INSTRUCTIONS FOR USE

Imegen® Specific Tuna ID Kit

Ref. IMG-309

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® Specific Tuna 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	NOV 2024	In section 1.3: modification of the positive control's description Content revision in 2.1.
V.05	SEP 2023	Contents review; modification of the storage temperature of the General Master Mix. Change of the manufacturer's identification, going from Imegen to Health in Code, S.L.
V.04	JUN 2020	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 analyzed 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® Specific Tuna ID Kit enables the user to detect the presence of Tuna DNA in food and feed samples.

Tuna DNA detection is done by real time PCR using two TaqMan™ probes. One of them, labelled with FAM™ or VIC™ dye, specifically detects one mitochondrial DNA sequence of Tuna. The second probe is labelled with Cy5® and detects an Internal Positive Control, which is used to rule out inhibitors in the sample and check the correct functioning of the assay.

The kit detects the following tuna species through the specific amplification of a specific region present in the mitochondrial genome.

- *Thunnus alalunga* (Longfin tuna)
- *Thunnus albacares* (Yellowfin tuna)
- *Thunnus obesus* (Bigeye tuna)
- *Katsuwonus pelamis* (Skipjack tuna)

01.3 Content and storage conditions of the kit

Imegen® Specific Tuna ID Kit contents the necessary reagents to perform 48 reactions:

Tuna 1 Master Mix	Master Mix with specific oligonucleotides, fluorophore-labelled hydrolysis probes (FAM™ probe for Tuna detection and Cy5® probe for the Internal Positive Control detection, IPC), synthetic plasmid including the specific IPC sequence and nuclease-free water: ➔ *Thunnus alalunga/Thunnus albacares* / Internal positive control (IPC)
Tuna 2 Master Mix	Master Mix with specific oligonucleotides, fluorophore-labelled hydrolysis probes (VIC™ probe for Tuna detection and Cy5™ probe for the Internal Positive Control detection, IPC), synthetic plasmid including the specific IPC sequence and nuclease-free water. ➔ *Thunnus obesus/Katsuwonus pelamis*
General Master Mix	Master Mix of PCR with nucleotides, $MgCl_2$, DNA polymerase and buffer needed to carry out RT- PCR.
Positive Control 1	Represents 1% of *Thunnus alalunga / Thunnus albacares* DNA.

Positive Control 2	Represents 1% of *Thunnus obesus / Katsuwonus pelamis* DNA.

Table 1. IMG-309 Imegen® Specific Tuna ID Kit components and description.

Reagents	Color indicator	Quantity	Conservation
Tuna 1 Master Mix*	Purple disk	360 µl	-20 °C
Tuna 2 Master Mix*	Yellow disk	360 µl	-20 °C
General Master Mix*	White disk	2 x 600 µl	-20 °C upon receipt. 2 - 8 °C after initial use. Store protected from light.
Positive control 1*	Purple lid	60 µl	-20 °C
Positive control 2*	Yellow lid	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

Imegen® Specific Tuna ID Kit is designed to determine, in a single PCR reaction, the presence or absence of tuna DNA and the internal positive control.

We recommend using, the positive control included in this kit for each run.

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

01. Thaw the Tuna 1 and Tuna 2 Master Mixes, the Positive Controls 1 and 2 and DNA 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 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 of Tuna 1 Master Mix or Tuna 2 Master Mix.

Reagents	Amount per reaction
Tuna 1 or Tuna 2 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 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-309 Imegen® Specific Tuna ID Kit probes and specifications.

Target	Receptor	Quencher
T. alalunga & T. obesus	FAM™	MGB
T. albacares & K. pelamis	VIC™	MGB
IPC	Cy5®	TAO

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 (FAM™ and VIC™) or "None" (Cy5®) depending on the probe. If the real time PCR system does not take into account the quenchers, select only the receptors (FAM™, VIC™ and Cy5®).
- 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 0.2 and to keep the default Baseline value to minimize the residual signal in the detection channels.

