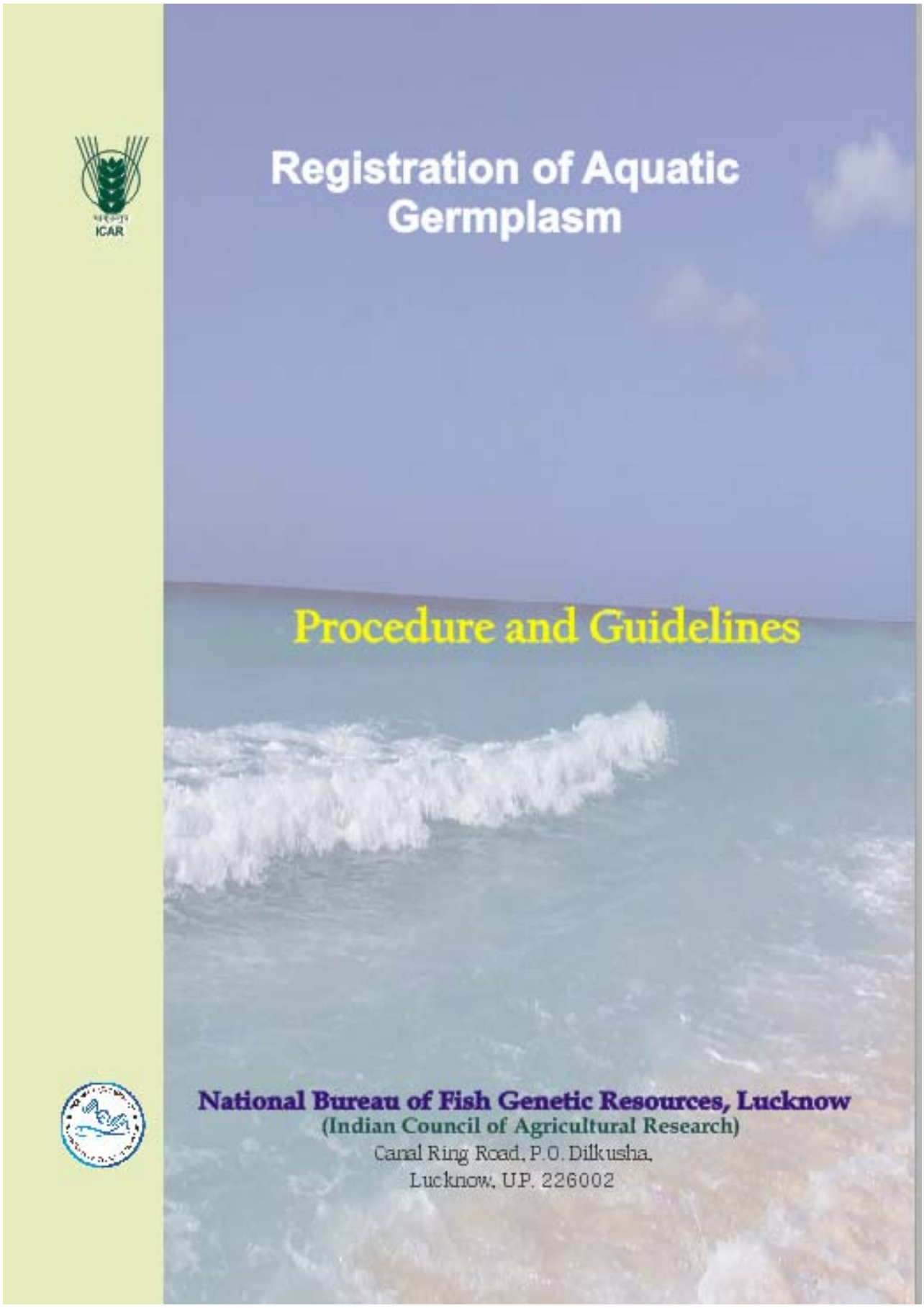
ICAR
Registration of Aquatic Germplasm
Procedure and Guidelines
National Bureau of Fish Genetic Resources, Lucknow
(Indian Council of Agricultural Research)
Canal Ring Road, P.O. Dilkusha,
Lucknow, U.P. 226002

CONTENTS

Background Information

India has rich aquatic biodiversity spread across different ecosystems. Of the 27,800 fish species reported, about 11% are found in Indian waters. Apart from finfish resources, nearly 2934 species of crustaceans, 5000 species of mollusks and 765 species of echinoderms also contribute to India's rich aquatic germplasm resources. India is the fourth largest producer of fish in the world and second largest producer of Inland fish. The total fish production is around 6 million tonnes. The fisheries sector provides employment to seven million fishermen and its share in GDP is around 1.4%. Majority of aquaculture production is supported by 3 species of Indian Major Carps and 1 species of shrimp.

