AQUA EXCEL 2020

AQUAculture infrastructures for EXCELlence in European fish research towards 2020 — AQUAEXCEL2020

D3.3: Booklet of cryopreservation procedures for the cryobanked species

Catherine Labbé. INRA

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 652831. This publication reflects only the view of the author, and the European Commission cannot be held responsible for any use which may be made of the information contained therein

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Executive Summary

The deliverable is part of Task 3.3, which aims to improve cryobanking practices within the consortium to better secure the genetic resources processed by the research facilities. One way to achieve this is to make available to the research community in AQUAEXCEL2020 all cryopreservation procedures already used by the various consortium partners.

Objectives: The objective of this deliverable is to produce a booklet of cryopreservation procedures commonly used in AQUAEXCEL2020 research facilities. These procedures will be freely accessible to any partner wishing to cryopreserve germinal material for the preservation of genetic resources. The name and contact of the author are included in each procedure, so that this person can easily be contacted to provide information and advice.

Rationale: A list of all cryopreserved material was established through a survey addressed to all consortium partners. From this list, a selection of procedures addressing a wide range of fish species (marine and fresh water) was established. The owners of the cryobanked collections were given a standard template and instructions to provide the procedures for each species they handled. The items in the template had been approved beforehand by the task partners during the second AQUAEXCEL2020 annual meeting. Almost all procedures were written by one actor and validated by a second actor.

The task leader collected the procedures, harmonized the content and information, and proposed the final version to the authors.

Procedures from some partners (UoS, NAIK, IEO) could not be obtained because the persons responsible retired or changed job and could not provide the procedures on time, or the procedure was no longer available from the partner and will have to be established from publications. However, this will be an open booklet and it is expected that by the end of the project, more procedures will be added, e.g., for Northern whitefish, Great Maraena and Atlantic halibut.

At present, the procedures are merged into a Portable Document Format (pdf) file. By the end of the project, they will be stored in the Central data repository of the consortium (D3.9, M58).

Main Results:

Sperm cryopreservation procedures are available for:

- Sterlet sturgeon (*Acipenser ruthenus*)
- Portuguese oyster (*Crassostrea angulata*)
- Common carp (*Cyprinus carpio*)
- Zebrafish (*Danio rerio*)
- Sea bass (*Dicentrarchus labrax*)
- Dusky grouper (*Epinephelus marginatus*)
- Rainbow trout (*Oncorhynchus mykiss)*
- Tilapia (*Oreochromis niloticus*)
- Brown trout (*Salmo trutta*)
- European catfish (*Silurus glanis*)
- Senegalese sole (*Solea senegalensis*)
- Gilthead seabream (*Sparus aurata*)
- Tench (*Tinca tinca*)
-

Germinal stem cell cryopreservation procedure are available for:

- Rainbow trout (*Oncorhynchus mykiss)*

Authors/Teams involved:

Procedure writing:
Yevhen HOROKHOVATSKYI and Marek RODINA: JU
Ana Luisa SANTOS and Elsa CABRITA: CCMAR
Catherine LABBE, Lionel GOARDON, Alexandra DEPINCE: INRA
Alain VERGNET: IFREMER

Sperm cryopreservation procedures (see Annex 2)

1. STERLET (ACIPENSER RUTHENUS) SPERM CRYOPRESERVATION
2. PORTUGUESE OYSTER (Crassostrea angulata) SPERM CRYOPRESERVATION
3. COMMON CARP (CYPRINUS CARPIO) SPERM CRYOPRESERVATION
4. ZEBRAFISH (Danio rerio) SPERM CRYOPRESERVATION
5. EUROPEAN SEABASS (Dicentrarchus labrax) SPERM CRYOPRESERVATION (CCMAR)
6. EUROPEAN SEABASS (Dicentrarchus labrax) SPERM CRYOPRESERVATION (IFREMER)
7. DUSKY GROUPER (Epinephelus marginatus) SPERM CRYOPRESERVATION
8. Rainbow trout (Oncorhynchus mykiss) testicular SPERM CRYOPRESERVATION
9. Nile tilapia (Oreochromis niloticus) SPERM CRYOPRESERVATION
10. Brown trout (Salmo trutta) SPERM CRYOPRESERVATION
11. EUROPEAN CATFISH (SILURUS GLANIS) SPERM CRYOPRESERVATION
12. SENEGALESE SOLE (Solea senegalensis) SPERM CRYOPRESERVATION
13. GILTHEAD SEABREAM (Sparus aurata) SPERM CRYOPRESERVATION
14. TENCH (TINCA TINCA) SPERM CRYOPRESERVATION
15. RAINBOW TROUT (Oncorhynchus mykiss) spermatogonial stem cell (SSCs) CRYOPRESERVATION

Conclusion

A total of fifteen standard procedures were collected, most of which related to sperm cryopreservation, while one procedure describes the cryopreservation of purified germinal stem cells. Other procedures are expected to be added until the end of the project.
The possibility of extending these procedures to species not yet available in the consortium will be raised at the AQUAEXCEL[2020] cryobanking workshop in October 2018.

Glossary

AQUAEXCEL[2020]: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020.

Document information

EU Project N°	652831	**Acronym**	AQUAEXCEL[2020]
Full Title	AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020		
Project website	www.aquaexcel.eu		

Deliverable	**N°**	D3.3	**Title**	Booklet of cryopreservation procedures for the cryobanked species
Work Package	**N°**	3	**Title**	**Common Standards and Tools**

Date of delivery	**Contractual**	12/09/2018 (Month 36)	**Actual**	28/09/2018 (Month 36)
Dissemination level	X	**PU Public, fully open, e.g. web**		
		CO Confidential, restricted under conditions set out in Model Grant Agreement		
		CI Classified, information as referred to in Commission Decision 2001/844/EC.		

Authors (Partner)	Yevhen HOROKHOVATSKYI and Marek RODINA: JU Ana Luisa SANTOS and Elsa CABRITA: CCMAR Catherine LABBE, Lionel GOARDON, Alexandra DEPINCE: INRA Alain VERGNET: IFREMER			
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Version log			
Issue Date	**Revision N°**	**Author**	**Change**
28/09/2018	1	Catherine Labbé	

Annex 1: Check list

Deliverable Check list (to be checked by the "Deliverable leader")

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time	X	*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW	X	*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW	X	
	The contributors (authors) correspond to those indicated in the DOW	X	
	The Table of Contents has been validated with the Activity Leader	X	*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL2020 deliverable template (title page, styles etc)	X	*Available in "Useful Documents" on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable	X	*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)	X	*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified	X	
	I have sent the final version to the WP Leader, to the 2nd Reviewer and to the Project coordinator (cc to the project manager) for approval	X	*Send the final draft to your WPLeader, the 2nd Reviewer and the coordinator with cc to the project manager on the 1st day of the due month and leave 2 weeks for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.*

Annex 2: Protocol booklet

AQUA EXCEL 2020

AQUAculture infrastructures for EXCELlence in European fish research towards 2020 — AQUAEXCEL2020

WP 3.3: Booklet of cryopreservation procedures for the cryobanked species

VERSION 1 _2018

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Work package number	**WP3**	**Mains actors**	**1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;**
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