Ct settings	Threshold	0.2
	Baseline	AUTO

The Ct and the cut-off are relative parameters directly influenced by the threshold level. Therefore, it is recommended to carefully analyse the signals in each of the detection channels (FAM, VIC and Cy5®) to establish the threshold at the beginning of the exponential phase and at a level higher than any residual signal.

It is recommended to analyse both the Ct "threshold cycle" and the shape of the amplification curve for each sample, and to verify that the results obtained for the controls are as expected:

- **Positive controls:** the result must always be positive in all amplification reactions, in the FAM™, VIC™ and Cy5® channel.
- **IPC (Internal Positive Control):** The result should be positive in the Cy5® channel in all the amplification reactions, including the PCR control (NTC) and the positive controls.
- **Negative controls:** There should only be amplification in the Cy5® channel where the internal positive control (IPC) is detected, which determines the absence of inhibition in the sample.

IPC	It should be checked that the IPC (Cy5®) is positive in all samples. A negative result in the IPC would indicate the presence of PCR inhibitors in the sample. Note also that the ICP result could be negative in those samples saturated with a lot of tuna DNA.
Master Mixes	Amplification in the FAM™ or VIC™ channel indicates presence of tuna DNA in the sample.

The presence of tuna in the sample is indicated by the amplification signal in the FAM™ and VIC™ channels, provided that the Ct value is less than or equal to the $Ct_{cut\text{-}off}$.

Table 5. IMG-309 Imegen® Specific Tuna ID Kit Probes and specifications.

Master Mixes	FAM	VIC
Tuna 1 Master Mix	*Thunnus alalunga*	*Thunnus albacares*
Tuna 2 Master Mix	*Thunnus obesus*	*Katsuwonus pelamis*

The $Ct_{cut\text{-}off}$ is the value that allows the user to determine if an amplification reaction is positive or negative. The $Ct_{cut\text{-}off}$ corresponds to the Ct value obtained in the Positive Control for each of the analysed species. It is recommended to analyse the Positive Control in each determination due to the variability between tests.

Establish the positive cut-off value for the test samples and assign results:

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

Table 6. Interpretation of the Cut off results in the real-time PCR assay.

Analyte	Sample result	Interpretation
Target DNA	No amplification	Not detected
	$Ct > Ct_{(cut\text{-}off)}$	Not detected
	$Ct \leq Ct_{(cut\text{-}off)}$	Detected
No template control / Extraction control	$Ct < Ct_{(cut\text{-}off)}$ or no amplification	Not detected (expected results)

NOTE: Any sample with a Ct equal to $Ct_{cut\text{-}off}$ contains approximately 1% tuna DNA.

In samples where no amplification in the FAM™ channel is seen, we can conclude that no tuna DNA is detected or that their 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 7. Results interpretation.

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

(*) If the presence of inhibitors is detected in the sample, we recommend checking if it is due to an excess of DNA in the reaction that may be saturating the PCR (the maximum recommended is 250ng). If the amount of DNA is correct, it is then recommended to repeat the DNA extraction. If the problem persists, please contact our technical department.

Tuna 1 Master Mix:

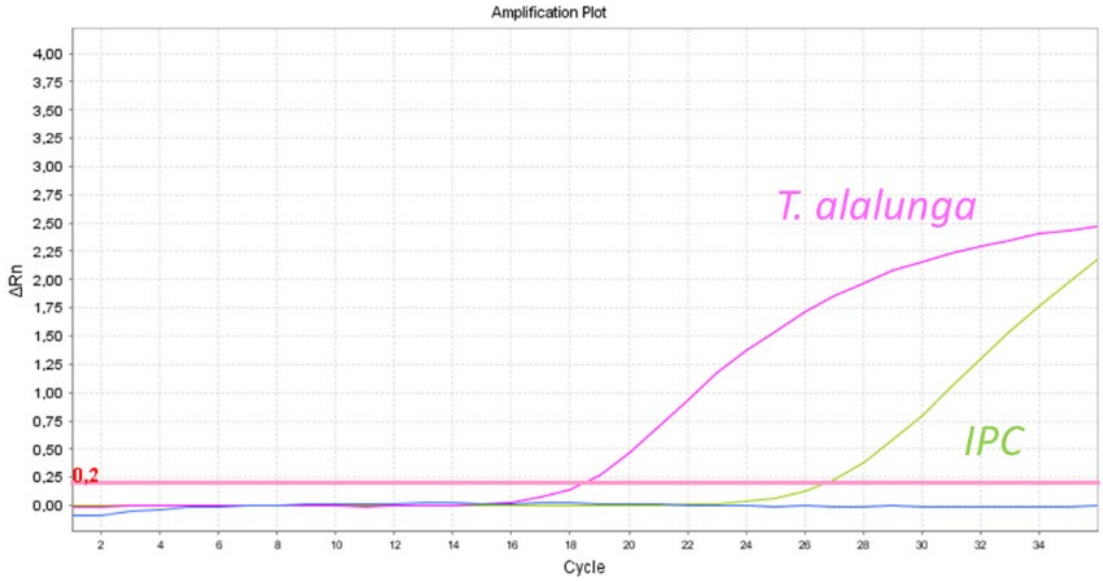

Figure 1. Positive sample (FAM): 100% T. alalunga (50 ng of total DNA).

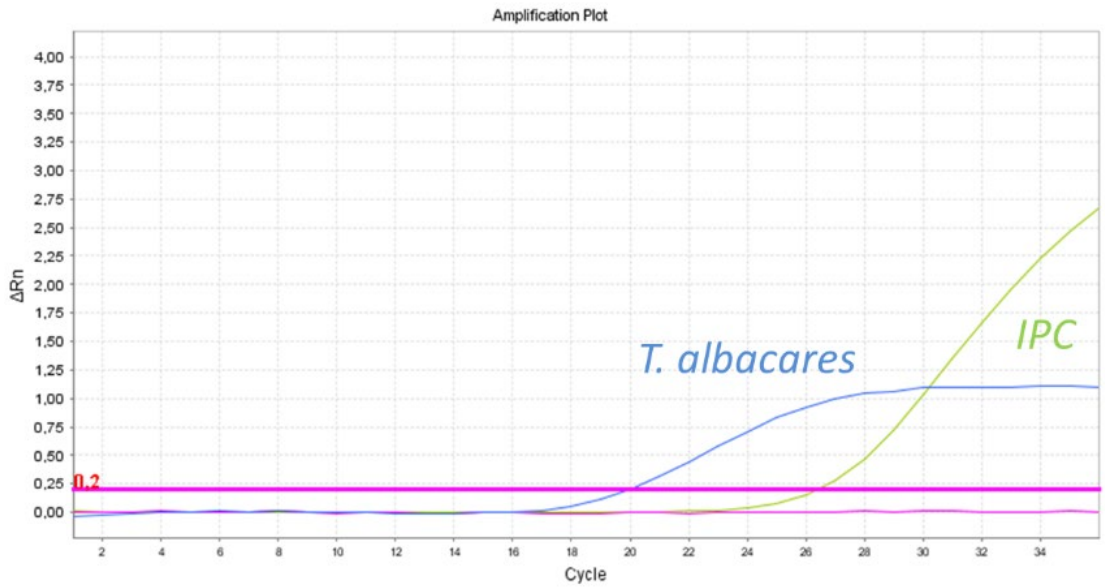

Figure 2. Positive sample (VIC): 100% T. albacares (50 ng of total DNA).

