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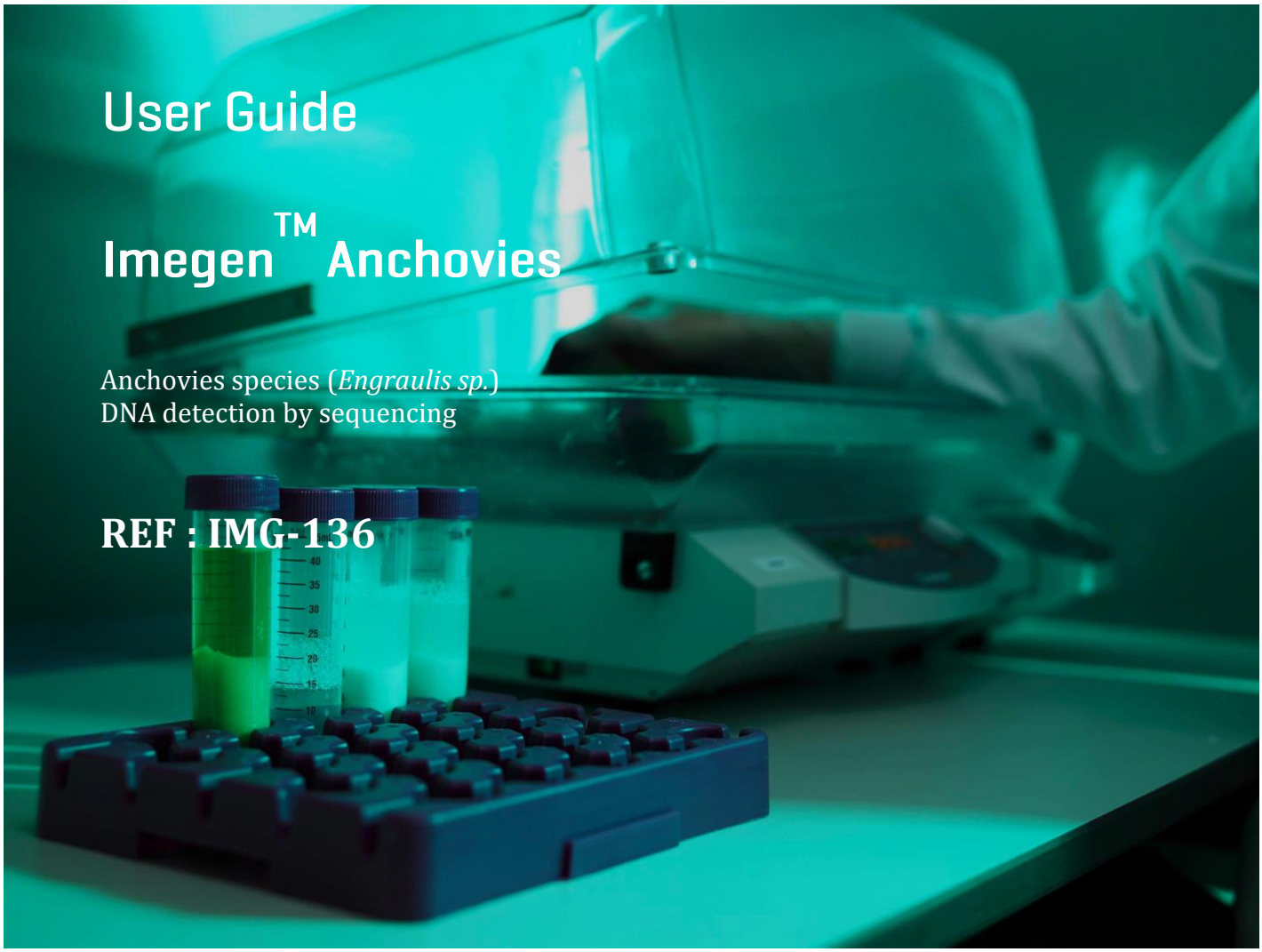

User Guide

Imegen™ Anchovies

Anchovies species (*Engraulis sp.*)
DNA detection by sequencing

REF : IMG-136

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All products sold by the Imegen are subjected to rigorous quality control. The ***imegen™ Anchovies 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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1. Product information

Kit description

imegen- Anchovies Kit *allows the identification of anchovies species present in a sample. The assay is done by PCR amplification and sequencing of a mitochondrial genome ATP6 gene region.*

Obtained sequences comparison with public data bases as GenBank and this region specific data bases allows the identification of the following anchovies species:

- *Engraulis encrasicolus*
- *Engraulis japonicus*
- *Engraulis ringens*
- *Engraulis anchoita*

Content and storage of the kit

The kit contains needed reagents to perform the PCR reactions:

- *Amplification primers for the PCR system.*
- *All needed reagents in order to perform the PCR.*
- *Sequencing primers.*

The kit contents the necessary reagents to perform 50 reactions:

Reagents	Color	Amount	Storage
Anchovies Master Mix	White pad	4x 478 µL	-20ºC
Anchovies PCR primers	Green pad	2x 137 µL	-20ºC
Anchovies Taq	Yellow cap	14 µL	-20ºC
Anchovies F primer	Blue pad	176 µL	-20ºC
Anchovies R primer	Red pad	176 µL	-20ºC

Table 1. *Kit components and storage temperature of* ***imegen- Anchovies Kit***

NOTE: Reagents required but not provided: reagents for sequencing reactions, capillary electrophoresis and consumables.

