INSTRUCTIONS FOR USE

Imegen® Roundup Ready Soya Quantification Kit

Ref. IMG-274

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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 Soya 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.06	SEP 2023	Contents review; modification of the storage temperature of the General Master Mix
V.05	AUG 2022	Change of the manufacturer's identification, going from Imegen to Health in Code, and update of the front page (logo ISO 9001 removed).
V.04	JUL 2019	Contents review

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01 Product information

01.1 General description

A considerable proportion of the general public experiences negative health effects triggered by certain components contained in their habitual diet. Susceptible persons can develop an intolerance (e.g. against lactose, due to the lack of a digestive enzyme), and others suffer from adverse reactions mediated by the immune system. The latter is characterized by the action of IgE antibodies against the offending food and is known as food allergy. The symptoms caused by allergic reactions range from rather mild manifestations to life threatening events (anaphylactic shock).

Usually, the recommended option to treat food allergies is to eliminate from the diet the food ingredients that cause the hypersensitivity. Allergic persons need to know whether the food items they purchase contain allergenic ingredients; they have to rely on the truthfulness of information given on the label of prepared and packaged food items. National and supra-national legislation has been put in place which requires food business operators to declare whether ingredients with a known allergenic potential have been used during manufacturing. The Codex Alimentarius General Standard for the Labelling of Prepacked Food requires, for example, the mandatory labelling of the presence of eight classes of food ingredients that are known to cause hypersensitivity in susceptible consumers (cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, and tree nuts), while European Community legislation (Commission Directive 2007/68/EC) extends the list to include also celery, mustard, sesame seeds, lupin, and molluscs.

Analytical testing systems are needed by the food industry to enable them to test whether allergens are present in their raw materials, the finished products and whether production lines have been correctly sanitised, by the food inspection authorities for market surveillance and by academia to enable and stimulate research into food allergy and allergen detection. Molecular biology provides highly specific and sensitive procedures to detect the presence of allergen species.

Genetically modified organisms (GMOs) are widely distributed, with Soya and corn being two of the most extensively cultivated crops worldwide. Indeed, Roundup Ready transgenic Soya accounts for some 77% of the Soya cultivated worldwide, thus making it the most widespread transgenic event and therefore the one that generates the most problems.

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.

In order to preserve the health of consumers, Health in Code has been developed some kits to detect plant species and food allergens based on the latest DNA technology, used to verify the presence or absence of allergenic ingredients in any food.

01.2 Intended use

Imegen® Roundup Ready Soya Quantification Kit allows the percentage of Roundup Ready Soya in a sample to be determined with respect to total Soya *(Glycine max)*.

This kit uses Real-Time PCR technology and contains all the reagents required to quantify the Roundup Ready Soya 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 Roundup Ready Soya.

01.3 Content and storage conditions of the kit

Imegen® Roundup Ready Soya Quantification Kit contents the necessary reagents to perform 48 reactions.

Sample analysis comprises two real-time PCR simultaneous processes:

- One of them allows the total amount of Soya DNA in the sample.
- The other, allows the total amount of Roundup Ready Soya DNA present in the sample.

RR Soya Master Mix	Master Mix with two specific oligonucleotides and a hydrolysis probe labelled with the FAM™ fluorophore to determine the the Roundup Ready Soya DNA amount in the sample. This reaction specifically amplifies the GTS-40-3-2 transgenic event in Roundup Ready Soya. This fragment is event-specific, in other words it amplifies the binding region between the Soya genome and the construct introduced in Roundup Ready Soya.
Soya Master Mix	Master Mix with two specific oligonucleotides and a hydrolysis probe labelled with the FAM™ fluorophore to determine the total Soya DNA amount in the sample. This reaction specifically amplifies an endogenous Soya gene known as lectin.

General Master Mix	Master Mix of PCR with nucleotides, $MgCl_2$, DNA polymerase and buffer needed to carry out RT- PCR.
RR Soy Standard*	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 Roundup Ready Soya with respect to the total Soya present in the sample to be calculated.

Table 1. IMG-274 Imegen® Roundup Ready Soya Quantification Kit components and description.

Reagents	Color indicator	Quantity	Conservation
RR Soya Master Mix*	Green pad	360 µl	-20 °C
Soya Master Mix*	Purple pad	360 µl	-20 °C
General Master Mix*	White pad	2 x 600 µl	-20 °C upon receipt. 2 - 8 °C after initial use. Store protected from light
RR Soy standard*	Green cap	6 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® Roundup Ready Soya Quantification Kit allows the determination of the percentage of Roundup Ready Soya in relation to the total Soya present in a sample.

Two absolute quantifications are performed during the course of the relative quantification of Roundup Ready Soya present in a sample. The first of these determines the total amount of Soya present in the sample and the second determines the amount of Roundup Ready Soya.

Preparation of the amplification reactions includes:

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

To estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples and controls to be simultaneously analysed, and then considering one more reaction, or increase a 10% the volume of each reagent.