Natural aquatic germplasm resources are important as majority of the genetic resources for food still come form the wild due to low domestication level in fisheries sector. In other words, capture fishery is equally important as cultivation for nutritional security across the globe. This is in contrast to the animal farming and agriculture where domesticated varieties only contribute to food basket. Therefore, management of fisheries resources draw parallel to that followed in wild life and forestry besides the agriculture. While it is true that certain aspects of biodiversity and genetic resources policy can apply equally to plants or animal or fish, however, significant differences need to be taken into account as well. Besides a source of food, aquatic germplasm resources are also an important source of various products of commercial value and to sustain other related trades like ornamental fishes. The challenge is to secure the IPRs related to aquatic germplasm so that the country is able to maintain its stake on its natural wealth and their potential benefits. There is urgent need to develop repositories of genetic resources that store the registered germplasm accessions. The repositories will also store the accessions of genetic stocks discovered/ varieties developed. Such repositories will maintain accessions for future use to retrieve information as well as whole or part of genome, if the species is not found in nature. The documented information with the registered can also serve as means to protect the traditional knowledge. To harness the potential of biotechnological innovations, it will be essential that DNA, whole and modified such as gene constructs is also part of the repositories. Besides, protecting IPR, these will also provide material for future research.

The species level accessions can be secured through building DNA and tissue Bank as a fast mode to store the material for long term. This can be used to retrieve the genetic information and for genetic manipulation in future with technological advancement. Sperm / cells and live gene bank accessions can be made only for prioritized and selected fish species. In the animals, the strains/ breeds of natural populations have been defined besides the developed varieties. Similarly, in fisheries there is need to identify genetic stocks / races in wild populations of cultivable fish species of India through concentrated effort using molecular markers, morphological and production traits. Some work done in the past at NBFGR, indicate that there different genetic stocks of fish exist in their natural population. Under XI plan, NBFGR will propose a Network Programme to intensify the effort.

The Present draft document is proposed to define type of accessions and set the procedures for registration of fish germplasm accessions. The draft also provide outline of the protocol, to be provided to applicants for use in collection of tissue / voucher accession.

Part A

1. Type of Accessions

	1.	2.
S. No.	**Type of Accession**	**Specific Mode of Accession Submission**
1	Species	Voucher specimen Tissue Samples DNA Isolate
2	Genetic Stock/ Elite/ adapted Germplasm	Cryopreserved sperm; Cell and Cell Lines; Live Animal
3	Developed Variety	Cryopreserved sperm; Cell and Cell Lines; Live Animal
4	Modified DNA Material	DNA material
5	Live Fish	Cryopreserved sperm; Cell and Cell Lines; Live Animal
6	Fish Samples under Germplasm Exchange	Voucher specimen Tissue Samples DNA Isolate

Under all the types of Accessions:

Voucher specimen and Tissue Samples will be essentially submitted along with photograph and other details/documents required as per FORM 1A. As far as possible, Total DNA isolate can be provided, however, if the submitting institute does not have facility, repository will make arrangement.

For Identified Genetic stocks/Strains from natural populations, proven elite germplasm or improved variety from domesticated stocks of aquaculture species:
In addition to Voucher specimen, Tissue Samples & DNA isolates Specific modes of accession given above in the table will be used.

Modified DNA Material :

Voucher specimens/Tissue/Total DNA isolates of the fish which is source of gene will be provided along with the DNA material submitted as accession.

For every species, at least there should be 3 samples / isolates for which corresponding voucher specimens is also submitted. Ten samples for each species from one location for species level identification/accessions.

The germplasm under exchange for farming; at least tissue samples of 50 specimens or DNA isolates will be deposited with 3 voucher specimens. If the germplasm is collected location wise, the sample size should be considered as per location.

2. Descriptors:

a. Available Taxonomic keys to be used as descriptors of species.

b. Biological traits reported in the Standard Ref. to be used for comparison of the accessions under registration.

Day, F., F.L.S. and F.Z.S., 1958 Fishes of India, Volumes 1-2. William Dawson and Sons Ltd., London.

Talwar, P.K. and A.G. Jhingran, 1991 Inland fishes of India and adjacent countries. vol 1. A.A. Balkema, Rotterdam. 541 p.

