SAMPLING PROTOCOLS FOR THE GBYP BIOLOGICAL SAMPLING

SAMPLING PROTOCOL FOR GENETICS (M or F)

MATERIALS

Product	Catalog Code
100 tubes 5mL with screw cap (SARSTEDT)	60.558.001
Surgical scissors, tweezers, disposable scalpels	
Non-denatured Ethanol 96%	
Gloves	
S-Monovette® racks D17, 50 apertures(SARSTEDT)	93.852

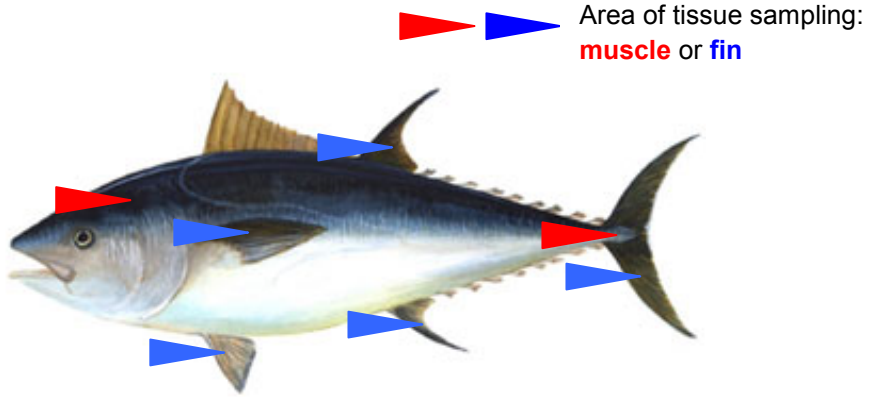
SUMMARY OF SAMPLING:

Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal), with two replicates for each individual.

Tissue type: white skeletal muscle near caudal fin when possible. Alternative: other muscle (e.g. from head) or fin clip from pectoral, 2nd dorsal, anal, pelvic or caudal fin.

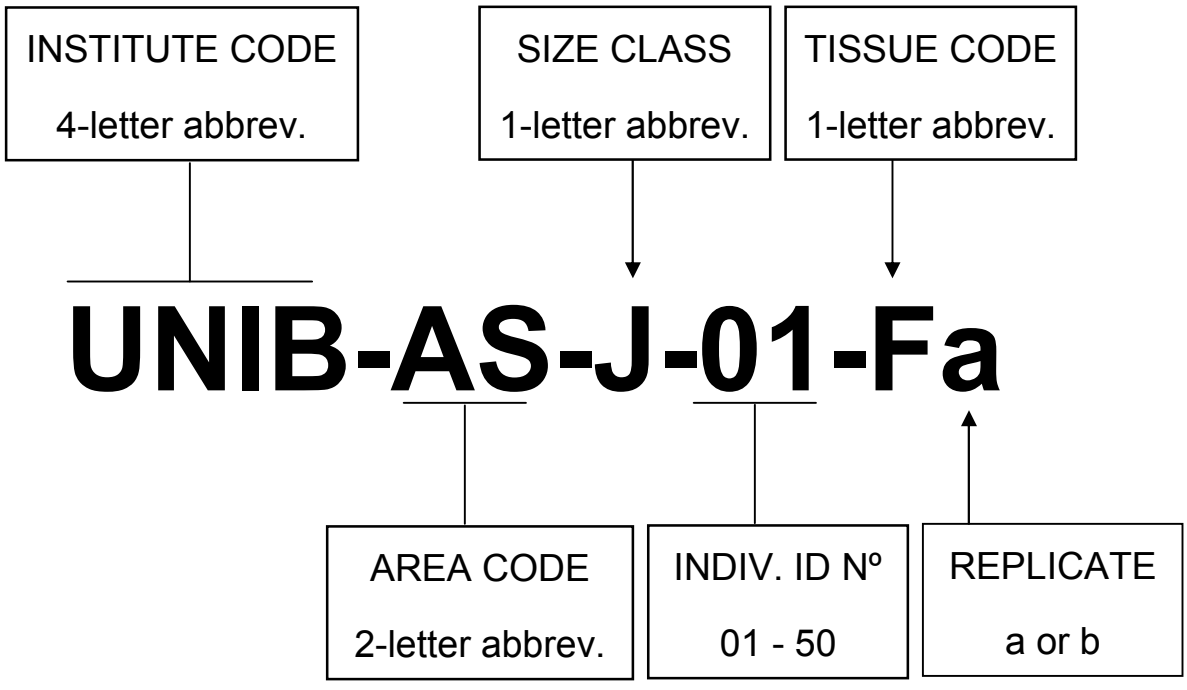
Volume of the muscle sample: 0.5 cm^3

Size of the fin clip: 1 cm^2



1. Labeling procedure and codes

We'll use the same code to label all samples. Sample labels must be visible on the tube. Please, refer to the sampling scheme given in the proposal and use the codes in Appendix 2. The following example shows how samples will be labelled.