Tuna 2 Master Mix:

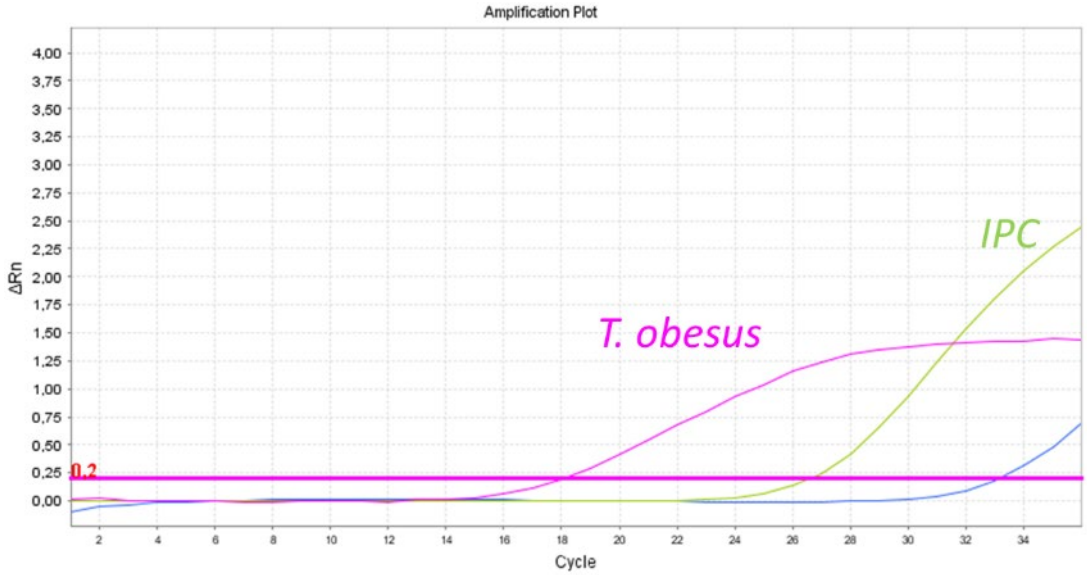

Figure 3. Positive sample (FAM): 100% T. obesus (50 ng of total DNA)

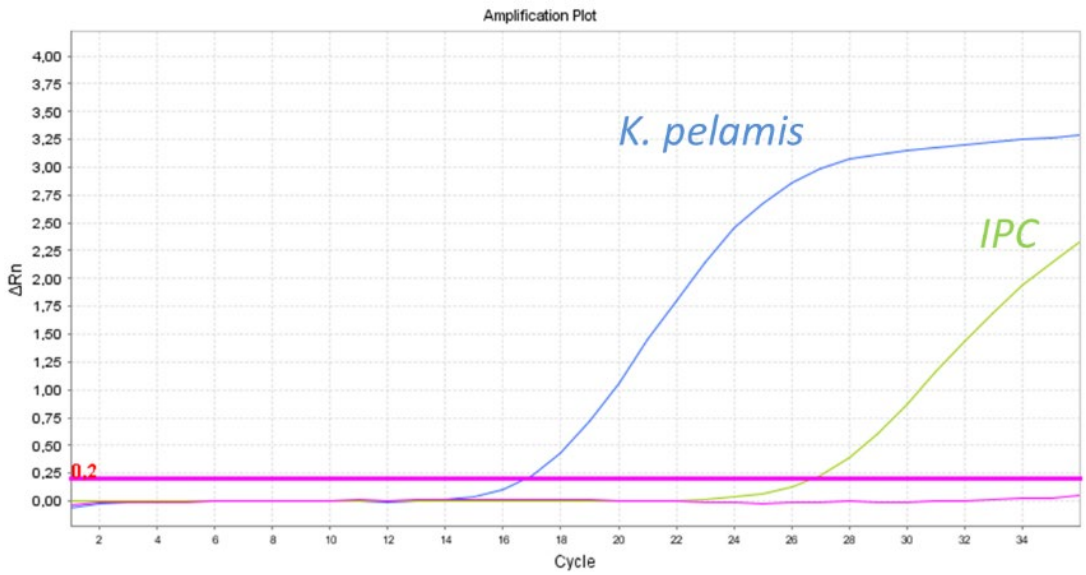

Figure 4. Positive sample (VIC): 100% K. pelamis (50 ng of total DNA).

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 8. Possible results and their interpretation.