List of protocols

- CRYO_SPERM_Acipenser ruthenus_JU_2018.pdf
- CRYO_SPERM_Crassostrea angulata_CCMAR_2018.pdf
- CRYO_SPERM_Cyprinus carpio_JU_2018.pdf
- CRYO_SPERM_Danio rerio_INRA_2018.pdf
- CRYO_SPERM_Dicentrarchus labrax_CCMAR_2018.pdf
- CRYO_SPERM_Dicentrarchus labrax_IFREMER_2018.pdf
- CRYO_SPERM_Epinephelus marginatus_CCMAR_2018.pdf
- CRYO_SPERM_Oncorhynchus mykiss_INRA_2018.pdf
- CRYO_SPERM_Oreochromis niloticus_INRA_2018.pdf
- CRYO_SPERM_Salmo trutta_INRA_2018.pdf
- CRYO_SPERM_Silurus glanis_JU_2018.pdf
- CRYO_SPERM_Solea senegalensis_CCMAR_2018.pdf
- CRYO_SPERM_Sparus aurata_CCMAR_2018.pdf
- CRYO_SPERM_Tinca tinca_JU_2018.pdf
- CRYO_SSC_Oncorhynchus mykiss_INRA_2018.pdf

Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

STERLET (*ACIPENSER RUTHENUS*) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL[2020] project, thanks to the contribution of

University of South Bohemia in Ceske Budejovice
Faculty of Fisheries and Protection of Waters,
Research Institute of fish Culture and Hydrobiology

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Procedure written by Yevhen Horokhovatskyi, JU, Czech Republic, 2018

Version 1_JU_2018

1. Objectives of the procedure and areas of application

Cryopreservation of sturgeon sperm for cryobanking of valuable genetic resources, and for laboratory and farm use

2. Bibliographical reference(s) for the described protocol (*if available*)

One example of the procedure description and application is presented in the following publication in another sturgeon species

Glogowski J, Kolman R, Szczepkowski M, Horváth Á, Urbányi B, Sieczyński P, Rzemieniecki A, Domagała J, Demianowicz W, Kowalski R *et al*: **Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol**. *Aquaculture* 2002, **211**(1–4):367-373.

Other related publications from JU are:

Horokhovatskyi Y, Rodina M, Asyabar HD, Boryshpolets S, Dzyuba B: **Consequences of uncontrolled cooling during sterlet (Acipenser ruthenus) sperm cryopreservation on post-thaw motility and fertilizing ability**. *Theriogenology* 2017, **95**:89-95.

Dyuba B, Boryshpolets S, Shaliutina A, Rodina M, Yamaner G, Gela D, Dzyuba V, Linhart O: **Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet Acipenser ruthenus during sequential stripping**. *Aquaculture* 2012, **356-357**:272-278.

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (4-5 years and older) fish male, during natural spawning season (April-May). The water temperature should be 15 °C. To induce spermiation,
fish should be injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution. The concentration of carp pituitary extract in physiological salt vary between 1-5 mg/ml depending on fish body weight, while dose for 1 kg of fish is 4 mg. Inject the fish in the muscular tissue along the side of the dorsal fin at a 45-degree angle pointing the needle towards the head of the fish. The maximal volume of suspension that can be injected to one side of the dorsal fin should not exceed 1 ml. Twenty-four hours after injection collect the sperm.

3.2. Fish anaesthesia before manipulation

The anaesthesia of fish is not needed.

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Contact information (address, city, country)**: Zátiší 728/II, 38901 Vodnany, Czech Republic**	

3.3. Extender composition for sperm storage (*if needed*)

Not needed

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by using individual plastic syringe (10-20 ml) with an attached 4-mm plastic catheter inserted into the urogenital ducts.

During sperm collection do not fill all volume of the syringe, leaving half of the volume of air inside and store on ice (2–4 °C) for maximum 2 hours before freezing. Avoid contamination of sperm with water or faeces.

Usually, the volume collected from 5 years old males varies between 5 ml and 20 ml, and the spermatozoa concentration ranges between 0.1×10^9 and 2×10^9 spermatozoa/mL semen.

4. Sperm quality assessment

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

Spermatozoa activation solution		For 100 ml solution
Tris (121.1 g/mol)	10 mM	121 mg
pH 8.0		

Adjust pH to 8.0 by adding HCl. To prevent spermatozoa from sticking to the microscope slide, add to the activation solution either 0.1% (w/v) BSA or 0.25% (w/v) Pluronic acid F-127 (catalog number P2443, Sigma-Aldrich, USA).

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C). Activate spermatozoa movement by mixing sperm and activation solution at an approximate ratio of 1:100. For fresh sperm, place 50 µl of activation solution on a glass slide under the microscope and add 0.5 µl sperm by mixing thoroughly for 2 s. The number of spermatozoa per field of view should range between 70 and 100. Moving through different levels of the droplet, estimate sperm motility percentage. The glass coverslip in this case is not needed.

Motility is, usually, subjectively estimated under a dark-field microscope (20x), at a room temperature (18-20 °C). For fresh sperm, place 50 µl of activation solution on a glass slide under a microscope and add 0.5 µl sperm by mixing thoroughly for 2 s. The number of

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spermatozoa per frame should be in range between 70 and 100. Moving through different levels of activation solution droplet, estimate sperm motility percentage.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (>80%) can be used for cryopreservation.

5. Sperm cryopreservation procedure

5.1. Cryoprotectant composition

For 100 ml: add the components to 80 ml of distilled water and adjust the pH to 8.1 with HCl. Thereafter, add 10 ml of methanol and adjust the volume of the cryoprotectant solution to 100 ml with distilled water.

Cryoprotectant composition		For 100 ml solution
KCl (74.55 g/mol)	0.25 mM	1.9 mg
Sucrose (342.29 g/mol)	23.4 mM	801 mg
Tris (121.1 g/mol)	30 mM	363 mg
Methanol	10% (volume)	10 ml
pH 8.1		

5.2. Sperm manipulation before cryopreservation

Before freezing dilute one volume of sperm with one volume of cryoprotectant solution (at 4 °C). During the 10 min equilibration time, place the obtained suspension into 0.5 plastic straws (www.imv-technologies.com, Ref: 014650 White). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing them.

5.3. Freezing device and container type

Whereupon, put filled straws on a 3 cm thick styrofoam raft (dimensions: 40 × 20 × 3 cm) and transfer them to a styrofoam box (dimensions: 52 × 33 × 30 cm), filled to a depth of 10 cm with liquid nitrogen. The detailed illustration is presented in the following publication Horokhovatskyi et al, 2017
https://doi.org/10.1016/j.theriogenology.2017.03.007.

After a 10-min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen.

5.4. Cooling programme (if available)

Styrofoam box gives fast but uncontrolled cooling rate.

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6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 40 °C for 6 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary from sample to sample.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

PORTUGUESE OYSTER (*Crassostrea angulata*) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

Center for Marine Sciences – CCMAR

Procedure written by Ana Luisa Santos, CCMAR, Portugal, 2018

Version 1_CCMAR_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Portuguese oyster sperm for cryobanking of valuable genetic resources, and for laboratory and farm use.

2. Bibliographical reference(s) for the described protocol

One example of the procedure description and application is presented in the following publications:

M.F. Riesco, F. Felix, D. Matias, S. Joaquim, M. Suquet, E. Cabrita, First study in cryopreserved Crassostrea angulata sperm, General and Comparative Endocrinology 245 (2017) 108-115.
https://doi.org/10.1016/j.ygcen.2016.05.003

3. Oyster manipulation

3.1. Oyster hormonal treatment

No hormonal treatment is required to induce spermiation. Sperm is collected through scalpel incisions in the gonad from mature oyster males during natural spawning season (May-September). The water temperature should be in range 20-22 °C.