The first 4 codes (e.g. "UNIB-AS-J-O1") have to be unique for each fish. The last code (e.g. "Fa") indicates the tissue type (O, S, G, F, or M) and replicate (a or b).

2. Preparation of the material before sampling

- Before sampling, prepare 100 5mL-tubes with screw cap with at least 2.5mL of Non-denatured Ethanol 96%; each microtube must be labeled with Sample ID according to the labeling code reported above (step 2). Label the microtubes with pens containing water-resistant ink, as well as including a waterproof paper inside the tube with the code written with pencil (as ethanol may dissolve the ink).
- Operator has to wear cleaned gloves.

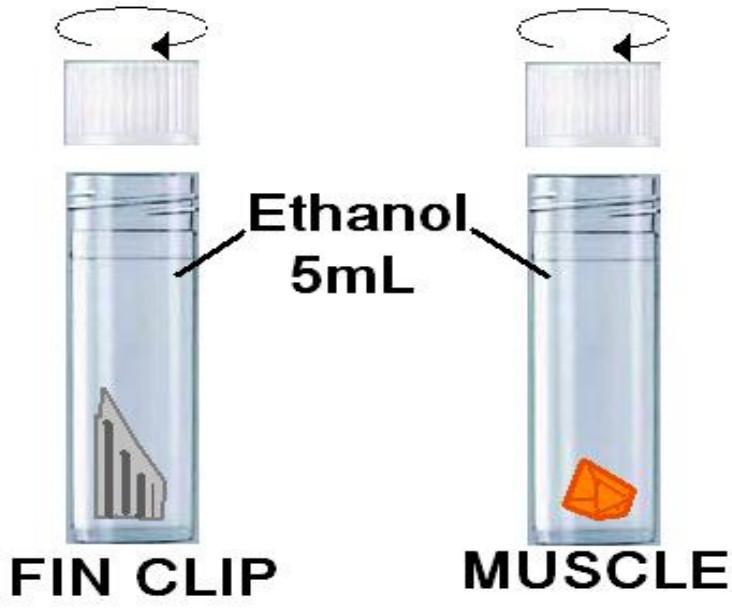
3. Sampling procedure

IMPORTANT: Sampling of tissue should be carried out twice from the same individual. Mark the replicates as "a" and "b". One replicate ("b") must be stored in your lab, while the other ("a") should be shipped to AZTI following the shipping instructions (see step 5).

- Cut a 0.5 cm^3 muscle sample or a 1 cm^2 fin clip from each individual. In the case of larvae, collect the entire larvae.
- Put the tissue clip into the ID labeled microtube with ethanol 96%. Ensure the tissue volume is no more than 10% of the liquid volume.
- Clean surgical instruments for each sampled animal with water or commercial ethanol and dry it with a new scrip each time.
- Store the microtube containing the tissue at -20°C. If it is not possible make sure that temperature does not exceed 4 ° C.

If your fish is already frozen:

- Take the sample from each frozen individual and put it directly into the microtube with ethanol 96%.
- The ice contained in the sample may dilute the ethanol. Therefore, change the ethanol after 1-2 days, in order to ensure proper sample preservation.



4. Fill sampling data form

The code of the sample must be clearly visible on the microtube.

- Fill the "GBYP2011data.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Nicolas Goñi (ngoni@azti.es) and Igaratza Fraile (ifraile@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the rack of microtubes with tissue samples in a storage box with the reference "GBYP 2011".
- Ship the box by express courier to:

Nicolas Goñi / Igaratza Fraile
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

Ship the samples by Express Courier mail. Deliver them as "Sea water samples".

