INSTRUCTIONS FOR USE

Imegen® Quant Species Kit

Ref. IMG-272

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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® Quant Species 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.05	OCT 2024	Transcription errors: figure 1 and AA.1. Content revision in section 2.1.
V.04	SEP 2023	Contents review. Change of the manufacturer's identification, going from Imegen to Health in Code, S.L. Modification of the storage temperature of the General Master Mix
V.03	JUL 2019	Contents review

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01

Product information

01.1 General description

The identification of meat species presence in food samples is an essential step in order to verify the origin of the used and traceability of the used raw materials, as well as to evaluate the quality control for the handling and cleaning process of production lines by which it passed.

The Health in Code species ID product works by PCR amplification of a specific tag located in the mitochondrial genome of the evaluated species. The particularity of this procedure is due to the fact that the mitochondrial genome is an ideal target since it can be analysed to ensure a specific detection of the desired species and at the same time excluding the detection of other related species. Since there are multiple copies of the mitochondrial genome in each cell, the sensitivity that this detection will have is up to 100 times greater than a test that only target a specific locus in a single copy within the nuclear DNA genome.

During the last decade there had been publicly known cases related to the deceptions that consumers suffer at the time of buying meat and receive other species that are not the ones that they are paying for. EU authorities revealed the presence of uncontrolled meat in food products, and because of this, the food industry authorities have developed food safety management systems to improve the resilience of supply chain to food fraud, mostly directed to prevent the fraud opportunity. Despite the fact, food fraud does not impose a health hazard., but in some ways, they are more dangerous because the raw materials and quality control actions are unknown and untraceable.

The possibility to have a fast and accurate method to determine the authenticity of the ingredient used for food preparation is now available but the precision of the results will be something important to take in consideration at the time of evaluating the food processing. The importance of this phenomenon also lies in economic and commercial problems for both the consumer and the production company Customers want to be sure about the origin of the product they are consuming, also the concern of the contained risk for health. DNA analysis allows a valuable and conscious identification of plants and animal derivatives, by efficiently detecting contaminations or fraud related to inaccurate declaration on the label of the species constituting the food.

01.2 Intended use

Imegen® Quant Species Kit allows the percentage of one species (see the list of species available below) DNA in a sample to be determined with respect to total animal DNA.

The DNA quantification is performed by real time PCR using TaqMan™-MGB probes. Imegen® Quant Species Kit contains the standard with which the samples can be compared to determine the percentage of one species.

Sample analysis comprises two real-time PCR simultaneous processes:

- **One of them allows the total amount of one species DNA in the sample**
- **The other, allows the total amount of animal DNA present in the sample**

To perform the reaction to determine the total DNA amount of one species you need to use one of the kits listed below:

- **Imegen® Beef ID Kit (IMG-264)**
- **Imegen® Pork ID Kit (IMG-265)**
- **Imegen® Poultry ID Kit (IMG-270)**
- **Imegen® Chicken ID Kit (IMG-266)**
- **Imegen® Turkey ID Kit (IMG-267)**
- **Imegen® Equine ID Kit (IMG-263)**
- **Imegen® Sheep ID Kit (IMG-268)**
- **Imegen® Goat ID Kit (IMG-175)**
- **Imegen® Fallow Deer ID Kit (IMG-177)**

Each kit contains the Beef/Pork/Poultry/Chicken/Turkey/Sheep/Goat/Fallow Deer or Equine master mix that includes two primers and a hydrolysis probe labelled with FAM™ fluorophore. This reaction amplifies one specific mitochondrial DNA sequence corresponding to the species referred by the kit.

To perform the reaction to determine the DNA amount of animal in the sample you need to use this kit which includes two primers and a TaqMan™-MGB probe labelled with the FAM™ fluorophore. The reaction specifically amplifies a highly conserved mitochondrial genomic region from animal species.

Imegen® Quant Species Kit includes a plasmid DNA standard containing a copy of each of the targets used during analysis. The standard concentration is $2x10^7$ DNA copies/µl. A comparison of the results obtained with the samples and this standard allows a relative quantification to be made and therefore the percentage of the selected species, with respect to the animal mitochondrial DNA in the sample to be calculated.