3.2. Oyster anaesthesia before manipulation

No anaesthesia is required to sperm collection.

3.3. Extender composition for sperm storage

The extender used for this species is artificial seawater (ASW).

3.4. Sperm collection and storage

Using an oyster knife, bivalves are opened and sex is microscopically determined. Sperm is collected by a dry method, directly from gonad using a scalpel doing small cuts in gonad. A micropipette is used to collect sperm into an eppendorf tube. Immediately, sperm is filtered with a 20 µm and 100 µm sieves and diluted 1:10 in artificial seawater. For fresh sperm, concentration and total motility is assessed. According to prior concentration, sperm is diluted in order to have a final concentration between 1 to 2 $x10^9$ spermatozoa/ml.

4. Sperm quality assessment

The sperm quality is assessed using several parameters. Motility and cell viability are the most widely used.

4.1. Sperm motility activation solution

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Contact information: CCMAR, University of Algarve, Campus of Gambelas, Faro, Portugal	

Usually no activation solution is required since the sperm is already activated.

4.2. Sperm motility assessment

Motility is assessed using Computer Assisted Sperm Analysis (CASA system) and ISAS software (ISAS, Proiser, Valencia, Spain). Samples are carried out in a Makler chamber under a 10x negative-phase contrast objective coupled with a digital camera (Basler A312f C-mount, Germany) set for 25 fps.
Samples need to be diluted 1:10 in artificial seawater prior the analysis. Motility analysis are performed by placing 10 µl of diluted sperm into a Makler chamber. After activation, sperm moves for several days.

4.3. Sperm quality threshold

Only samples with motility higher than 40% and final concentration between 1 to 2 $x10^9$ spermatozoa/ml should be cryopreserved.

4.4. Sperm Viability assessment

Two methods to assess oyster sperm viability (fluorescence microscope or flow cytometer) can be used.

For fluorescence microscope analysis, mix 15 µl of diluted sperm, 0.5 µl SYBR Green (final concentration 100 nM) and 1.5 µl propidium iodide and observe in a fluorescence microscope. Count at least 100 cells, distinguishing live (SYBR green positive, green cell) and dead cells (PI stained, red cells).

For flow cytometer analysis, dilute 5 µl of sperm in 500 µl of 1% NaCl buffer. Add 2 µl propidium iodide (PI) at a concentration of 2.4 mM to the suspension. Analyse in a flow cytometer after 5 min incubation in the dark.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Cryoprotectant solution		For 100 ml solution
Extender solution (ASW)		80 ml
DMSO (78.13 g/mol)	20%	20 ml

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute sperm in cryoprotectant solution 1:1 (v:v). During the 10 min equilibration time, place the obtained suspension into 0.5 ml plastic straws (www.minitube.com, Ref: 13408). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing.

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Contact information: CCMAR, University of Algarve, Campus of Gambelas, Faro, Portugal	

5.3. Freezing device and container type

A portable programed biofreezer (Asymptote Grant EF600, UK) programmed with a freezing rate of 6°C/min from 0 to -70°C) is used for freezing straws. After freezing ends, straws are directly stored in a liquid nitrogen (LN_2) container.

5.4. Cooling programme

Asymptote Grant EF600

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 37°C for 10 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa decreases with time.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

Procedure written by (name, email)**:**	**Procedure validated by** (name, email):
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COMMON CARP (CYPRINUS CARPIO) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of
University of South Bohemia in Ceske Budejovice
Faculty of Fisheries and Protection of Waters,
Research Institute of fish Culture and Hydrobiology

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Procedure written by Yevhen Horokhovatskyi, JU, Czech Republic, 2018

Version 1_JU_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Common carp sperm for cryobanking of valuable genetic resources, and for laboratory and farm use

2. Bibliographical reference(s) for the described protocol (*if available*)

One example of the procedure description and application is presented in the following publication: https://doi.org/10.1016/0044-8486(84)90159-5

Adobe Acrobat Document

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (2 years and older) fish male, during natural spawning season (May-June). The water temperature should be in range 18-20 °C. To induce spermiation, fish should be injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution. The concentration of carp pituitary extract in physiological salt vary from can be within 1-5 mg/ml depending on fish body weight, while dose for 1 kg of fish is 1 mg. Inject the fish in the muscular tissue along the side of the dorsal fin at a 45-degree angle pointing the needle towards the head of the fish. The maximal volume of suspension that can be injected to one side of the dorsal fin should not exceed 1 ml. Twenty-four hours after injection collect the sperm.

3.2. Fish anaesthesia before manipulation

The anaesthesia of fish is not needed.

3.3. Extender composition for sperm storage (*if needed*)

Not needed

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage into individual plastic (10-20 ml) syringes leaving half of the volume of air inside and store on ice (2–4 °C) for maximum 2 hours before freezing. Avoid contamination of sperm with water, urine or faeces.

Usually, the volume collected from 2 years old males varies between males (up to 5 ml), and the spermatozoa concentration ranges between 10×10^9 and 20×10^9 spermatozoa/mL semen.

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4. Sperm quality assessment

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

For 100 ml: add the components to 80 ml of distilled water and adjust the pH to 8.2 with HCl. Thereafter, adjust the volume of the spermatozoa activation solution to 100 ml with distilled water.

Spermatozoa activation solution		For 100 ml solution
NaCl (58.44 g/mol)	45 mM	263 mg
KCl 74.55 (g/mol)	5 mM	37 mg
Tris (121.10 g/mol)	30 mM	363 mg
pH 8.2		

Adjust pH to 8.2 by adding HCl. To prevent spermatozoa from sticking to the microscope slide, add to the activation solution either 0.1% (w/v) BSA or 0.25% (w/v) Pluronic acid F-127 (catalog number P2443, Sigma-Aldrich, USA).

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C). Activate spermatozoa movement by mixing sperm and activation solution at an approximate ratio of 1:5000. For fresh sperm, place 50 µl of activation solution on a glass slide under the microscope and add sperm using the tip of dissecting needle by mixing thoroughly for 2 s. The number of spermatozoa per field of view should range between 70 and 100. Moving through different levels of the droplet, estimate sperm motility percentage. The glass coverslip in this case is not needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (>80%) can be used for cryopreservation.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

For 100 ml: add the components to 80 ml of distilled water and adjust the pH to 8.1 with HCl. Thereafter, add 16 ml of ethylene glycol and adjust the volume of the cryoprotectant solution to 100 ml with distilled water.

Cryoprotectant solution	For 100 ml solution

NaCl (58.44 g/mol)	59 mM	345 mg
KCl (74.55 g/mol)	6.3 mM	47 mg
$CaCl_2$* $2H_2O$ (147.02 g/mol)	0.68 mM	10 mg
$MgCl_2$ (95.21 g/mol)	1.2 mM	11 mg
$NaHCO_3$ (84.00 g/mol)	27 mM	227 mg
Sucrose (342.29 g/mol)	3.4 mM	116 mg
D-mannitol (182.20 g/mol)	69 mM	1257 mg
Tris (121.10 g/mol)	118 mM	1859 mg
Ethylene glycol	16% (volume)	16 mL
pH 8.1		

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute one volume of sperm with one volume of cryoprotectant solution (at 4 °C). During the 10 min equilibration time, place the obtained suspension into 0.5 mL plastic straws (www.imv-technologies.com, Ref: 014650 White). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing them.