Notes

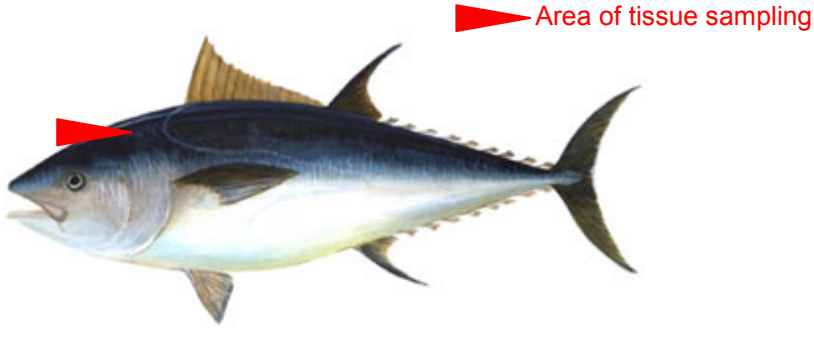
SARSTEDT website : http://www.sarstedt.com

SAMPLING PROTOCOL FOR OTOLITHS (O)

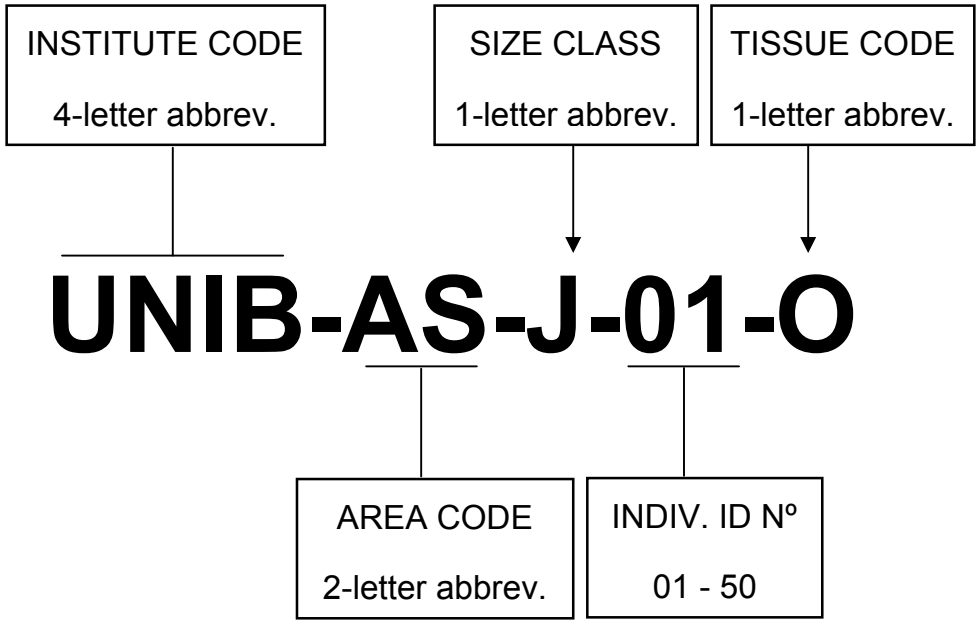
MATERIALS

Product	Catalog Code
Gloves (laboratory gloves)	
Knife, handsaw or circular saw	
Non-metallic forceps (plastic or ceramic)	
50 Microtubes 2mL with O-ring cap (SARSTEDT)	72.694.007
Storage boxes (SARSTEDT)	95.64.997

SUMMARY OF SAMPLING:
Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal)
Tissue type: otoliths.



1. Labeling procedure and codes
We will use the same labeling procedure used for genetic samples, the tissue code being here O (for otoliths). Labels must be visible on the microtube.



2. Sampling procedure

- The otoliths can be sampled once all other tissues are sampled. Change into clean gloves (disposable lab/examination gloves) for each fish. If tuna heads have been previously frozen, ensure that thawing is complete before starting the extraction. Extracting otoliths from partly frozen canals could break them.

- Cut into the fish head CAREFULLY to make certain that you don't break the otoliths. The easiest way is to cut the head with a large knife in the frontal plane above the supraorbital ridge (Fig.1a). At first attempts, it is better not to cut too close from the eyes, and to do successive small cuts then until the brain appears.

- **It is very important to work carefully because the otolith can easily be damaged at this stage!** CAREFULLY remove the brain (Fig.1b). Otoliths will be located at the back of the brain cavity, inside semi-circular canals (Fig.1c). Gently prospect into the canals. The posterior end of the otolith is the most fragile. Use small forceps to CAREFULLY extract the otolith from the bony capsules and GENTLY remove the membrane surrounding the otolith immediately after extraction (the membrane is harder to remove after it has dried).

Fig.1: Extraction of sagittal otoliths from a bluefin tuna head

- Clean the otolith with deionized water and store in plastic vials with the corresponding code.

