INSTRUCTIONS FOR USE

Imegen® Fish Allergen ID Kit

Ref. IMG-276

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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® Fish Allergen ID 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
V.05	JUL 2023	General Master Mix's temperature update
V.04	APR 2021	Change of kit's name, addition of "Allergen" in capital letter and manufacturer's name and address update

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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 sanitized, 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.

In order to preserve the health of consumers, Health in Code S.L. 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.

References:

Paschke, A. & Ulberth, F. Anal Bioanal Chem (2009) 395: 15.
https://doi.org/10.1007/s00216-009-2989-0

01.2 Intended use

In order to preserve the health of consumers, it 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.

Identification of fish presence in food samples is an essential step in order to verify the origin and traceability of the used raw materials, as well as a necessary quality control for handling and cleaning processes of production lines.

Imegen® Fish Allergen ID Kit allows determining the presence of DNA of Fish in any food.

Fish DNA detection is done by real time PCR using three hydrolysis probes. Two of them, labelled with FAM™ dye, specifically detect mitochondrial DNA sequence of Fish. The third probe is labelled with VIC™ and detects an Internal Positive Control, which is used to rule out inhibitors in the sample and check the correct functioning of the assay.

Health in Code S.L. is certified by BV against the standard UNE-EN ISO 9001 "Quality management systems" for the design, development, manufacture and commercialization of kits for genetic analysis (Certification number ES090493-1).

01.3 Content and storage conditions of the kit

Imegen® Fish Allergen ID Kit contents the necessary reagents to perform 48 reactions:

Fish Master Mix	Master Mix with specific oligonucleotides, fluorophore-labelled hydrolysis probes (two FAMTM probes for Fish detection and VICTM probe for the Internal Positive Control detection, IPC), synthetic plasmid including the specific IPC sequence and nuclease-free water.
General Master Mix	Master Mix of PCR with nucleotides, MgCl2, DNA polymerase and buffer needed to carry out real-time PCR.
Positive Control	DNA sample containing 0.01% of Fish DNA.

Table 1. IMG-276 Imegen® Fish Allergen ID Kit components and description.

Reagents	Color indicator	Quantity	Conservation
Fish Master Mix*	Yellow pad	360 µl	-20 ˚C
General Master Mix*	White pad	600 µl	-20 ºC upon receipt. 2-8ºC after first use. Keep protected from light.
Positive control*	Yellow cap	60 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

A PCR master mix should be prepared in order to perform the analysis with Imegen® Fish Allergen ID Kit:

The PCR Master Mix contains:

- **Fish Master Mix**
- **General Master Mix**

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

01. Thaw the Fish Master Mix, the Positive Control vial and samples.
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 make calculations taking into account the number of samples to be simultaneously analyzed, and then considering one more reaction, or increase a 10% the volume of each reagent.

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

Reagents	Amount per reaction
Fish Master Mix	7.5 µL
General Master Mix	12.5 µL

04. Vortex and spin the 1.5 mL tube and dispense 20 µl into corresponding wells or tube of 0.2 ml.
05. Add 5 µl of each DNA sample at 10 ng/µl, 5 µl of Positive Control and 5 µl of the Negative Controls* into the appropriate wells.

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

(*) 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-276 Imegen® Fish Allergen ID Kit probes and specifications.

Target	Receptor	Quencher
Fish	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/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:

- **Positive control:** the result must always be positive in all amplification reactions, both in the FAM™ channel as VIC™.
- **Negative controls:** amplification should only be detected in the VIC™ channel. In this channel an internal positive control (IPC) is detected, which determines the absence of inhibition in the sample.

IPC	It must be checked that the IPC (VIC™) is positive in all samples, with a Ct similar to the Positive Control. A negative result in the IPC indicates the presence of inhibitors in the sample. It should be noted that IPC result may be negative in samples where a lot of Fish DNA (FAM™) is detected, because the PCR reagents are exhausted before amplification of the IPC begins.
Fish DNA	Amplification in the FAM™ channel indicates presence of Fish DNA in the sample.