5.3. Freezing device and container type

Whereupon, put filled straws on a 3-cm thick styrofoam raft (dimensions: 40 × 20 × 3 cm) and transfer them to a styrofoam box (dimensions: 52 × 33 × 30 cm), filled to a depth of 10 cm with liquid nitrogen. The detailed illustration is presented in the following publication https://doi.org/10.1016/j.theriogenology.2017.03.007.

Adobe Acrobat Document

After a 10-min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen.

5.4. Cooling programme (if available)

Styrofoam box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 40 °C for 6 s.

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6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary from sample to sample.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

Zebrafish (Danio rerio) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of **INRA LPGP research unit,** with Pierre Millon (INRA) and Timea Kollar (Univ Gödöllö, Hungary) during an AQUAGAMETE STSM.

Food and Agriculture COST Action FA 1205

AQUAGAMETE

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Procedure written by Catherine Labbé, INRA, France, 2018

Version 1_INRA_2018

1. Objectives of the procedure and areas of application

Cryopreservation of valuable genetic material using sperm as genetic support.

2. Bibliographical reference(s) for the described protocol (*if available*)

Several procedures were published, but the following describe a protocol using bovine straws, adapted to cryobanking facilities. It was adapted from :

Yang H., Carmichael C., Zoltan M. Varga, Tiersch T.R., 2007, Development of simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish Danio rerio, Theriogenology, vol.68, p 128-136.

3. Fish manipulation and sperm collection

3.1. Fish hormonal treatment

Sperm is collected on males with good physical condition, aged 4 months or more. No hormonal treatment is required, but the night before collection, males may be conditioned with females in the same tank, separated by a mesh (but not mandatory if the males are in good condition).

3.2. Fish anaesthesia before manipulation

The males are anaesthetized with tricaine 50 mg/L or phenoxy-2 ethanol 250µL/250 mL tank water, according to the recommendation of the local animal welfare committee.

3.3. Extender composition for sperm storage (*if needed*)

The collected volumes are very small (1-4 µL), and sperm may have to be diluted for handling. Dilution induces spermatozoa swelling whatever the osmolality of the medium (tested up to 500 mOsm/kg). One of the best medium, although not perfect, is HBSS 300 (Yang et al, 2007).

HBSS300 (Hank's balanced salt solution) is a solution that keeps sperm of zebrafish immobilized. This solution is at 300mOsmol/kg and pH 8 (no need to adjust the pH).

Prepare a 2X HBSS300, called HBSS600

For 250 mL :			
NaCl	(58.44 g/mol)	274 mM	4 g
KCl	(74.55 g/mol)	10.8 mM	0.2 g
MgSO4, 2 H2O	(246.47 g/mol)	2.0 mM	0.12g
CaCl2, 2 H2O	(147.02 g/mol)	2.6 mM	0.096g
Na2HPO4	(358.14 g/mol)	0.5 mM	0.045 g
KH2PO4	(136.1 g/mol)	0.88 mM	0.03 g
NaHCO3	(84.01 g/mol)	8.4 mM	0.18g
Glucose	(180.16 g/mol)	11 mM	0.5g

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HBSS 300 is made by mixing 1 volume HBSS 600 with 1 volume distilled water.

3.4. Sperm collection and storage

Anaesthetized fish are fished, rinsed with tank water to remove anaesthesia from the skin, and placed in a small grove made with wet paper.
To prevent sperm activation upon collection the genital papilla and the pelvic fins are rinsed with HBSS 300 (be careful of the gills).

Sperm collection can be done under a binocular, but it is not mandatory for practised manipulators. With tiny forceps whose ends are protected with pads (to prevent skin damage), press the flanks mildly (from mid body to the pelvic fins), and aspirate sperm at the same time with a plastic pipet. The liquid should be whitish (diluted sperm) or white (concentrated sperm).

Dilute sperm with 15 µL HBSS300 (rinse the pipet) and store few hours on ice.

4. Sperm quality assessment prior to cryopreservation

The sperm quality parameter the most widely used is the motility percentage. It is recommended to count sperm concentration as well with this low producing species.

4.1. Sperm motility activation solution

Tap water with 5 mg/mL BSA to prevent spermatozoa from sticking to the slide.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C).

- On the glass slide, mix 1 µL diluted sperm with 20 µL tap water with 5 mg/mL
- Add the coverslip (not mandatory)
- Immediately estimate the percentage of spermatozoa with a rapid and straightforward movement, on several frames at different height of the droplet.

The number of spermatozoa per field of view should range between 100 and 200. Too many cells may induced overestimation of the motility percentage, whereas too less lead to underestimation. Automated motility assessment devices can also be used, but they are not mandatory with regards to the information needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (> 90%) can be reliably used for cryopreservation. When the samples are very precious, lower quality is acceptable, but beware that survival after thawing will be lower.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

50 volumes HBSS 600
16 volumes MeOH 100%
10 volumes de sucrose 1,26 M dans H2O (mw 342.3, 4.31 g/10 mL water)
24 volumes distilled water

In practise, for 1 mL extender, mix 500 µL HBSS 600, 160 µL MeOH, 100 µL sucrose and 240 µL water.

5.2. Sperm manipulation before cryopreservation

WORK ON ICE

1 volume diluted sperm
+ 1 volume cryoprotectant solution

In a 250 µL straw, aspirate cryoprotectant solution (will wet the cotton without loosing the precious sperm sample), then air, then sperm into cryoprotectant, then air. Avoid too much air or the straw will float in the LN2 tank. Adjust with cryoprotectant volume.

Cryoprotectant

Sperm + cryoprotectant

5.3. Freezing device and container type

The classical raft method is very bad for zebrafish sperm. Use a programmable freezer.

5.4. Cooling programme (if available)

+ 2°C to -8.5 °C at -10°C/min
Hold 6 min at -8.5 °C
-8.5°C to -80°C at -10 °C/min

Store the straws into liquid nitrogen

6. Sperm thawing procedure

6.1. Thawing device and programme

The straws are frozen in a water bath at 20°C°C for 10 s. This temperature and duration allows that the straw content is rapidly thawed, whereas the inside temperature does not rise above 4°C (MeOH is a very toxic molecule at room temperature).

6.2. Sperm washing (if needed)

Not recommended, as zebrafish spermatozoa are very fragile upon thawing. Thank to the high dilution rate upon fertilization, the cryoprotectant toxicity does not alter egg quality.

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6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing.
Cut the straw on each side of the sperm sample, and empty the straw section with a pipet.

6.4. Thawed sperm quality assessment

Sperm quality can be assessed by motility percentage (5-60 %) as described in 4.2. Because motility is hardly correlated to fertilization rate, it is recommended to perform a fertility test on a fraction of the straw collection.
Storage duration in liquid nitrogen does not alter sperm quality, provided that care is taken to maintain the straw temperature below -150°C (beware of the straw heating during sorting and manipulation of the collections).