3. Decontamination and storing procedure

Otoliths must be carefully cleaned prior to processing for microchemical analyses. Decontamination procedure suggested by Rooker et al. (2008) is recommended.

- Immerse the otoliths into deionized water to hydrate biological residues adhering to the otolith surface. Remove these residues using small forceps.

- Then, immerse the otoliths into 0.1% nitric acid for 5 min. to remove surface contamination.

- Clean the otoliths again with deionized water to remove the remaining nitric acid.

- Finally, dry otoliths in a fume hood during 24 hours and store them by pairs in plastic vials with their corresponding labels. Cleaning otoliths immediately after collection reduces the amount of time required to prepare them for sectioning.

4. Fill sampling data form

The code of the sample must be visible on the microtube.

- Fill the "GBYP2011data.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Nicolas Goñi (ngoni@azti.es) and Igaratza Fraile (ifraile@azti.es). The newly

labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the vials in a storage box with the reference “GBYP 2011”.
- Ship the box by Express Courier to:

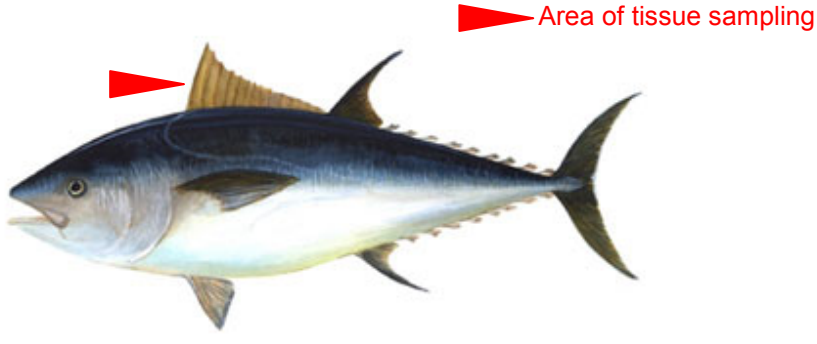
Nicolas Goñi / Igaratza Fraile
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

SAMPLING PROTOCOL FOR FIRST DORSAL FIN RAYS, SPINES (S)

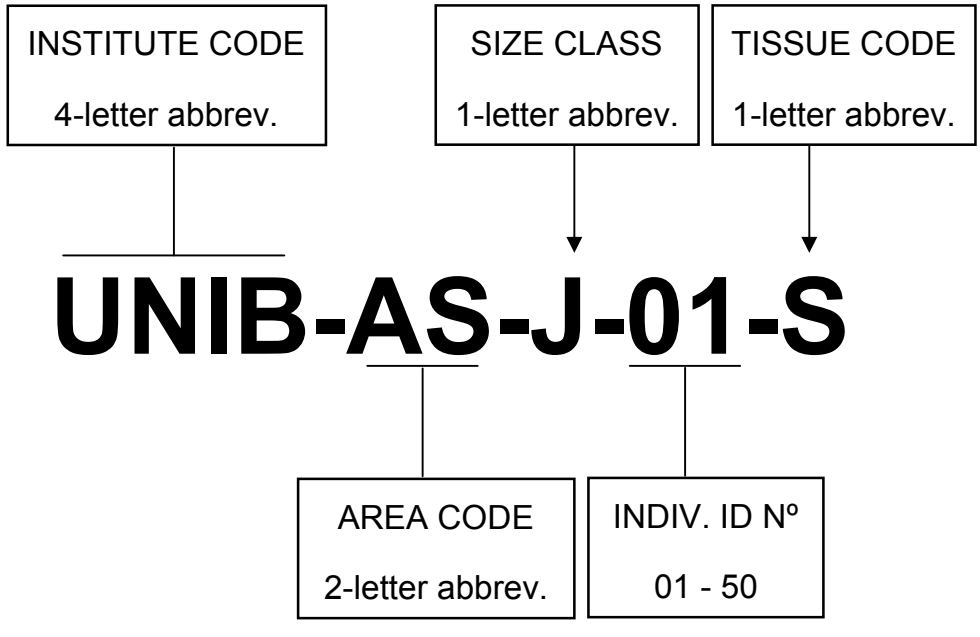
MATERIALS

Product	Catalog Code
Gloves (laboratory gloves)	
Knife, forceps	
Paper envelopes	

SUMMARY OF SAMPLING:
Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal).
Tissue type: first dorsal fin ray.



1. Labeling procedure and codes
We will use the same labeling procedure used for other tissues, the tissue code being “S” (for spines). The label must be visible on the envelope.