To establish a positive cut-off value (0.001%) for the test samples:

$$Ct_{(cut-off)} = Ct_{(Positive\ Control)} + 6.64$$

Table 5. Cut-off values. (1) For fresh or minimally processed meats samples, the cut-off value corresponds approximately 0.001% of Fish DNA, when DNA concentration is 10ng/uL.

Sample Ct value	Sample result
$Ct > Ct_{(cut-off)}$	Negative
$Ct \leq Ct_{(cut-off)}$	Positive

(*) For fresh or minimally processed meat samples, the cut-off value corresponds to approximately 0.001% fish DNA, when the DNA samples concentration is 10 ng/µl.

In samples where no amplification in the FAM™ channel is seen, we can conclude that no Fish DNA is detected or that its amount in the sample is below than the detection limit.

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.

Fish Master Mix		Interpretation
Fish (FAM™ channel)	IPC (VIC™ channel)	
-	+	No Fish DNA is detected
+	+	Fish DNA is detected
-	-	PCR inhibitors presence in the sample*
+	-	Sample with big amount of Fish DNA

* If presence of inhibitors in the sample is detected, we recommend checking whether there has been an excess of DNA in the reaction (the recommended maximum is 250 ng). If the amount of DNA is right, we recommend repeating DNA extraction. If the problem persists, please contact our technical department.

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	Master Mix		Interpretation
	Fish	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 Fish DNA

(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. Ensure the DNA concentration is as recommended, and if the problem persists purify the sample.

(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 Fish 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® Fish Allergen ID 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)
- StepOnePlus™ 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® Fish Allergen ID 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 (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. Animal species used during the specificity of IMG-276 Imegen® Fish Allergen ID Kit.

Meat species	Result
Atlantic salmon *(Salmo salar)*	Detected
Angler *(Lophius piscatorius)*	Detected
Atlantic cod *(Gadus morhua)*	Detected
European hake *(Merluccius merluccius)*	Detected
Atlantic halibut *(Hippoglossus hippoglossus)*	Detected
European bass *(Dicentrarchus labrax)*	Detected
European anchovy *(Engraulis encrasicolus)*	Detected
Sole *(Solea solea)*	Detected
Dab *(Limanda limanda)*	Detected
Brown trout *(Salmo trutta)*	Detected
Blue shark *(Prionace glauca)*	Detected
European eel *(Anguilla anguilla)*	Detected
Red bandfish *(Thymichthys politus)*	Detected
Muscovy duck *(Cairina moschata)*	Not detected
Duck *(genus Anas)*	Not detected
Prawn *(Aristaeomorpha foliacea)*	Not detected
Norway lobster *(Nephrops norvegicus)*	Not detected
Shrimp *(Crangon crangon)*	Not detected
Mussel *(Mytilus galloprovincialis)*	Not detected

Clam *(Ruditapes decussatus)*	Not detected
Cuttlefish *(Sepia officinalis)*	Not detected
Squid *(Loligo vulgaris)*	Not detected
Octopus *(Octopus vulgaris)*	Not detected
Human *(Homo sapiens)*	Not detected
Chicken *(Gallus gallus)*	Not detected
Turkey *(Meleagris gallopavo)*	Not detected
Beef *(Bos taurus)*	Not detected
Pork *(Sus scrofa domestica)*	Not detected
Horse *(Equus caballus)*	Not detected

AA.2 Detection limit

The detection limit has been calculated upon standard samples consisting of mixtures of raw Fish meat and other meat species. Imegen® Fish Allergen ID Kit can detect blends containing 0.001% (w/w) of fish DNA. The limit of detection in processed samples varies depending on the composition of the sample and the method of food processing.

To ensure the representativeness of the results, we recommend the use of a DNA extraction method that allows you to process a large amount of sample (10-20 g). If you do not have a procedure with these features, we recommend the use of **Imegen® Food Extraction Kit** (Part No: IMG-262).

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 authorized 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.