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	**1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;**
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

EUROPEAN SEABASS (Dicentrarchus labrax) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

Center for Marine Sciences – CCMAR

Procedure written by Ana Luisa Santos, CCMAR, Portugal, 2018

Version 1_CCMAR_2018

1. Objectives of the procedure and areas of application

Cryopreservation of European seabass sperm for cryobanking of valuable genetic resources, and for laboratory and farm use.

2. Bibliographical reference(s) for the described protocol

One example of the procedure description and application is presented in the following publication:

S. Martinez-Paramo, P. Diogo, M.T. Dinis, F. Soares, C. Sarasquete, E. Cabrita, Effect of two sulfur-containing amino acids, taurine and hypotaurine in European sea bass (Dicentrarchus labrax) sperm cryopreservation, Cryobiology 66 (2013) 333-338.
https://doi.org/10.1016/j.cryobiol.2013.04.001

S. Martinez-Paramo, P. Diogo, M.T. Dinis, M.P. Herraez, C. Sarasquete, E. Cabrita, Incorporation of ascorbic acid and alpha-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm, Theriogenology 77 (2012) 1129-1136.
https://doi.org/10.1016/j.theriogenology.2011.10.017

3. Fish manipulation

3.1. Fish hormonal treatment

Usually no hormonal treatment is required to induce spermiation. Sperm is collected by stripping from mature fish male, during natural spawning season (November-March). The water temperature should be around 13-15°C.

3.2. Fish anaesthesia before manipulation

Fish are anesthetised with 200 ppm of 2-phenoxyethanol.

3.3. Extender composition for sperm storage

Extender solution: Non-activating mineral medium (NAM Solution)		For 100 ml solution
NaCl (58.44 g/mol)	59.83 mM	349.64 mg
KCl (74.55 g/mol)	1.47 mM	10.96 mg
$MgCl_2$ (95.211 g/mol)	12.91 mM	122.92 mg
$CaCl_2$ (110.98 g/mol)	3.51 mM	38.95 mg
$NaHCO_3$ (100.115 g/mol)	20 mM	200.23 mg
Glucose (180.156 g/mol)	0.44 mM	7.93 mg
BSA (66430.3 g/mol)	1%	1 g
pH 7.7		

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage into with a 1 ml syringe without a needle. Avoid contamination of sperm with water, urine or faeces.
Immediately after collection, the sperm need to be diluted 1:6 (v/v) in NAM solution and maintained at 4ºC.

4. Sperm quality assessment

The sperm quality is assessed using several parameters. Motility and cell viability are the most widely used.

4.1. Sperm motility activation solution

Sperm motility is activated using artificial seawater.

Spermatozoa activation solution (Artificial Seawater)		For 100 ml solution
NaCl (58.44 g/mol)	513.3 mM	3 g
KCl (74.55 g/mol)	10.7 mM	79.77 mg
$CaCl_2$ (110.98 g/mol)	11.7 mM	129.85 mg
$MgSo_4$ (120.366 g/mol)	54.8 mM	659.61 mg
$NaHCO_3$ (84.007 g/mol)	11.6 mM	97.45 mg

4.2. Sperm motility assessment

Motility is assessed using Computer Assisted Sperm Analysis (CASA system) and ISAS software (ISAS, Proiser, Valencia, Spain). Samples are carried out in a Makler chamber under a 10x negative-phase contrast objective (Nikon E200, Tokyo, Japan) coupled with a digital camera (Basler A312f C-mount, Germany) set for 50 fps.
Motility analysis are performed activating 0.5 µl of sperm with 20 µl of artificial seawater. Quantify at 10, 20, 30 and 45 s post-activation motile spermatozoa (%), Curvilinear velocity (VCL; µm/s), straight line velocity (VSL; µm/s) and spermatozoa linearity (Lin; %).

4.3. Sperm quality threshold

No requirements followed

4.4. Sperm Viability assessment

Depending on the method used to assess sperm viability (fluorescent microscope or flow cytometer) sperm may need to be diluted after thawing.
For fluorescent microscope analysis, pre-diluted sperm is diluted 1:1000 in NAM solution. Incubate sperm with 0.25 µM SYBR and 18 µM PI (final concentration) for 5 min in the dark at 4 ºC.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Cryoprotectant solution		For 100 ml solution
Extender solution		90 ml
DMSO (78.13 g/mol)	10%	10 ml

5.2. Sperm manipulation before cryopreservation

For cryopreservation, extender is added to each sperm sample (1:6, v/v) (see 3.4). Add 10% DMSO (v/v, final concentration) to diluted sperm and load the mixture into 0.5 ml plastic straws (www.minitube.com, Ref: 13408). Equilibration time should not overpass 4 min. If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing them.

5.3. Freezing device and container type

Whereupon, put filled straws in a rack and freeze them at 6.5 cm above liquid nitrogen. After a 15 min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen and store it in a nitrogen container until use.

5.4. Cooling programme (if available)

No available

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 35°C for 15 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa drop drastically after thawing.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

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8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of
Ifremer Palavas, Laboratory L-3AS et laboratory L-SEA

Procedure written by Alain Vergnet, IFREMER, France, 2018

Version 1_IFREMER_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Seabass sperm for cryobanking of valuable genetic resources, and for laboratory and farm use

2. Bibliographical reference(s) for the described procedure (*if available*)

This procedure is derived from

Fauvel C, Boryshpolets S, Cosson J, Leedy JGW, Labbe C, Haffray P, Suquet M: **Improvement of chilled seabass sperm conservation using a cell culture medium**. *Journal of Applied Ichthyology* 2012, **28**(6):961-966.

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (2 years and older) fish male, in the middle of natural spawning season (January - March). The water temperature is less than 14 °C. An hormonal treatment is not needed, the fish give naturally good quantity and quality of sperm during this period.

3.2. Fish anaesthesia before manipulation

A small sedation is done in adding 100 ml of benzocaine (150g/ liter) by 1 m^3.

3.3. Extender composition for sperm storage (*if needed*)

Not needed

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage into individual plastic (5 ml) syringes leaving half of the volume of air inside and store in the fridge at 4 °C for maximum 2 hours before freezing. Avoid contamination of sperm with water, urine or faeces.
Usually, the volume collected from 2-year-old males varies from 0.5 ml to 2.5ml, and the spermatozoa concentration is around 50×10^9 spermatozoa/mL semen.

4. Sperm quality assessment

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

Marine water + BSA (20 mg/mL) is used for the activation solution.

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4.2. Sperm motility assessment

The motility is measured using a semen analyser CASA (IVOS II, IMV Technologies, Hamilthon Thorne). 7 µl of diluted sperm at 1:3 ratio with MarineFreeze medium (IMV Technologies commercial medium) for cryopreserved spermatozoa and with MarineSol (IMV Technologies commercial medium) for fresh sperm is mixed with 1 mL of activation solution. 3µl of the mix are put on a Leja slides (IMV Technologies) and analysed by the CASA IVOS II system, 3 times 1 second within 10 seconds after activation.

4.3. Sperm quality threshold

Sperm motility assessment is not done in routine for the moment. In 2019 we will qualify all the semen using a CASA IVOS II using a threshold at 80% of motile spermatozoa.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

The cryoprotectant solution used for Seabass is the commercial solution MarineFreeze (IMV Technologies) developed using culture cell medium (Fauvel et al 2012, Improvement of chilled seabass sperm conservation using a cell culture medium. J. Appl. Ichthyol., 28: 961–966.). The cryoprotectant solution is composed of a saline solution, glutamine, BSA and 13% DMSO.