2. Sampling procedure

Spine extraction

The spine used for ageing purposes is the first ray of the first dorsal fin. It is important to extract a complete spine from the base including the condyle where the spine inserts in the fish avoiding any damage of the spine base as it is an essential part for ageing. It is not difficult to remove the dorsal fin first ray from juveniles but for larger specimens it is recommended to use a sharp knife or scalpel to cut carefully the strong ligaments that support the spine base deep in the fins insertion in the body depression.

Spread out the first dorsal fin and cut the membrane joining the two first dorsal rays by using a knife (Figure 2). Then, push the spine forward and down progressively (Figure 3B), then cut and turn it alternately to the right and to the left until the ligament breaks (Figure 3C). Finally, the spine must be twisted and pulled out (Figure 3D). Care should be taken in order not to twist the spine in its base.

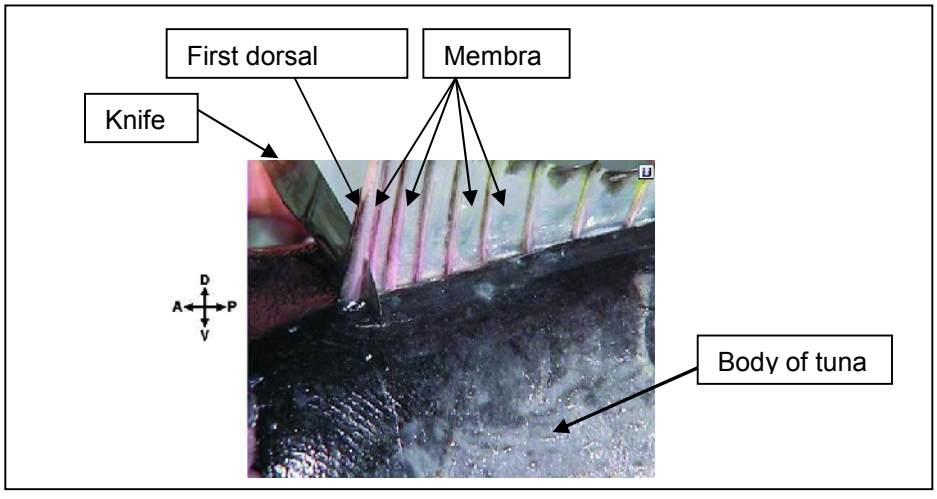


Figure 2. Insertion of the knife into the membrane separating the first two spines of the 1st dorsal fin (figure taken from Panfili *et al*., 2002).

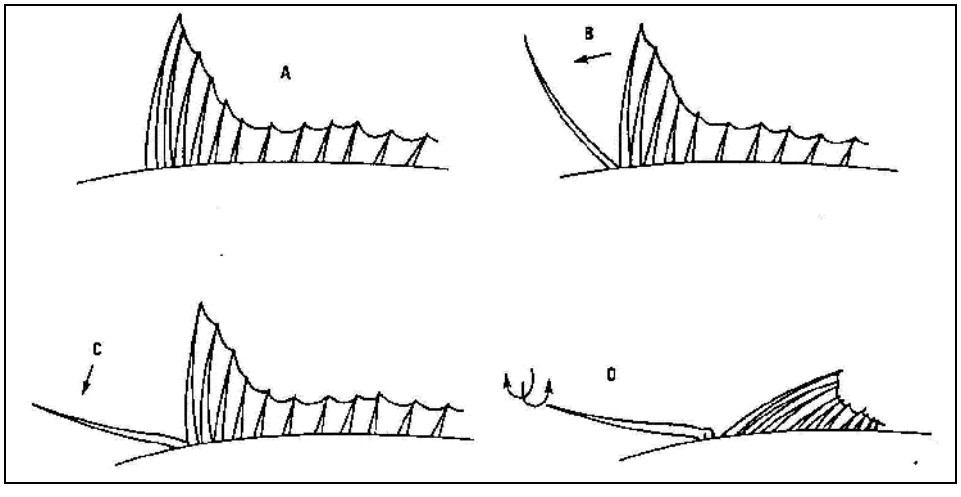


Figure 3. Technique of extraction of the first spine of the bluefin tuna dorsal fin. (Figures taken from Compeán-Jiménez, 1980).

3. Cleaning and storing procedure

It is recommended to carry out the whole cleaning step just after the spine has been extracted, as the connective tissue is still fresh and can be removed easily. It is recommended to remove carefully the remainder connective tissue with a scalpel and tweezers, avoiding causing any damage to the surface of the base of the spine. Moreover, the epidermis covering the spine must be also removed before carrying out the procedure of the spine preparation.

