# Census of Antarctic Marine Life (CAML)

**Sampling protocols for CAML barcoding studies**

Dr Bob Ward – Annie Lindgren – February 2006

Sampling protocols are quite widely applicable, and probably depend more on the size of the sampled animal than on its systematic affiliation. However, for convenience we have separated the following into invertebrate (concentrating on cephalopods) and vertebrate (fish) sections.

It is desirable to voucher any specimen intended to contribute its barcode towards a barcode library. This can be done after a tissue subsample has been removed for DNA analysis. A photograph of the specimen is also highly desirable. These steps will facilitate the resolution of any species identification queries that might arise post-barcoding. Subsequent to the establishment of a comprehensive barcode library, then future unknown specimens should be able to be identified to species by referencing their barcode to the barcode library.

For each specimen for the barcode library, the following should be recorded in a sample log: identification number, whether the specimen has been retained as a voucher, species (if known), geographic location, GPS location (latitude and longitude), depth (if known), collector name, identifier name (preferably with a score of taxonomic certainty for the specimen ranging from 1 (no doubt at all) to 5 (substantial uncertainty) – see Appendix), vessel name, date of collection and any incidental notes that might be of use later.

Whenever possible, five (or more, but five is a reasonable target) different individuals should be sampled from each species, preferably separated geographically so that a reasonable sampling of species level genetic diversity is obtained.

For tissue sampling, ensure that the scalpel blade and forceps are cleaned with ethanol between individuals, even individuals of the same species. It may also be necessary to clean the cutting surface with alcohol. This alcohol can be normal lab grade 70% ethanol.

Ensure that all sampling tools are free of formalin, as even small quantities can adversely affect the quality of the tissue.

Tissue may be stored frozen or in ethanol at room temperature or in a fridge. Storage ethanol is preferably 95-100% analytical grade. Good quality 70% ethanol would also be adequate, but low grade ethanol, contaminated with e.g. methanol, hinders DNA extraction.

After tissue sampling, the quicker the tissues get to the genetic processing lab the better.

**Invertebrates – Cephalopods**

1. Identify specimen to lowest taxonomic rank possible and place an ID tag with the specimen.
2. With clean tools, remove a section of tissue from the cephalopod, preferably from the mantle (away from nuchal or mantle cartilage sites) or from the gill. Gill tissue is often the best tissue to extract DNA, and is preferred. However, particularly in octopods and cirrates, gills can have important morphological information, so it is up to the discretion of the collector in such cases. Arm and fin tips are less preferred because there tends to be less DNA rich tissue in those sites.
3. The size of the tissue fragment will depend on the size of the animal, and the availability of tissue for collection. Ideally a fairly large fragment, e.g. 2 $cm^2$ should be retained and cut into 1-3 smaller chunks (to ensure better fixation). However, the small size of many specimens does not allow for such large pieces, so smaller pieces will suffice when necessary. Only a small sample of tissue is needed for a single DNA extraction, but it is always helpful to have enough material to re-extract DNA if necessary. Therefore, multiple samples should be taken if time and material allow. Place the tissue in a small cryovial or eppendorf tube (or other such screw-top container), usually with a total volume of between 1.5 and 3 mL. Screw top vials tend to be better than pop-top vials because they ensure a better seal. Pop-top vials have a tendency to open during shipping or transport, leading to contamination and mixing up of material.
4. Place the extracted tissue in 70-100% ethanol. 100% ethanol is best, but many ships travel with 70%, which is acceptable as well. There is some debate as to the ratio of ethanol to tissue, the best method for short-term storage is simply to fill the vial to the top with ethanol and then change the liquid after 24 hours to reduce overall water levels. There should be at least three times as much ethanol as tissue.
5. A tag should be placed in the vial (not just written on the outside of the container) in order to decrease the risk of mix-ups or contamination. All specimens from which DNA is collected should be retained, fixed in 10% formalin-seawater mix and vouchered at a museum or university (along with collection information). It is imperative that the animal is retained and correctly linked to the tissue sample in order to facilitate future research.

**Invertebrates – general**

Small invertebrates might have to be retained whole, either frozen in ziplock bags or stored in 70-100% ethanol in screw-top vials. With specimens large enough that they can be tissue sampled, it is best to avoid sampling digestive tissue.

**Vertebrates – fish**

1. Remove a cube of white muscle from the side of the fish, ideally from the same side of every specimen to ensure one side is blemish-free. We use the right side.
2. The muscle sample must be labelled and either stored frozen (-20C is OK) in a ziplock plastic bag or stored in ethanol in a 2-3ml vial (preferably in a fridge). In the former instance a piece of muscle about $1cm^3$ or larger would be ideal, in the latter two small samples would be good, each c. $5mm^3$. If possible, change the liquid after 24 hours to reduce water level.
3. Tubes can be labelled on the outside with pencil but a tag should also be placed in the vial. Waterproof paper can be inserted in the tube or in the plastic bag.
4. Any retained voucher specimens can be stored for the short-term frozen. For long-term storage specimens should be fixed in formalin (length of time dependant on specimen size) than transferred to low-grade 70% ethanol for long-term storage.

For further discussion of tissue collection guidelines, see http://research.amnh.org/amcc and/or http://clade.acnatsci.org/rosenberg/archiving/method/index.html

**Note added in proof**

Where frozen samples are used for barcoding, quarantine restrictions may apply. Check the national requirements and any international restrictions. For example in Australia, the transfer of frozen samples between Quarantine Approved Premises requires a permit from the Australian Quarantine Inspection Service; the default level is Quarantine Containment Level 2.

For further information, see http://www.daff.gov.au

**Appendix**. Example of a ranking scheme for certainty of identification. This is the scheme used by the Australian National Fish Collection housed at the CSIRO Marine Laboratories in Hobart, Tasmania.

IDENTIFICATION LEVELS

As of July 1993, specimens in the CSIRO Fish Collection will be identified to one of five levels of reliability depending on the taxonomic expertise of the identifier involved and their intentions. A general definition of these levels follows:

Level 1: Highly reliable identification — Specimen identified by (a) an internationally recognised authority of the group, or (b) a specialist that is presently studying or has reviewed the group in the Australian region.

Level 2: Identification made with high degree of confidence at all levels — Specimen identified by a trained identifier who had prior knowledge of the group in the Australian region or used available literature to identify the specimen.

Level 3: Identification made with high confidence to genus but less so to species — Specimen identified by (a) a trained identifier who was confident of its generic placement but did not substantiate their species identification using the literature, or (b) a trained identifier who used the literature but still could not make a positive identification to species, or (c) an untrained identifier who used most of the available literature to make the identification.

Level 4: Identification made with limited confidence — Specimen identified by (a) a trained identifier who was confident of its family placement but unsure of generic or species identifications (no literature used apart from illustrations), or (b) an untrained identifier who had/used limited literature to make the identification.

Level 5: Identification superficial — Specimen identified by (a) a trained identifier who is uncertain of the family placement of the species (cataloguing identification only), (b) an untrained identifier using, at best, figures in a guide, or (c) where the status & expertise of the identifier is unknown.