01.3 Content and storage conditions of the kit

Imegen® Quant Species Kit contents the necessary reagents to perform 48 reactions:

Species Master Mix	Master Mix with specific oligonucleotides, fluorophore-labelled hydrolysis probes for real-time PCR detection of a highly conserved mitochondrial genomic region from animal species (total animal DNA target), for total animal DNA detection.
General Master Mix	Master Mix of PCR with nucleotides, $MgCl_2$, DNA polymerase and buffer needed to carry out RT- PCR.
Species Standard	Plasmid DNA quantitation standard containing the total animal DNA target and each of the individual animal specie targets used during analysis.

Table 1. IMG-272 Imegen® Quant Species Kit components and description.

Reagents	Color indicator	Quantity	Conservation
Species Master Mix*	Blue pad	360 µl	-20 °C
General Master Mix*	White pad	600 µl	-20 °C upon receipt. 2 - 8 °C after initial use. Store protected from light
Species Standard*	Blue cap	5 x40 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

To quantify one animal species DNA present in a sample is necessary one of the nine available animal species ID kits listed above with Imegen® Quant Species Kit.

Two absolute quantifications are performed during the course of the relative quantification of animal species, present in a sample. The first of these, determines the total amount of animal DNA present in the sample and the second determines the amount of each species DNA in the sample (Beef/ Pork/ Poultry/ Chicken/ Turkey/ Sheep/ Goat/ Fallow Deer or Equine), depending on the kit used.

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 Species Standard and prepare four 1:10 serial dilutions of this standard (see Figure 1). This process results in the quantitative standards with which the samples can be compared.

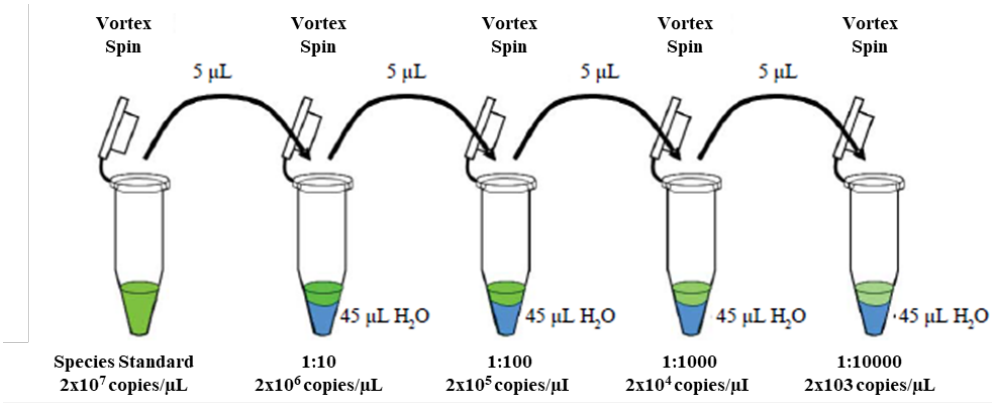

Figure 1. Four serial standard dilutions are made from Species Standard to perform two standard curves.

02. Vortex each reagent and keep cold.

03. Add into a 1.5 mL tube (one for each PCR master mix preparation), the following reagents (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
Species Master Mix	7.5 µL
General Master Mix	12.5 µL

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

05. Add 5 µl of each DNA sample at 10 ng/µl, 5 µl of each standard dilution and 5 µl of each control* into the appropriate wells.

06. Cover the well plate with optical film or the tubes with optical cover and spin in the centrifuge.

07. 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-272 Imegen® Quant Species Kit probes and specifications.

Target	Receptor	Quencher
Animal DNA	FAM™	MGB
Specific Species	FAM™	MGB
IPC	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 analyse the results, it is recommended to establish the Threshold at 0.1 and to keep the default Baseline value to minimise the residual signal in the detection channels.

Ct settings	Threshold	0.1
	Baseline	AUTO

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

- **Negative controls:** Amplification must be only detected in the VIC™ channel for animal species reaction of amplification. No amplification should be detected in either the reaction corresponding to Animal. Amplification in a negative control would indicate the presence of contamination and therefore that the assay should be repeated.