Before storing the sample, it is advisable to remove all tissue remains and dry the spine out on blotting paper. Spines are ideally stored dry in a paper envelope, which should be kept in a cool place (refrigerated) if they are not going to be cut immediately. NOTE: Do not use plastic bags for preserving them.

If the spine is too large to fit in the envelope, it can be cut in half and both pieces kept in the envelope, remembering that the piece forming the base of the spine is the most important since it is the part used for age interpretation.

4. Fill sampling data form

- Fill the “GBYP2011data.xls” file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Nicolas Goñi (ngoni@azti.es) and Igaratza Fraile (ifraile@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the envelopes in box with the reference “GBYP 2011”.
- Ship the box by Express Courier mail to:

Nicolas Goñi / Igaratza Fraile
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

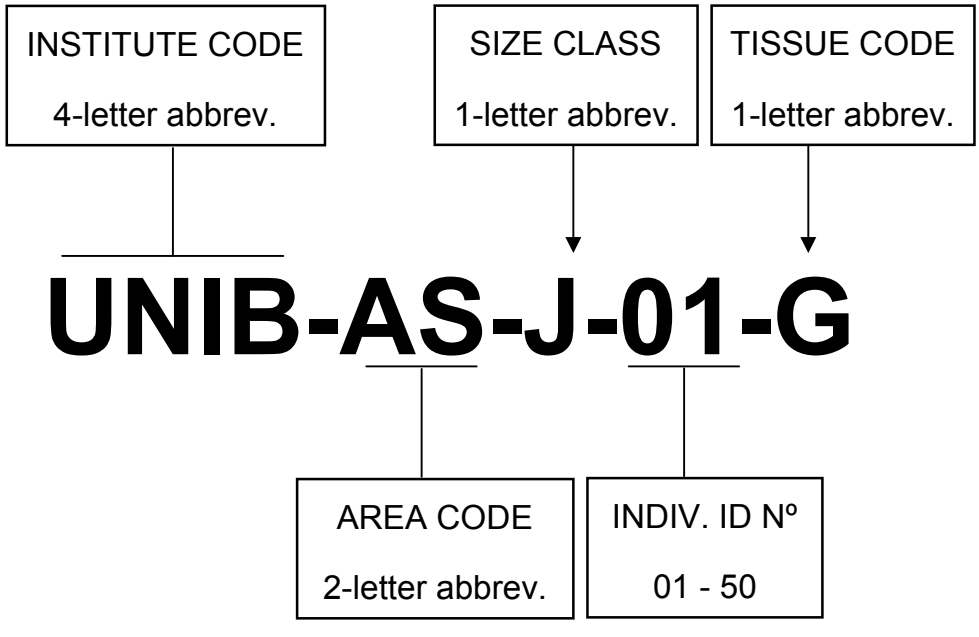
SAMPLING PROTOCOL FOR GONADS

MATERIALS

Product	Catalog Code
50 containers 70 mL assembled with yellow screw cap (SARSTEDT)	75.9922.744 (www.sarstedt.com)
Surgical scissors, tweezers, scalpel blades	
Fixator (Bouin, Hollande or 4% Formaldehyd)	
Gloves	
Parafilm	

SUMMARY OF SAMPLING:
Sample size: 50 individuals by region and size-class (medium and large individuals only, as defined in the sampling scheme given in the proposal).
Tissue type: gonad.
Size of the tissue sample: tissue sample length must be approximately half its width.

1. Labeling procedure and codes
We will use the same labeling as in previous procedures, the tissue code being "G" (for gonads). The label must be visible on the container.



2. Preparation of the material before sampling
- Bouin or Hollande fixator will be used for storing the gonad samples

- In case of not disposing of Bouin or Hollade fixator, a 4% formaldehyd solution will be used. To prepare 1L of 4% formaldehyd solution, use the following components:
  - → 9.4g of dibasic phosphate
  - → 4.7 g of monobasic phosphate
  - → 100 mL of 40% formaldehyd (usual concentration of commercial formaldehyd)
  - → non-salted water

Dilute the phosphate progressively in non-salted water, then add water up to 900 mL, then complete with 100 mL of 40% formaldehyde.

- Before sampling, each container must be labeled using formalin-resistant ink. For additional safety, we will also use submersible paper tags labelled with pencil, and put them into the containers.

- The operator has to wear cleaned gloves.