5.2. Sperm manipulation before cryopreservation

The sperm kept at 4 °C is put in a Falcon 15 ml tube with Cryoprotectant solution at the ratio of 1:3. The falcon tube is gently agitated until the sperm seems well mix in the middle. There is no need to have an equilibrium time but we need to work in a cold place to avoid the warming of the straws during the filling. The straws are CBS High Security sperm straw of 0.5 ml (Cryo Bio System, IMV Technologies group). They are filled and sealed at both extremities using a Semi-automatic filling and sealing system for CBS High Security straws PACE (Cryo Bio System, IMV Technologies group).

5.3. Freezing device and container type

We have experimented 2 ways to freeze the straws, vapour freezing unit and a Micro-Digitcool programmable automatic Freezer (IMV Technologies)

For the vapour freezing, the filled straws are quickly rowed on a freezing rack (IMV Technologies). The rack is introduced in the vapour freezing unit and put on a Styrofoam raft

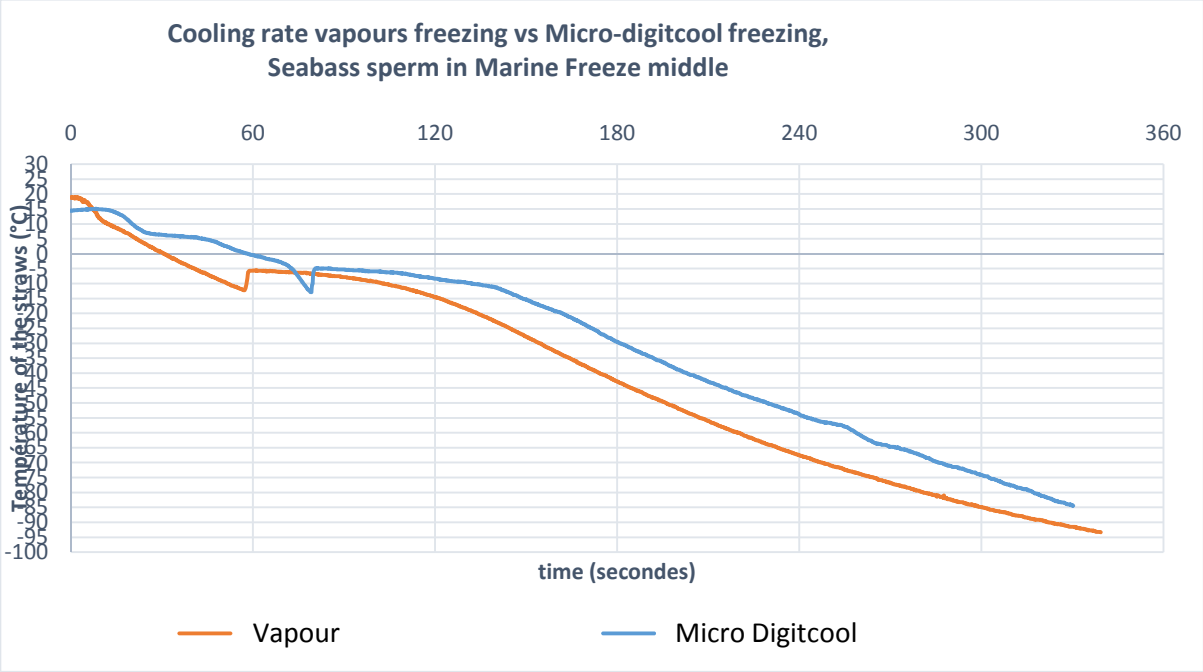
floating on 10 cm depth of liquid nitrogen. The straws stay at minimum 6 minutes at 7 cm of the liquid nitrogen level before transferring them directly into liquid nitrogen.

For Micro-Digitcool programmable automatic Freezer, the filled straws are quickly rowed on a freezing rack (IMV Technologies). The rack is introduced in the Micro-Digitcool and placed on the bottom of the unit. The programmable freezing unit is closed and the freezing programme is run. At the end of the freezing programme, the straws are transferred directly to the liquid nitrogen.

5.4. Cooling programme (if available)

The vapour freezing give fast but uncontrolled cooling rates.

The Micro-Digitcool programme starts with an initial temperature at 15 °C, with a cooling rate of -20 °C*min^{-1} until the straws reach -90 °C. At the end of the programme the straws are kept at -90 °C.



Even if the two techniques give the same cooling rate -20 °C*min^{-1} when the temperature is under 0 °C, the straws done with the Micro-Digitcool give a better motility rate after thawing, 67% vs 41%. This difference could be explained by the difference of cooling rate, from ambient temperature (+15 °C to +20 °C) to the temperature of 0 °C, 2 times faster with the vapour freezing. This point has to be improved.

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 28 °C for 20 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

DUSKY GROUPER (*Epinephelus marginatus*) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

Center for Marine Sciences – CCMAR

Procedure written by Ana Luisa Santos, CCMAR, Portugal, 2018

Version 1_CCMAR_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Dusky grouper sperm for cryobanking of valuable genetic resources, and for laboratory and farm use.

2. Bibliographical reference(s) for the described protocol

One example of the procedure description and application is presented in the following publications:

E. Cabrita, S. Engrola, L.E.C. Conceicao, P. Pousao-Ferreira, M.T. Dinis, Successful cryopreservation of sperm from sex-reversed dusky grouper, Epinephelus marginatus, Aquaculture 287 (2009) 152-157.
https://doi.org/10.1016/j.aquaculture.2008.10.019

M.F. Riesco, C. Raposo, S. Engrola, S. Martinez-Paramo, S. Mira, M.E. Cunha, E. Cabrita, Improvement of the cryopreservation protocols for the dusky grouper, Epinephelus marginatus, Aquaculture 470 (2017) 207-213.
http://dx.doi.org/10.1016/j.aquaculture.2016.12.027

3. Fish manipulation

3.1. Fish hormonal treatment

Sperm is collected from mature fish male between 1st of June and the 1st of July. The water temperature should be in range 21-23 °C. To induce spermiation, fish should be induced hormonally with 25 µg/kg GnRHa implants. Implants are introduced in the abdominal area, being fish anaesthetised. 24-48 hours after implants are introduction sperm is collect.

3.2. Fish anaesthesia before manipulation

Individuals need to be sedated in the breeding tank with 75 ppm 2-phenoxyethanol and then anaesthetised in a 50 l tank with 200 ppm 2-phenoxyethanol for 10 min.

3.3. Extender composition for sperm storage

The extender used for this species is a 1% NaCl solution (~300 mOsm/Kg).

Extender solution		For 100 ml solution
NaCl (58.44 g/mol)	1%	1 g

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3.4. Sperm collection and storage

With fish anaesthetised, dry out urogenital area with paper towel and collect the sperm by abdominal massage using a syringe (without needle). Avoid contamination of sperm with water, urine or faeces. Introduce sperm into eppendorfs tubes and store it in a Styrofoam rack on ice (to avoid direct ice contact) until further analysis (within 1h).
The sperm volume collected can vary from 5 to 400 µl, and the spermatozoa concentration ranges between 1.2×10^9 and 16.3×10^9 spermatozoa/ml.