Controls	Specific Tuna Master Mix		Interpretation
	Tuna	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 sheep 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 Tuna 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® Specific Tuna 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)
- 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® Specific Tuna 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 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 9. Specificity of Master Mix Tuna 1 from IMG-309 Imegen® Specific Tuna ID Kit.

Tuna 1 Master Mix (Thunnus alalunga / Thunnus albacares)	
Meat species	**Result**
Longfin tuna *(Thunnus alalunga)*	Detected
Yellowfin tuna *(Thunnus albacares)*	Detected
Bigeye tuna *(Thunnus obesus)*	Not detected
Skipjack tuna *(Katsuwonus pelamis)*	Not detected
Atlantic bluefin tuna *(Thunnus thynnus)*	Not detected
Clam *(Ruditapes decussatus)*	Not detected
Crab *(Cancer pagurus)*	Not detected
European crayfish *(Astacus astacus)*	Not detected
Prawn *(Aristaeomorpha foliacea)*	Not detected
Octopus *(Octopus vulgaris)*	Not detected
Cuttlefish *(Sepia officinalis)*	Not detected
European eel *(Anguilla anguilla)*	Not detected
Dab *(Limanda limanda)*	Not detected
European bass *(Dicentrarchus labrax)*	Not detected
Sole *(Solea solea)*	Not detected
Atlantic cod *(Gadus morhua)*	Not detected
European hake *(Merluccius merluccius)*	Not detected

European pilchard *(Sardina pilchardus)*	Not detected
Angler *(Lophius piscatorius)*	Not detected
Brown trout *(Salmo trutta)*	Not detected
Swordfish *(Xiphias gladius)*	Not detected
Beef *(Bos taurus)*	Not detected
Pork *(Sus scrofa domestica)*	Not detected
Horse *(Equus caballus)*	Not detected
Goat *(Capra aegagrus hircus)*	Not detected
Sheep *(Ovis aries)*	Not detected

Table 10. Specificity of Master Mix Tuna 2 from IMG-309 Imegen® Specific Tuna ID Kit.

Tuna 2 Master Mix (Thunnus alalunga / Thunnus albacares)	
Meat species	**Result**
Longfin tuna *(Thunnus alalunga)*	Not detected
Yellowfin tuna *(Thunnus albacares)*	Not detected
Bigeye tuna *(Thunnus obesus)*	Detected
Skipjack tuna *(Katsuwonus pelamis)*	Detected
Atlantic bluefin tuna *(Thunnus thynnus)* [1]	Detected
Clam *(Ruditapes decussatus)*	Not detected
Crab *(Cancer pagurus)*	Not detected
European crayfish *(Astacus astacus)*	Not detected
Prawn *(Aristaeomorpha foliacea)*	Not detected
Octopus *(Octopus vulgaris)*	Not detected
Cuttlefish *(Sepia officinalis)*	Not detected
European eel *(Anguilla anguilla)*	Not detected
Dab *(Limanda limanda)*	Not detected
European bass *(Dicentrarchus labrax)*	Not detected
Sole *(Solea solea)*	Not detected
Atlantic cod *(Gadus morhua)*	Not detected
European hake *(Merluccius merluccius)*	Not detected
European pilchard *(Sardina pilchardus)*	Not detected
Angler *(Lophius piscatorius)*	Not detected
Brown trout *(Salmo trutta)*	Not detected

Swordfish *(Xiphias gladius)*	Not detected
Beef *(Bos taurus)*	Not detected
Pork *(Sus scrofa domestica)*	Not detected
Horse *(Equus caballus)*	Not detected
Goat *(Capra aegagrus hircus)*	Not detected
Sheep *(Ovis aries)*	Not detected

[1]The *T. obesus* amplification systems also amplifies DNA from *T. thynnus.*

AA.2 Detection limit

The detection limit has been calculated upon standard samples consisting of mixtures of raw specific tuna meat and other species. Imegen® Specific Tuna ID Kit can detect blends with as little as 1% (w/w) of specific tuna meat. The limit of detection in processed samples varies depending on the composition and 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 (Ref 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 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.