3. Sampling procedure

- Extract the gonads from the peritoneal cavity and weight them.

- Cut a slice from one of the gonads. The length of the slice must be approximately half of its width. Then cut a portion of 1/8 of the slice and weight it. This will be the gonad sample.

- Put the gonad sample into the labeled container, add fixator and cap the container.

- In case of using Bouin fixator, remove the fixator after 12 hours and fill the container with 70% ethanol.

- Check that the containers are properly closed and ensure their closure with parafilm.

- Clean surgical instruments for each sampled animal.

- Store the tubes at ambient temperature.

4. Fill sampling data form

- Fill the "GBYP2011data.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Nicolas Goñi (ngoni@azti.es) and Igaratza Fraile (ifraile@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the containers with tissue samples in a storage box.
- Ship the box to:

    Nicolas Goñi / Igaratza Fraile
    AZTI-Tecnalia Marine Research Division
    Herrera kaia portualdea z/g
    20110 PASAIA
    SPAIN

Ship the samples by Express Courier mail. Deliver them as "Sea water samples".

Filling the GBYP2011 SAMPLING DATA Template:

Below is an extract of the "GBYP2011data.xls" file:

INDIVIDUAL FISH CODE					TISSUE COLLECTED			
PARTNER CODE	AREA	FISHING GEAR	INDIVIDUAL ID NUMBER	SIZE CLASS CODE	TISSUE CODE1	TISSUE CODE2	TISSUE CODE3	TISSUE CODE4

SAMPLING DATA										
Date [dd/mm/yyyy]	Latitude	Longitude	Length [cm]	Length Type	Weight [Kg]	Weight Type	Sex	Reproductive Stage	Collector	Notes

And below are the alternative categories and codes that are needed to fill in the template:

Body measurement:

- Fork length (FL): this is the straight line from the end of the upper jaw (end of the snout) to the posterior of the shortest caudal ray (fork of the caudal fin) (Figure 1). This can be best measured using a **caliper** or alternatively with a tape measure, although it must be kept straight while measuring. The fish should be placed on a flat surface in a horizontal position. In the case of very large specimens in which this measurement is very difficult to make, one of these other lengths may be used:

- First dorsal length (LD1): this is the straight line from the end of the upper jaw (end of the snout) to the base of the first dorsal spine (the start of the first dorsal fin) (Figure 1).

- Curved fork length (CFL): this is the length from the upper jaw (end of the snout) to the fork by an imaginary longitudinal line, with the corresponding fish curvature (Figure 1).

- **Head length (LHead):** This is the straight line from the end of the upper jaw (end of the snout) to the posterior border of the operculum. (Figure 1).

The type of measurement being used must be clearly specified, as well as the measurement unit (e.g. cm). FL and CFL are measured to the lower centimeter (a specimen of 70,8 cm or 70,2 cm would correspond to the 70 cm range), LD1 is measured to the lower half centimeter (a specimen of 30,4 cm measures as 30 cm and one of 30,7 cm corresponds to 30,5 cm).

