in the process of sample handling, contamination of the reagents or environmental contamination. Thoroughly clean the laboratory where the PCR process was performed, as well as equipment. If necessary, use new aliquots of the reagents used in the PCR. Prepare the PCR reaction containing the Positive Control last to avoid cross contamination.

04 Limitations

04.1 Equipment

Imegen® Roundup Ready Alfalfa 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® Roundup Ready Alfalfa 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. See the results in the table below:

Table 8. Specificity of IMG-315 Imegen® Roundup Ready Alfalfa Quantification Kit.

GM-Crops	Result
MON00101-8[1]	Detected
MON00163-7[1]	Detected
MON89788[2]	Not detected
A2704[2]	Not detected
A5547[2]	Not detected
DP356043[2]	Not detected
MON87701[2]	Not detected
MON87769[2]	Not detected
MON87705[2]	Not detected
CV127[2]	Not detected
MON87708[2]	Not detected
DP305423[2]	Not detected
DAS58416[2]	Not detected
RR Soy[2]	Not detected
Bt11[3]	Not detected
NK603[3]	Not detected

MIR604[3]	Not detected
MON89034[3]	Not detected
T45[4]	Not detected
GT74[4]	Not detected
RF1[4]	Not detected
RF3[4]	Not detected
MON810[3]	Not detected
MIR162[3]	Not detected
GA21[3]	Not detected
HERCULEX[3]	Not detected

Non-GM Samples	**Result**
Alfalfa *(Medicago sativa)*	Not detected
Maize *(Zea mays)*	Not detected
Celery *(Apium graveolens)*	Not detected
Carrot *(Daucus carota)*	Not detected
Lentil *(Lens culinaris)*	Not detected
Walnuts *(Juglans regia)*	Not detected
Sesame *(Sesamum indicum)*	Not detected
Oats *(Avena sativa)*	Not detected
Rice *(Oryza sativa)*	Not detected
Cotton *(Gossypium hirstium)*	Not detected
Wheat *(Triticum)*	Not detected
Rapeseed *(Brassica napus)*	Not detected
Pork *(Sus scrofa)*	Not detected
Cow *(Bos taurus)*	Not detected
Sheep *(Ovis aries)*	Not detected
Deer *(Cervidae)*	Not detected
Buffalo *(Bubalus bubalis)*	Not detected
Atlantic cod *(Gadus morhua)*	Not detected
Common dab *(Limanda limanda)*	Not detected
Human *(Homo sapiens)*	Not detected

AA.2 Detection and quantitation limit

The limit of relative quantification (LOQrel) of the Imegen® Roundup Ready Alfalfa Quantification Kit has been established in 0.1% (w/w) for both the J101 and the J163 events, 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 (Acc, J101 and J163).

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.