Kapoor, D., R. Dayal and A.G. Ponniah, 2002 Fish biodiversity of India. National Bureau of Fish Genetic Resources Lucknow, India.775 p.

FAO Species identification sheets

c. Karyotype Profile and species discriminating DNA markers profile as and wherever or whenever is known.

Accession codes:

Two types of accession codes will be applied as identifier for each accession.

Primary code:

Field code at the time of collection.

Alpha-numeric

Species to be identified as with predefined abbreviation.

Secondary code : This accession Code will be given at repository

Fish species code:

Fish Biodiversity Database of NBFGR is the basis for the purpose.

5 digit numeric code: Species Specific

The accession Code

Species specific code followed by (-); alphabetic code of river system / basin (-) river- Sl. No. of accession.

Multiple accessions within river will be distinguished by Sl. no. of accession

Example

Farraka

34323	-	GN	-	HG	001

34323	-	GN	-	HG	010

Howarah

34323	-	GN	-	HG	011

34323	-	GN	-	HG	020

The accession codes given above will read the following information

Out of total 20 accessions of *Labeo rohita* (34323) collection from Hooghly (HG) river which is part of Ganga river system (GN) with serial no 1to 10 are collections from **Farraka** and 11-20 collections from **Howrah**.

The river systems and main tributaries will need to be coded in alphabetical abbreviation (2-3 digits)

c. In case of improved variety, the alphabetic code for river will be replaced by the variety name code (e.g. Jayanti: **JY)**

d. For genetic stocks, besides species descriptors, stock descriptor based on individual genotype data using molecular markers or morphological/ Biological descriptors for stock/ variety (Wherever available) will be used. To discriminate stock, atleast at one locus in genotype data or haplotypes or with respect to one trait, significant differences must be proven.

e. Tissue, DNA isolates and voucher specimen of each sample will be identified by the same accession code

f. The Accession code will be generated as line barcodes for physical labeling for reading through scanner.

4. Formats for providing Information along with accessions

Form IA Species information, With Tissue/ DNA Accessions, Universal, with all submissions.

Additional Forms (To be developed)

Form IB Cryopreserved Sperms/ Cells /Cell Lines

Form 1C Modifed DNA

Form 1D Live Accession

Form 1E Varieties /Strains/ Genetic Stocks

Form 1F If Accessions are part of sample under Germplasm Exchange

5. Management of information

Custom developed software based database of accession will be developed to manage the information.

In future, the database can be linked in public domain accessible through login information and password?

The database will be expanded in future to incorporate

- Links to other databases.
- Incorporate species specific DNA profile as and when available.

6. Dissemination of Information in Public Domain

1 **E- Repository** link at institute site that provide updated information. Online submission of information can also be worked out.

2 Publication: Different possible options

(a) Initiate Journal on Aquatic Genetic Resources.

(b) NBFGR Newsletter can provide a regular column to publish the repository information.

(c) Initiate a Journal on Germplasm Resources that provide information of accession from all sources like plants, microbes, Animal, Fish etc. besides publishing original articles on related topics. The scope of Journal can be wide, which may include besides AgroBiodiversity, other areas such as Forestry, Wild Life etc. also

The information published in such Journals will be in a specified format.

7. Expansion of Collection Base of Accessions at Species Level

(a) Wide publicity to invite researchers / NGO's / institutes / colleges who can volunteer to contribute to collections. To facilitate, detachable

registration form will be attached to Protocol book (like a prospectus of the school).

(b) Such contributors can register with the information as likely available species and the area. After registration the Contributors are provided start up kit, including protocol book or even techniques can be demonstrated.

(c) Regional level researchers can be identified who can act as centers for providing the material, inputs to collections / center for collection of samples before shifting to main repository.

(d) Database carry the name of collector against the accession, any sequence submitted in gene bank has the name as coauthor, any publication arises carry due acknowledgement to such collectors.

II. Registration of Genetic Stocks and Improved Strains

a. Genetic Stocks from wild populations

i The germplasm accessions discovered as distinct genetic stocks will be registered upon request of the discoverer and submission accompanied with relevant documentary evidences and data.