The recommended protocol for preparation of reactions is showed below:

01. Thaw a vial of RR Soya 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.

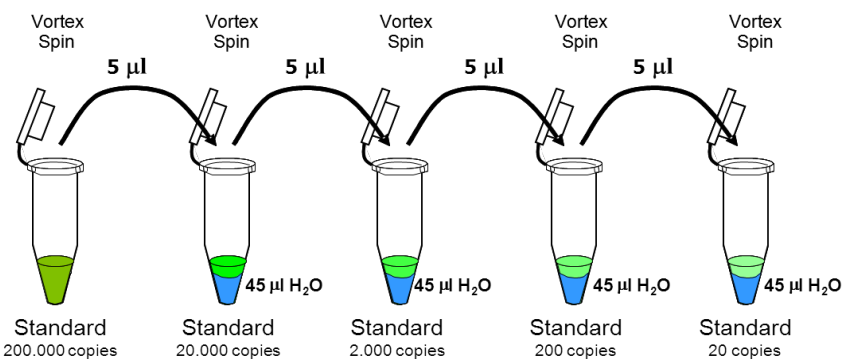

Figure 1. Four serial standard dilutions are made from RR Soy Standard to perform two standard curves.

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 1.2 mL tube, the following reagents:

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

Reagents	Amount per reaction
RR Soya Master Mix or Soya Master Mix	7,5 µL
General Master Mix	12,5 µL

05. Vortex and spin the 1.5 mL tubes and dispense 20 µL per well or tube of 0.2 ml (see the example for three samples in figure 2).

06. Add 5 µL of each sample DNA (10-25 ng/µL) to the corresponding wells:
- Total Soya reactions and,
- RR Soy amplification reactions

07. Add 5 µL of each standard dilution to the corresponding wells:
- Total Soya reactions and,
- RR Soy amplification reactions

08. Add 5 µl of each control (negative control and DNA extraction control) to the corresponding wells:
- Total Soya reactions and,
- RR Soy 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.

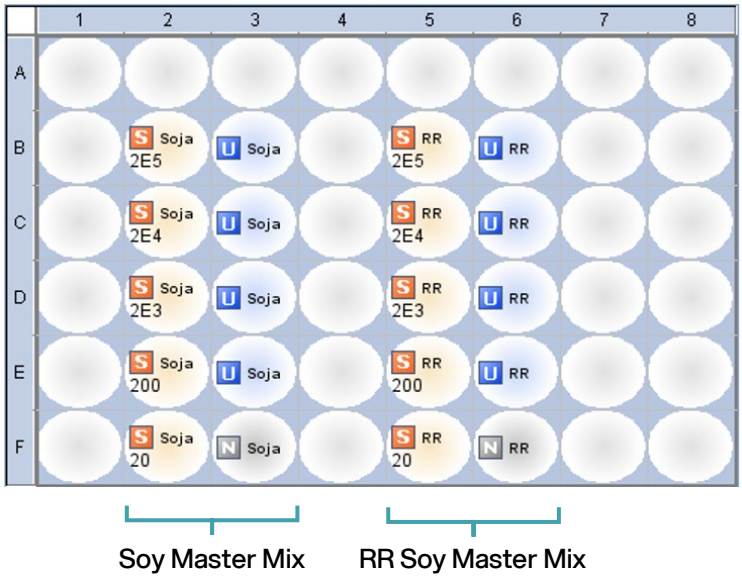

Figure 2. Proposed design analysis for 2 samples obtained from the same DNA extraction round.

(*) 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-274 Imegen® Roundup Ready Soya Quantification Kit probes and specifications.

Target	Receptor	Quencher
Soya	FAM™	MGB
RR Soya	VIC™	MGB

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

- **Reaction volume:** 25 µL.
- **Targets:** FAM™ and VIC™.
- 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:** No amplification should be detected in either the reaction corresponding to Soya or that corresponding to Roundup Ready Soya. Amplification in a negative control indicates the presence of contamination and therefore that the assay should be repeated.
- **RR Soy standard:** Amplification should be detected for the five points corresponding to the Soya standard and the five points corresponding to the Roundup Ready Soya standard. Furthermore, the curves obtained using the standard points should meet the following requirements:

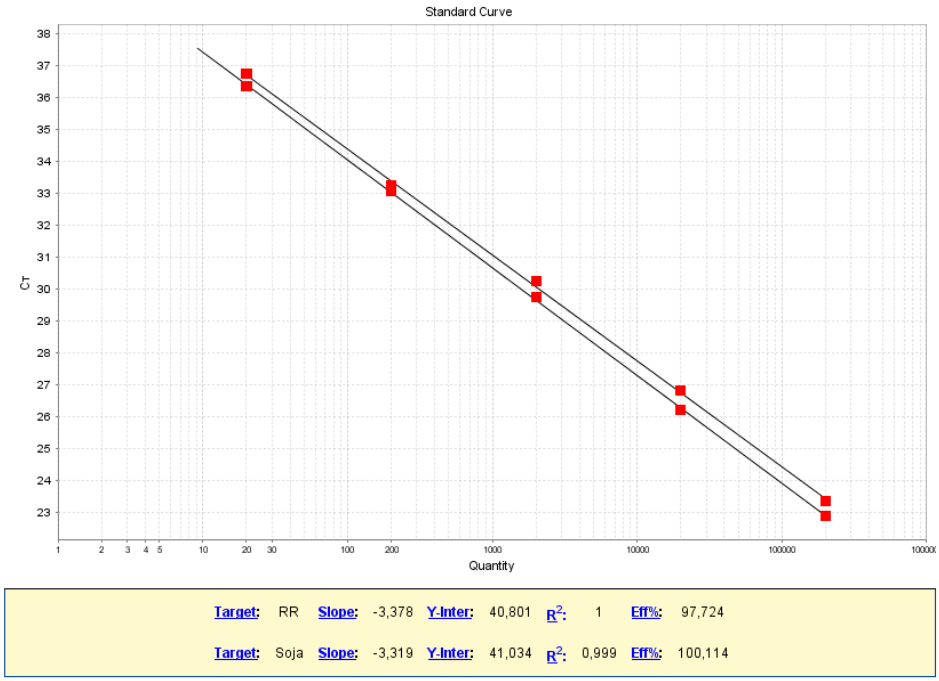

Figure 3. Standard curves for total Soya and RR Soy targets. Red dots represent the dilutions of the standard.

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 analysed.

If duplicated have been performed, the results for both replicates should be similar.

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

- **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 Soya.

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

$$\%RR\ Soy = \frac{Nº\ of\ copies\ of\ RR\ x\ 100}{Nº\ of\ soy\ copies}$$

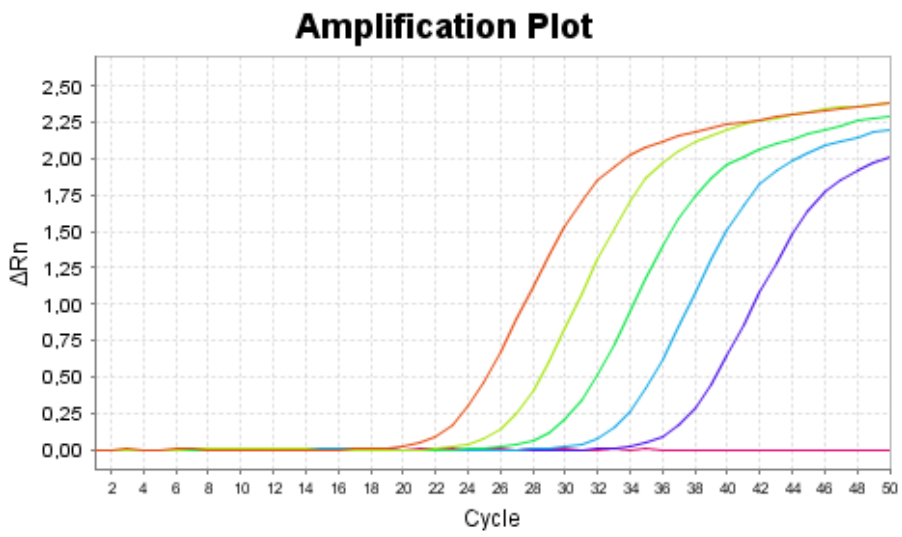

Figure 4. Amplification curves for each of the dilutions of the RR Soy Standard using RR Soy master mix.

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.

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

*It is possible that the inability to detect Soya 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.

04 Limitations

04.1 Equipment

Imegen® Roundup Ready Soya 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 Soya 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

As well as the theoretical specificity analyses performed when designing the oligonucleotides and probes used in the kit, specificity assays have been performed using DNA samples extracted from different transgenic and non-transgenic vegetable species, and a synthetic plasmid included in the kit. The synthetic plasmid, manufactured by GeneScript®, contains a unique sequence which is amplified by the Roundup Ready Soya system and the endogenous Soya gene, lectin. See the results in the table below:

Table 8. Specificity of IMG-274 Imegen® Roundup Ready Soya Quantification Kit.

Vegetable species	Result
Maize *(Zea Mays)*	Not detected
Soya *(Glycine max)*	Detected
Rape *(Brassica napus)*	Not detected
Cotton *(Gossypium hirsutum)*	Not detected
Beetroot *(Beta vulgaris)*	Not detected

AA.2 Detection and quantitation limit

To establish the limit of detection (LOD) for the Imegen® Roundup Ready Soya Quantification Kit, real-time PCR assays were performed using samples containing different DNA copy numbers. The **limit of detection** for the kit is established at **3 DNA copies.**

The **limit of quantitation** (LOQ) for the Imegen® Roundup Ready Soya Quantification Kit was determined using the standard provided with the kit, which consists of a synthetic plasmid containing targets for the endogenous Soya gene known as lectin

and for the transgenic event GTS-40-3-2 present in Roundup Ready Soya, and was set at **20 copies of DNA.**

Imegen® Roundup Ready Soya Quantification Kit allows relative quantifications of up to 0.01% (w/w) of Roundup Ready Soya to be determined with respect to total Soya in a sample.

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.