Equipment and material required but not supplied

In the following table the equipment requirements for using ***imegen-Anchovies Kit*** *are shown:*

Equipment	
1	PCR Thermal Cycler
2	Micropipettes (10 µl, 20 µl and 200 µl)
3	Vortex

Materials	
1	0.2 ml sterile tubes
2	1.5 ml sterile tubes
3	Powder-free latex gloves

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2. PCR

PCR reactions preparation

To estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples and controls (we recommend to include positive and negative 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 amplification reactions is showed below:

1. *Thaw the Anchovies Master Mix, PCR primers, the Taq and samples DNA.*
2. *Vortex each reagent and keep cold.*
3. *In a 1.5 ml tube, add the following amounts:*

Reagents	Amount per reaction
Anchovies Master Mix	34.8 µL
Anchovies PCR primers	5 µL
Anchovies Taq	0.25 µL

***Table 2.** Reagents amount per reaction*

4. *Vortex the 1.5 mL tube and spin it.*
5. *Dispense 40 µL of the PCR Master Mix in each 0.2 mL tube or well.*
6. *Add 10 µl of DNA sample at 10 ng/µl into the appropriate 0.2 mL tubes or wells.*

* *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.*

PCR amplification program

The following instructions must be followed in order to setup the amplification program:

- *Optimal program:*

Fields	Step 1 Enzyme activation	Step 2 PCR			Step 3	
Cycle Number	1 initial cycle	40 cycles			1 Final cycle	
		Denaturation	Primers binding	Extention	PCR Finishing and holding step	
Temperature	95°C	95°C	55°C	72°C	72°C	4°C
Time	10 minutes	30 seconds	30 seconds	30 seconds	10 minutes	∞

***Table 3**. Optimal PCR program*

After PCR amplification program, PCR products must be analysed by 2.5% agarose gel electrophoresis in order to verify the presence of a 147 bp amplified in all samples and positive controls, and its absence in negative controls.

3. Sequencing reactions

PCR products purification

The amplified fragments must be purified using a purification protocol that ensures the removal of surplus amplification primers and other PCR reagents.

Sequencing reactions preparation

*From one purified PCR product, two sequencing reactions are prepared: one with the forward primer (**Anchovies F primer**) and the other with the reverse (**Anchovies R primer**).*

Sequencing reactions are performed in a final volume of 10 µl. To estimate the amount of reagents required, it should be taken into account the number of samples and controls to be analysed simultaneously, and then considering a further reaction or increasing by 10% the volume of each reagent.

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

1. *In a 1.5 ml tube, add the following amounts (reagents not supplied):*

Reagents	Amount per reaction
BigDye 3.1	1 µL
BigDye 5x Buffer	2 µL
Nuclease free water	4.4 µL

***Table 4**. Sequencing reagents amount per reaction*

2. *Vortex and spin the sequencing Master Mix.*
3. *Dispense 7.4 µL of the Sequencing Master Mix in each 0.2 mL tube or well.*
4. *Add 1 µl of purified PCR product into the appropriate 0.2 mL tube or well.*
5. *Add 1.6 µl of corresponding primer (Anchovies F primer or Anchovies R primer) into the appropriate 0.2 mL tube or well.*

Sequencing amplification program

Sequencing amplification reactions must be summited to the following PCR program:

Fields	Step 1 Enzyme activation	Step 2 PCR			Step 3
Cycle Number	1 initial cycle	30 cycles			1 Final cycle
		Denaturation	Primers binding	Extention	Holding step
Temperature	95°C	96°C	50°C	60°C	4°C
Time	1 minutes	10 seconds	5 seconds	4 minutes	∞

***Table 5**. Sequencing Optimal PCR program*

After sequencing PCR amplification program, sequencing PCR products should be stored at 4°C until the purification step.

Sequencing products purification and capillary electrophoresis

Sequencing reactions must be purified using a purification protocol that ensures removal of Dye and other sequencing reagents excess.

Once purified, the sequencing reactions should be subjected to capillary electrophoresis. Depending on the used sequencer model, manufacturer's recommended polymer should be used. In order to setup the electrophoresis conditions, it should be taken into account that the size of the amplified products is 147 bp.

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4. Results analysis

It is recommended to perform an alignment of the direct and reverse sequences of each sample to verify the obtained sequence, although it is also possible to perform the analysis using only one of the sequences of each sample.

The last 30 bp obtained in each sequencing reaction must be removed before comparison with databases, since these regions correspond to the region where the amplification primers are designed and are not of the analysed sample.

The obtained sequences should be compared to own sequences or public databases to identify the analysed anchovy species.

*It has been described a group of E. japonicus, that using this analysis, cannot be differentiated from E. encrasicolus, thus, presence of these species must be verified through further analysis of other DNA region [**IMG-145 imegen-Anchovies II kit**].*

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