The genetic stock is a group of individuals reproductively isolated from other group of conspecific individuals.

ii. The distinct genetic stock will be considered for registration if there is sufficient evidence that allele/haplotype frequencies significantly differ from another neighboring subpopulation atleast at one locus.

iii. The genetic stock thus considered distinct must be submitted with standard morpho-meristic data and production traits also, if claimed for the production value.

iv. The genetic stocks differences based on only morpho-meristic characteristics will also be considered, if data is supported with sufficient sample size (>50) per location and done through use of standard methodologies & parameters.

v. The appropriate Performa, to derive information from applicant for registration of genetic stock will be developed as Forrm 1B. Form 1C will be used for production traits, if some genetic stock is claimed for superior production value wrt trait.

vi. The desired information will include sample size (>50) per location; only codominant & mtDNA markers will be allowed; no. of markers (loci) used; Results to prove that pairs of loci did not suffer from linkage disequilibrium and are neutral; results to prove that the allele frequencies at locus that differ from the nearest neighbors examined with same set or parameters and other details of analysis.

vii. Both Marker data and morpho – meristic characters will be used as descriptors.

b. Improved strains developed for Aquaculture.

i. Fish improved strains will be a group of fish of common origin and of one species, similar in genetically determined economic and biological properties and morphological characteristics, demanding similar requirement as to natural and production conditions and capable of reproduction.

ii. Commercial exploitation of varieties will be for culture purpose for food or ornamental trade developed through selective breeding/hybridization.

iii. Under no circumstances, these will be meant for releasing in open waters.

iv. Appropriate performa to derive the information from applicant will be developed so as to precisely conclude for verification of the claim.

v. The “applicant” shall mean a breeder who is entitled to file an application for the protection of a improved strains, the applicant shall be entitled file such an application only if the variety or breed has been discovered, developed or created by his own breeding research, in his own name and on his own account. An applicant may be represented by several breeders provided that the variety or the breed has been discovered, developed or created by their material support.

vi. Each improved strains should be labeled by a name which is its general name

vii. The applicant shall be granted, on the basis of his application, a Registration

Certificate in respect of variety or a breed as framed in definitions

viii. Registration Certificate shall certify:

(a) The creation of improved strains;
(b) The name of the improved strains, with an indication of the species (genus);
(c) The holder of the Registration Certificate by stating the improved strains trade name , only for Indian citizens, farmers or companies registered in India.
(d) The dates of commencement and termination of the protection of the rights in respect of the variety

ix. CONDITIONS FOR THE GRANTING BREEDER'S CERTIFICATE

The conditions for the granting of the Breed's Certificate shall comply if the germplasm is

(a) The germplasm shall be deemed distinct if it clearly differs in one or more assessment characteristics from any other breed whose existence is a matter of common knowledge at the date of priority. It should be Distinct by way of at least one major trait or property form any other breed that is commonly known of upon the date of the filing of the application.
(b) Uniform to a level that is adequate to the biological properties of the species concerned.
(c) Stable in its major traits, while respecting the peculiarities ensuing from the environment in which the animals are bred.
(d) New
(e) Sufficiently large in number for reproduction
(f) The animal breed shall be deemed reproducible if its assessment characteristics remain unchanged through several generations

(g) The denomination must, at the date of priority, enable the animal breed to be identified

(h) should not be liable to mislead, it must be different from the denomination of an

existing breed of the same or a closely related fish species and its use must not be contrary to public policy or morality.

The created or discovered and developed, germplasm, lines or hybrids shall be liable for registration

Part B

PROTOCOLS FOR

COLLECTION OF

GERMPLASM ACCESSIONS

CONTENTS

Background information

Foundation of precise genetic analysis is laid from the time of sampling itself. When the target species for the study are wild populations, the researcher has to be extra careful about the source of the fish. There can be two commonly encountered points of apprehension:

1. The specimens (commercial species) are not mixed with that from farmed stocks. This may not be problem for the species not grown in culture.

2. The fish for sale may not be actual from same location but could be transported from other sources nearly or even distant. e.g. Large size catla from Punjab (Indus river) are sent to Guwahati for fetching higher price. Such situation will give false picture after analysis.

The following tips can help:

a) Repeated cross-questioning to more than one fisherman / auctioneers taking care not to annoy them.
b) Reach the actual sites at the time of fishing, even in the odd hours.
c) The heap of fishes with heterogenous size of target fish or with heterogenous compositions with non-cultured species is more likely to be from wild than otherwise.
d) Get the samples from landing centre close to fishing site, where fishermen bring the catch directly for first auction.
e) Normally, traders who buy the catch, they may sell directly or pool the fish from other sources to send them to larger markets in cities. Hence the markets especially in cities deserve to be avoided.

In case of ambiguity about source, avoid taking sample.