4. Sperm quality assessment

The sperm quality is assessed using several parameters. Motility and cell viability are the most widely used.

4.1. Sperm motility activation solution

Sperm motility is activated using seawater.

4.2. Sperm motility assessment

Motility is assessed using Computer Assisted Sperm Analysis (CASA system) and ISAS software (ISAS, Proiser, Valencia, Spain). Samples are carried out in a Makler chamber under a 10x negative-phase contrast objective coupled with a digital camera (Basler A312f C-mount, Germany) set for 50 fps.
Samples need to be diluted 1:9 in a non-activating medium (1% NaCl, ~300 mOsm/Kg) prior the analysis. Motility analysis are performed activating 1 µl of sperm into a Makler chamber with 9 µl of seawater at 4ºC.

4.3. Sperm quality threshold

No requirements followed.

4.4. Sperm Viability assessment

Dilute 5 µl of sperm in 500 µl of 1% NaCl buffer. Add 2.5 µl propidium iodide (PI) at a concentration of 2.4 mM and 0.5 µl SYBR-14 at a concentration of 0.1 mM to the suspension. Analyse in a flow cytometer after 5 min incubation in the dark.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Cryoprotectant solution		For 100 ml solution
Extender solution		90 ml
DMSO (78.13 g/mol)	10%	10 ml
BSA (66430.3 g/mol)	10 mg/ml	1 g
Optional*: Taurine (125.14 g/mol)	50 mM	625.7 mg

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*Taurine improves cell viability in dusky grouper.

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute sperm in cryoprotectant solution 1:9 (v:v). During the 4 min equilibration time, place the obtained suspension into 0.5 ml plastic straws (www.minitube.com, Ref: 13408). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing.

5.3. Freezing device and container type

Put filled straws in a horizontal rack 3 cm above liquid nitrogen (N_2) in a covered Styrofoam box. Sperm freezing is performed in liquid nitrogen vapour during 10 min. After this time end, straws are immersed in liquid nitrogen and stored in N_2 container.

5.4. Cooling programme (if available)

No available

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 25°C for 30 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary with time.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

Rainbow trout (Oncorhynchus mykiss) testicular SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of **INRA PEIMA infrastructure and INRA LPGP research unit**

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Procedure written by Catherine Labbé, INRA, France, 2018

Version 1_INRA_2018

1. Objectives of the procedure and areas of application

Cryopreservation of testicular sperm from neomales. The neomales are obtained after masculinization of females, in order to obtain all-X spermatozoa. Those spermatozoa are used to produce an all female progeny in aquaculture. In research practices, the neomales are also required to ensure propagation of isogenic lines, produced from gynogenesis and thereby siring all-female progenies.
The masculinization treatment is tuned so that neomales will not develop sperm ducts. Therefore, the testis of mature neomales have to be collected and the mature sperm extracted.

The cryopreservation procedure can also be successfully applied to sperm collected by stripping from normal males.

2. Bibliographical reference(s) for the described protocol (*if available*)

Neomale production was described in the Reprofish documents :
https://www.reprofish.eu/reprofish_eng/content/download/3307/.../Sex+control.pdf

The following procedure was not published by INRA, due to contracts with IMV Technologies. However, neomale sperm cryopreservation was studied and described in
Judycka, S., Ciereszko, A., Dobosz, S., Zalewski, T., Dietrich, G.J., 2017. Effect of dilution in sperm maturation media and time of storage on sperm motility and fertilizing capacity of cryopreserved semen of sex-reversed female rainbow trout. General and Comparative Endocrinology 245, 89-93.

Ciereszko, A., Dietrich, G.J., Nynca, J., Krom, J., Dobosz, S., 2015. Semen from sex-reversed rainbow trout of spring strain can be successfully cryopreserved and used for fertilization of elevated number of eggs. Aquaculture 448, 564-568.

Dietrich, G.J., Nynca, J., Dobosz, S., Zalewski, T., Ciereszko, A., 2014. Application of glucose-methanol extender to cryopreservation of semen of sex-reversed females rainbow trout results in high post-thaw sperm motility and fertilizing ability. Aquaculture 434, 27-32.

Robles, V., Cabrita, E., Cunado, S., Herraez, M.P., 2003. Sperm cryopreservation of sex-reversed rainbow trout (Oncorhynchus mykiss): parameters that affect its ability for freezing. Aquaculture 224, 203-212.

3. Fish manipulation and sperm collection

3.1. Fish hormonal treatment

Sperm is collected during the spawning season of the rainbow trout. It will depend on the strain (fall, winter, spring).

3.2. Fish anaesthesia before manipulation

When the neomales are devoid of sperm ducts, spermatozoa have to be collected from the testis after euthanasia of the sedated animals.
The neomales are anaesthetized with tricaine 100 mg/L prior to euthanasia by a blow on the head, according to the recommendation of the local animal welfare committee.

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3.3. Extender composition for sperm storage (*if needed*)

Testicular sperm requires at least 2h maturation into the STORFISH medium. This medium can also be used for sperm storage at 4°C under oxygen atmosphere for several days. However, it is better to cryopreserve the spermatozoa the day of the collection.

The 10X StorFish extender can be purchased at the IMV Technologies company, under the ref 018500
https://www.imv-technologies.com/our-solutions/fish/detail/product/storfish-1-litre-qsp-10-litres-.html

3.4. Sperm collection and storage

- Remove the testis from the animal.
- Remove the blood and the blood vessels lining one side of the testis;
- Weight the testis
- Transfer the testis in a plastic dish on ice.

From this step on, testis and spermatozoa should be handled at 4°C (on ice).

- Add 9 mL cold StorFish per g testis. For sperm storage, the dilution should be 9 mL/g.
- Cut the testis into 0.5 cm^2 pieces (more or less depending on the size of the testis)
- Allow the mature spermatozoa to leak freely from the opened testicular canals. A gentle shaking can be applied. Avoid squeezing the testis.
- After about 10 min, filter the sample onto a 350 µm nylon mesh, to get rid of the bigger tissue pieces.
- Store the sperm for 1 h (minimum) at 4°C (or on ice), to allow final maturation of the released spermatozoa.

4. Sperm quality assessment prior to cryopreservation

The sperm quality parameter the most widely used is the motility percentage. However, when the spermatozoa are collected during the spawning season, a test prior to cryopreservation is not always mandatory (unless sperm is stored for more than 18h).

4.1. Sperm motility activation solution

The 10X solution is ActiFish, obtained from IMV Technology under the ref 018274
https://www.imv-technologies.com/our-solutions/fish/detail/product/actifish.html

Once diluted 10 times, this 1X solution at 300 mOsm/kg and is devoid of potassium, thereby allowing motility activation in salmonid species.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C).

- Dilute the testicular sperm after filtration 50 times
- On the glass slide, mix 1 µL diluted sperm with 20 µL ActiFish 1X containing 5 mg/mL BSA to prevent spermatozoa from sticking to the glass slide
- Add the coverslip (not mandatory)

- Immediately estimate the percentage of spermatozoa with a rapid and straightforward movement, on several frames at different height of the droplet.