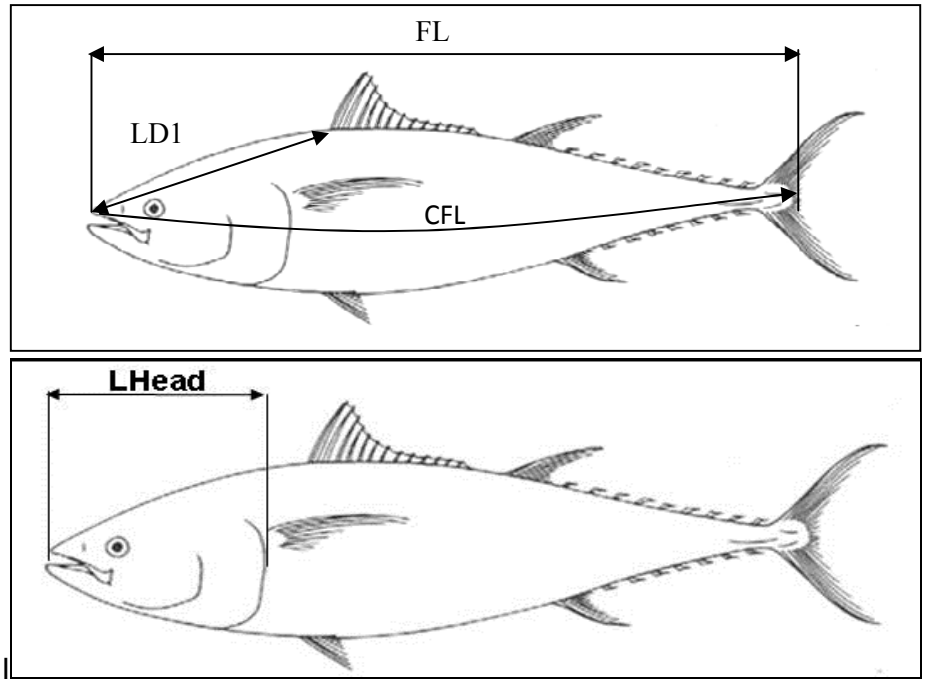


Figure 1. Types of measurements of bluefin tuna: Strait fork length (SFL), First dorsal length (LD1), Curved fork length (CFL) and head length (LHead).

Codes

Institute code	
AZTI	AZTI-Tecnalia
FMAP	Federation of Maltese Aquaculture Producers
HCMR	Hellenic Centre for Marine Research
IEO	Intituto Español de Oceanografía
IFRE	French Research Institute for Exploration of the Sea (IFREMER)
INHR	Institut National de la Reserche Halieutique
IPIM	Instituto de Investigação das Pescas e do Mar (IPIMAR)
ISTA	Istambul University
CYPR	Cyprus Marine Science Foundation
NECT	Necton Marine Research Society
NRIF	National Research Institute of Far Seas Fisheries (NRIFSF)
UNIB	University of Bologna
UNIC	University of Cagliari
UNIG	University of Genova
UCA	University of Cadiz

Area code	
LS	Levantine Sea: Turkish PS (medium-large) and larvae
EG	North Egypt coast (medium-large): PS if in activity in that area
CR	Crete (medium-large fish): Greek LL
SY	Gulf of Syrta (medium-large): French, Italian and Libyan PS
MA	Malta (medium-large): Maltese LL
SI	South of Sicily and Ionian Sea (medium-large): Italian PS and LL
AS	Adriatic Sea (small): Croatian and Italian PS
TU	Gulf of Gabes (small): Tunisian PS
BA	Balearic (medium-large): French and Spanish PS
TY	Tyrrhenian (medium-large): Italian PS and (small): Italian handline
SA	Sardinia (medium-large): Italian Trap
GL	Gulf of Lion (small): Spanish, French artisanal fleets, French sport
LI	Ligurian: Italian artisanal fleet
SS	Southern Spain (juveniles & medium size): Spanish LL
AL	North African coasts (medium size): Algerian PS
GI	Gibraltar (small, medium-large): Moroccan and Spanish HL, Portuguese & Spanish traps, Spanish BB
PO	Portuguese traps
BB	Bay of Biscay (small and medium): Spanish BB & French MWT
MO	Western coast of Africa (medium-large): Morrocan Trap
MC	Madeira – Canary Islands (medium-large): Portuguese & Spanish BB
CA	Central and North (medium-large): Japanese & Taiwanese LL
AZ	Azores (small-medium): Portuguese artisanal fishery

fishing gear	
PS	purse-seine
LL	longline
HL	handline
T	trap
BB	baitboat
MWT	midwater trawl
SU	survey (for larvae)

Size class		
V	Larvae	
0	Age 0	<=3 kg
J	Juveniles	>3 & <=25 kg
M	Medium	>25 & <=100 kg
L	Large	>100 kg

Tissue code	
O	otoliths
S	spines
G	gonad (maturity)
F	fin (genetic)
M	muscle (genetic)

Length Type	
FL	straight fork length
CFL	curved fork length
LHead	head length
LD1	first dorsal length

Weight Type	
T	total
GG	gilled/gutted

Maturation stage	
I (IMMATURE)	Small ovaries with no visible oocytes. Translucent pink color.
IM (INITIAL MATURATION)	Ovaries occupy 1/4 to 3/4 of the peritoneal cavity. Pale yellow color.
M (MATURE)	Ovaries occupy 3/4 of the peritoneal cavity. Visible vascularization. Yellow/orange color. Opaque and visible oocytes.
S (SPAWNING)	Ovaries in maximum developmental stage. Orange color. Very developped vascularization. Translucid ovocytes visible through the gonad surface.
R (RESTING)	Flaccid ovaries. Purple color. Occasionally visible translucid oocytes and opaque oocytes in advanced developmental stage, corresponding to the next spawning.
PS (POST SPAWNING)	Flaccid ovaries. Purple color. Gonadal wall thick and very vascularized (very visible capillaries). No ovocytes in advanced developmental stage.