For successful sampling of wild stocks, researcher is dependent on fishermen. In our experience, fishermen are very cooperative, when approached with consideration for their sensitivities. Hence, while dealing with fishermen is patient and persuasive, win confidence, compensate for extra efforts he is taking for you and make your sampling programme as per his routine fishing schedule rather than imposing your convenience.

The later is very important as fishing schedule is as per market compulsions which if not met will leave unsold fish and loss to fisherman.

After source of specimen is decided, decision need to be made whether it is fit for sampling. A live or just dead fish can be an optimum specimen to collect samples. However, unfortunately in larger water bodies, this is not always possible. Reservoirs and river fishing is done through gill nets and fish size is also normally large. Most of the non-air breathing fish species will reach dead at the time of landing. Decision of whether the specimen is worth sampling or not, is very crucial. Besides conventional checks like redness of gills etc., first try to draw blood. If blood is available, it may be worth to sample for other tissues too. In our experience, blood could be drawn from caudal vein from the fish dead for approx. 5-6 hrs. And such samples have yielded fairly good results for DNA as well as allozyme genotyping.

The samples required for population differentiation are collected from different geographical locations at far off places, where repeated visits are not possible. So all types of tissues required, i.e. Blood, Muscle, Gill, Fin Clip are collected simultaneously from the same fish at each location to make it cost as well as labour effective.

Materials required for collection of blood, gill and liver

1. **Camera** for taking the fish photographs**.**

2. **A weighing balance** (small).

3. **Scissors and scalpels with blades** for dissection of fish**.**

4. **1.5 ml tubes** (a) with 1.25 ml 95% ethanol and sealed with parafilm to avoid evaporation of ethanol.
   (b) with micropore tape for labeling, on which code number to be written with ball pen.

5. **Parafilm** (for sealing the tubes) after collection of sample.

6. **Syringes and needles**
   (USE FRESH SYRINGE AND NEEDLE FOR EVERY FISH)

   Disposable syringe of 1 or 2 ml

   Size of the needle depends on species to be sampled, according to average size of fish.
   Needle for small fish No. 22, 1 inch.
   for big fish No. 20, 1.5 inch.

7. **Protocol** for collection of Blood, Gill and Fin Clip.

8. **Parafilm** (for sealing the tubes) after collection of sample.

9. **Pieces of aluminium foils** for labeling of fish/ **Pieces of white cotton cloth** for labeling of fish

10. **Methanol / Formalin** to store the fish.

11. **Worksheets** to enter the observations and data.

12. Ball pens/ Water resistant marker pen/ Lead Pencil.

No. of fish to be collected per riverine location : 10 for testing
(As much possible in case of highly endangered fish)

Source of Material

Item	**Manufacturing Company**
Eppendrof Tubes(1.5ml)	Tarson
Cryobox	Tarson
Syringes/ Hypodermal needles	Dispovan or any other
Parafilm	S.D. Fine Chemicals /Sigma

1. Please Note that Tubes should be autoclaved, even pressure cooker can be used for the purpose. Prior to autoclaving tubes should be wrapped in aluminum foil. Do not touch inner side of cap and rim of tube with hand.

2. In case if any center find it difficult to get these material, please inform NBFGR along with name and address of reliable courier service operating in your area, preferably a national courier. The boxes with autoclaved and filled tubes with kit will be sent from NBFGR.

Collection of Tissue Accessions

STEPS

Identify the fish on the basis of taxonomic characters and simultaneously fill the work sheet (list I) for every fish collected for all the parameters given in it. .

Take out the blood with syringe and needle and store in 95% ethanol (protocol given below).

3. Weigh the fish and note down in the work sheet for this purpose.

4. Take a photograph of the fish by placing the code number along with the fish, so that it can be identified easily on a later date.

Protocol for collection of blood

USE FRESH SYRINGE AND NEEDLE FOR EVERY FISH

Fill the 1.5 ml tubes with 1.25 ml 95% ethanol.

Rinse the syringe and the needle with heparin from vial by taking heparin in and out of the syringe and needle, back into the vial.

Collect the blood from individual fish with heparinized syringe (1 or 2 ml) and needle from caudal vein.

Remove the needle from syringe with blood.

Put 0.25 ml of blood, upto the mark of 1.5 ml on the tube, from syringe directly into tube with 95% ethanol .

Close the cap of the tube and immediately mix by shaking vigorously (avoid clotting of blood).

Seal the tubes with parafilm.
(Cut the parafilm into small strips and separate the two layers (paper and parafilm). Seal the tube with parafilm strip by placing along the tube cap and stretch parafilm while rotating the tube. Then press the stretched parafilm along the tube cap).