- **Species Standard:** Amplification should be detected for the five points corresponding to the Species standard and the five points corresponding to the beef/ pork / poultry/ turkey/ chicken/ sheep/ goat/ fallow deer or equine standard.

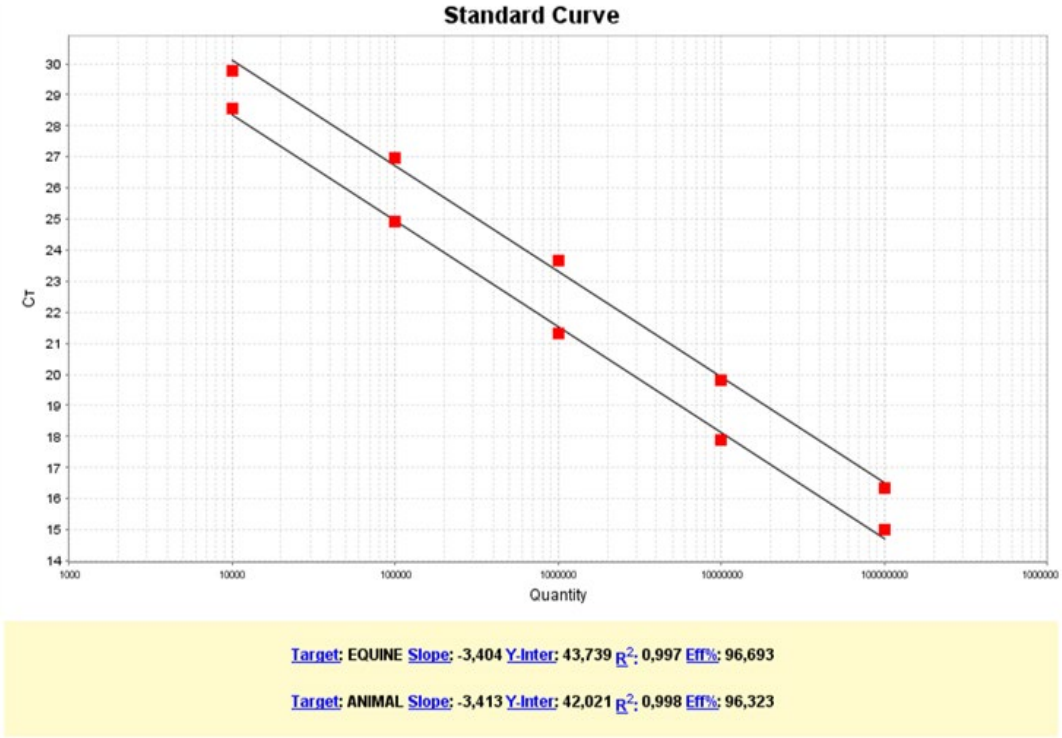

Figure 3. Standard curves for animal and, in this case, equine targets. Red dots represent the dilutions of the standard.

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.

Two results are possible for each amplification reaction of both beef / pork / poultry / turkey / chicken / sheep / goat / fallow deer or equine DNA and animal DNA:

- **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 beef/ pork/ turkey/ chicken/ sheep/ goat/ fallow deer or equine DNA.
- **Not Quantifiable:** No amplification is detected in the sample, or the amplification detected is lower than the last point on the curve.

The following formula should be used to calculate the percentage of beef/pork/poultry/turkey/chicken/sheep/goat or equine (species) DNA with:

$$\%\ Species\ DNA\ =\ \frac{Species\ DNA\ x\ 100}{Animal\ DNA}$$

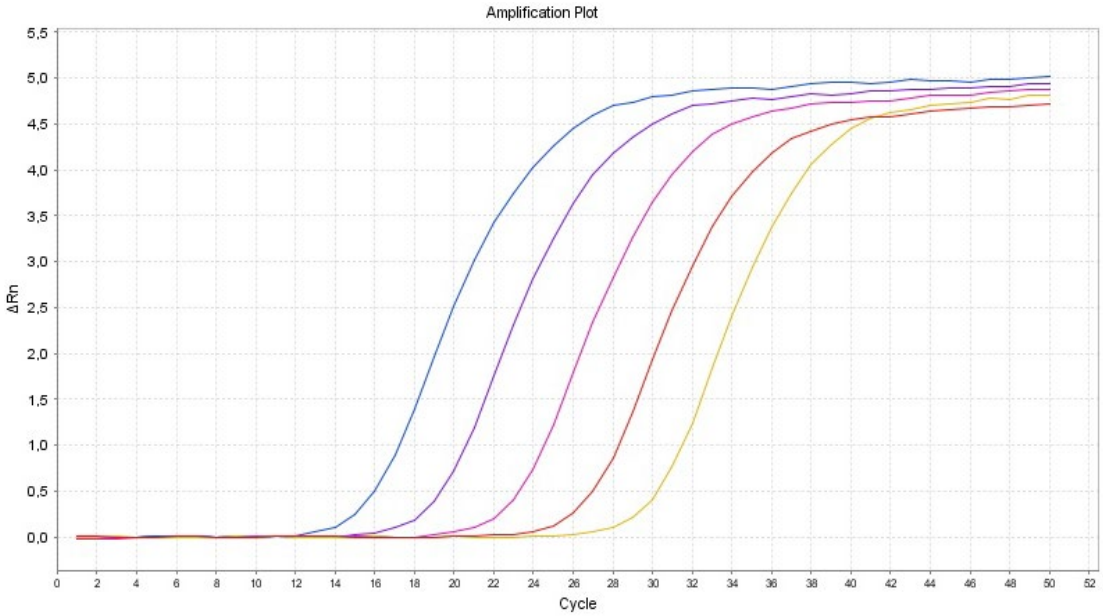

Figure 4. Amplification curves for each of the dilutions of the Species Standard using Species master mix.

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 6. Results interpretation.

Animal	Animal Species*	Interpretation
Quantifiable	Not quantifiable	No specific animal species detected in the sample or the amount of specific animal species detected in the sample is lower than the limit of quantification
Quantifiable	Quantifiable	The amount of specific animal species DNA with respect to total animal DNA in the sample is X%
Not quantifiable	Not quantifiable	The amounts of specific animal species and animal DNA detected in the sample are lower than the limit of quantification

(*) One of the follow animal species: beef, pork, poultry, turkey, chicken, sheep, goat or equine.

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 7. Possible results and their interpretation.

Controls	16S Master Mix	Animal-Specific Master Mix		Interpretation
		Animal-specific	IPC	
Positive control	+	+	+	Expected result
	-	-	-	[1] PCR Amplification Failure
Extraction Negative Control	-	-	+	Expected result
	+	+	+	[2] Contamination in the DNA extraction procedure
PCR Negative Control	-	-	+	Expected result
	+	+	+	[3] PCR contamination with animal DNA

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

(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 animal 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® Quant Species 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® Quant Species 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-272 Imegen® Quant Species Kit.

Species	Result
Horse *(Equus caballus)*	Detected
Mule *(Equus asinus x Equus caballus)*	Detected
Donkey *(Equus asinus)*	Detected
Beef *(Bos taurus)*	Detected
Pork *(Sus scrofa domestica)*	Detected
Water buffalo *(Bubalus bubalis)*	Detected
Fallow deer *(Dama dama)*	Detected
Chicken *(Gallus gallus)*	Detected
Turkey *(Meleagris gallopavo)*	Detected
Goat *(Capra aegagrus hircus)*	Detected
Duck (Genus *Anas*)	Detected
Ostrich *(Struthio camelus)*	Detected
Goose *(Anser anser)*	Detected
Human *(Homo sapiens)*	Detected
Rice *(Oryza sativa)*	Not detected
Wheat *(Triticum aestivum)*	Not detected
Soya *(Glycine max)*	Not detected
Maize *(Zea mays)*	Not detected
Tomato *(Solanum lycopersicum)*	Not detected
E. Coli *(Escherichia coli)*	Not detected

AA.2 Detection limit

The detection limit has been calculated with standard samples consisting of mixtures of raw animal meat and other species. Imegen® Quant Species Kit can detect as little as 0.05% of specific animal species DNA with respect to total animal DNA in a sample. The limit of detection in processed samples varies depending on the composition and food processing.

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:

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