Label the tube with fish number with ball pen.

Keep the tube with blood in the box, serially.

Write with **ball pen** the name of species and the centre on the box , where tape has been put for the purpose.

Protocol for collection of Muscle/Gill / Fin Clip

USE CLEAN FORCEPS AND SURGICAL BLADE FOR EVERY FISH

a. Muscle

1. Use no. 11 (vary according to the size of fish) scalpel blade held on shaft.
2. Make incisions (not more than 5-6 mm deep).
3. Remove skin flap.
4. Cut small white muscle piece using surgical blade or small fine scissors.

b. **Gill**

1. Cut a piece of gill, approx. 5-10 filaments, and put it in 95% ethanol in 1.5 ml

Eppendorf tubes.

2. Dirt and any visible parasites should be removed from tissues as these can affect genetic analyses.

c. Fin Clip

1. Collect a fin clip (pectoral, pelvic, adipose or caudal) from each fish being sampled. Take a fin clip using a clean knife, scalpel, or scissors, cleanly remove a whole or partial fin from the fish being sampled.

2. The adipose fin in some fishes present can also be used as sample. Do not take more than one square centimeter of fin tissue.

3. Dirt and any visible parasites should be removed from tissues as these can affect genetic analyses.

Fixing and storage of tissues.

1. Put the tissue in 95% ethanol (1ml) in Eppendroff tube.

2. Seal the tubes with parafilm.
   (Cut the parafilm into small strips and separate the two layers (paper and parafilm). Seal the tube with parafilm strip by placing along the tube cap and stretch parafilm while rotating the tube. Then press the stretched parafilm along the tube cap).

3. Alternately, Cryovials with screw caps and ‘O” ring can be effective method of storage without necessity for parafilm sealing.

4. Label the tube with fish number with ball pen.

5. Keep the tube with tissues in a cryobox serially.

6. Write with **ball pen** the name of species and the centre on the box , where tape
has been put for the purpose.

7. Store in refrigerator, till to be sent to Repository.

Wherever refrigerator is not available especially under field conditions, it is possible to hold these sealed tubes at room temperature till these are brought to laboratory. Exposure to heat and sunlight should be avoided.

Primary (Field) Code number of individual fish

Primary code:

- Field code at the time of collection.
- Alpha-numeric
- Species to be identified as with predefined abbreviation

Example

For *Labeo dyocheilus*, collected by Garhwal University, Srinagar is

LD08001

where LD = ***L****abeo* ***dyocheilus***

08 = Code No.of centre of Garhwal University, Srinagar

001 = 1st fish collected from 1st site

Code no. of fishes from 1st site 1 - 10

2nd site 10 -20

3rd site 20 -30

and so on

Even if number of fishes sampled from 1st site is less than 10, code no. of fish sampled from 2nd site has to start from 10, and so on.

The collaborators can send the list of species likely to be collected to NBFGR, to obtain the alphabetic codes and serial no.for collection from the particular river.

1. Clean scissors and forceps after dissection of each fish, to avoid contamination between the fishes.

2. Before putting liver in the tube, make sure that sufficient (approximately 1 ml) 95% ethanol is present in the tube .

3. Assign the code for each fish according to the example cited above.

4. Label the tubes and the boxes at the appropriate places **with ball pen only**.

5. Fill the work sheet for every fish collected for all the parameters given in it.

VOUCHER SPECIMEN

1. Minimum of three fish of each species to be preserved for depositing long with tissue samples.

2. In case species exhibit sexual dimorphism, two each of male and female to be preserved.

3. For identification, make a folded aluminum foil with label and insert in the mouth deep, so that it does not come out.

4. The specimens can be preserved in formalin solution in plastic jars or of appropriate size.

5. Different species can be put in one jar and jar should be numbered and name of centre pasted on it.

6. The following information to be sent along with samples

Taxonomic information for each species either of the

- Identification sheet (photocopy)
- Reference of author or book

c. Good photograph of the fish taken on appropriate background

Material and Documents to be submitted to NBFGR, Lucknow

1. **Boxes with tubes filled with fixed blood, liver, gill and fin clip.**

2. **Passport information of the species to be submitted with the accessions.**

FORM A (I to V) Download

3. Form for submission of Accessions.

FORM B (B1 & B2) Download

4. Specimen preserved in formalin along with the list.

ANY DOUBTS CAN BE CLARIFIED FROM

Director

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