The number of spermatozoa per field of view should range between 100 and 200. Too many cells may induced overestimation of the motility percentage, whereas too less lead to underestimation. Automated motility assessment devices can also be used, but they are not mandatory with regards to the information needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (> 90%) can be reliably used for cryopreservation. When the samples are very precious, lower quality is acceptable, but beware that survival after thawing will be lower.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Most of the collections at INRA were cryopreserved with Cryofish from IMV Technologies, a saline solution to which egg yolk and DMSO are added:
Cryofish 8 volumes, egg yolk 1 volume, DMSO 1 volume.

Since 2017, the new IMV Technology media without animal proteins was used, Freezefish (ref 026520), to which methanol (MeOH) was added:
Freezefish 9 volumes, MeOH 1 volume.

One advantage of using MeOH (over DMSO) is that at thawing, viability of the thawed spermatozoa can last up to 60 min in the cryopreservation medium, thereby allowing some lag time prior to fertilization.

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute 1 volume of sperm with 3 volumes of cryoprotectant solution (at 4 °C) and mix gently.

Use 0.5 mL bovine straws (www.imv-technologies.com, Ref: 014650 White), or 0.5 mL CBS straws (ref CBS 014650 from IMV Technologies).

The straws are usually filled with the MRS1 DUAL automatic machine (IMV Technologies ref 022989 230 V). They can also be filled with a P1000 pipette when a small number of straws is to be filled. If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions.

No equilibration time is mandatory, and no damage is induced by a 60 min storage time on ice

5.3. Freezing device and container type

The straws layered on a 100 straws rack (IMV Technology ref 007117) are set on a 3-cm thick styrofoam raft floating above 10 cm liquid nitrogen in a closed insulated box (L*l*h= 760*400*350

mm). After a 10-min exposure to liquid nitrogen vapour, the straws are plunged into liquid nitrogen and stored.

5.4. Cooling programme (if available)

Insulated box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

The straws are frozen in a water bath at 37°C for 10 s. These temperature and duration allows that the straw content is rapidly thawed, whereas the inside temperature does not rise above 4°C (MeOH and DMSO are very toxic molecules at room temperature).

6.2. Sperm washing (if needed)

Not recommended, as trout spermatozoa are very fragile upon thawing. Thank to the high dilution rate upon fertilization, the cryoprotectant toxicity does not alter egg quality.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing when DMSO is used. With methanol, a lag time of 60 min is allowed without impairment of the fertilisation rate (Horvath, A., Labbe, C., Jesensek, D., Hoitsy, G., Bernath, G., Kaczko, D., Bokor, Z., Urbanyi, B., 2015. Post-thaw storage of sperm from various salmonid species. Journal of Applied Ichthyology 31, 119-124.)

6.4. Thawed sperm quality assessment

Sperm quality can be assessed by motility percentage (5-60 %) as described in 4.2. Because motility is hardly correlated to fertilization rate, it is recommended to perform a fertility test on a fraction of the straw collection.
Storage duration in liquid nitrogen does not alter sperm quality, provided that care is taken to maintain the straw temperature below -150°C (beware of the straw heating during sorting and manipulation of the collections).

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

Nile tilapia (Oreochromis niloticus) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of **INRA LPGP research unit**

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Procedure written by Catherine Labbé, INRA, France, 2018

Version 1_INRA_2018

1. Objectives of the procedure and areas of application

Preservation of pure tilapia strains, for aquaculture or research applications.

The cryopreservation procedure is described for sperm obtained by stripping of the males. However, because of the small amount of sperm that is obtained (0.3 mL), the procedure can also be successfully applied to sperm collected from mature testis, dilacerated in SFMM (see composition below) and frozen the same way as sperm obtained by striping.

2. Bibliographical reference(s) for the described protocol (*if available*)

The following procedure was not published by INRA, due to contracts with IMV Technologies. However, tilapia sperm cryopreservation was studied and described in

Chao NH, Chao WC, Liu KC, Liao IC: **The properties of tilapia sperm and its cryopreservation**. *J Fish Biol* 1987, **30**:107-118.

Rana KJ, McAndrew BJ: **The viability of cryopreserved tilapia spermatozoa**. *Aquaculture* 1989, **76**:335-345.

3. Fish manipulation and sperm collection

3.1. Fish hormonal treatment

No treatment is required. Sperm should be collected on the dominant male in order to optimize milt quantity

3.2. Fish anaesthesia before manipulation

Sperm striping can be carried on without anaesthesia, by expert hands in order to reduce the handling time and the stress to the fish.

When testicular sperm is collected, males are anaesthetized with tricaine 100 mg/L prior to euthanasia by a blow on the head, according to the recommendation of the local animal welfare committee.

3.3. Extender composition for sperm storage (*if needed*)

Testicular sperm is manipulated into the STORFISH extender. Stripped sperm is used without dilution. It is important to cryopreserve the spermatozoa the day of the collection.

The 10X STORFISH extender can be purchased at the IMV Technologies company, under the ref 018500
https://www.imv-technologies.com/our-solutions/fish/detail/product/storfish-1-litre-qsp-10-litres-.html

3.4. Sperm collection and storage

FOR STRIPPING:

The sperm is collected with a catheter at the urogenital pore, while applying gentle pressure on the abdomen.

FOR TESTICULAR SPERM
- Remove the testis from the animal.
- Remove the blood and the blood vessels lining one side of the testis;
- Weight the testis
- Transfer the testis in a plastic dish on ice.

From this step on, testis and spermatozoa should be handled at 4°C (on ice).
- Add 4 mL cold StorFish per g testis.
- Cut the testis into 0.5 cm^2 pieces (more or less depending on the size of the testis)
- Allow the mature spermatozoa to leak freely from the opened testicular canals. A gentle shaking can be applied. Avoid squeezing the testis.
- After about 10 min, filter the sample onto a 40 µm nylon mesh, to get rid of the bigger tissue pieces.
- Centrifuge the sperm suspension 250 g, 4°C, 15 min and dilute the cells with STOREFISH (1 volume sperm + 9 volumes extenderl)
- Store the diluted sperm at 4°C (or on ice) prior to cryopreservation (the same day).

4. Sperm quality assessment prior to cryopreservation

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

Water with 5 mg/mL BSA to prevent spermatozoa from sticking to the glass.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C).

- Dilute the sperm 50 times
- On the glass slide, mix 1 µL diluted sperm with 20 µL water containing 5 mg/mL BSA
- Add the coverslip (not mandatory)
- Immediately estimate the percentage of spermatozoa with a rapid and straightforward movement, on several frames at different height of the droplet.

The number of spermatozoa per field of view should range between 100 and 200. Too many cells may induced overestimation of the motility percentage, whereas too less lead to underestimation. Automated motility assessment devices can also be used, but they are not mandatory with regards to the information needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (> 80%) can be reliably used for cryopreservation. When the samples are very precious, lower quality is acceptable, but beware that survival after thawing will be lower.

5. Sperm cryopreservation procedure

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5.1. Composition of the cryoprotectant solution

The saline solution is from Mounib (1978):
$KHCO_3$ 100 mM (mw 100 g/mol), sucrose 125 mM (mw 342.3 g/mol), reduced gluthatione 6.5 mM (mw 307.3 g/mol) in distilled water.

The Cryofish from IMV Technologies can also be used.

To the saline, 10 % (vol /vol) egg yolk and 10 % methanol (MeOH) are added:
Cryofish or Mounib 8 volumes, egg yolk 1 volume, MeOH 1 volume.

We checked that DMSO, dimethylacetamide and propylene glycol do not provide the same cryoprotection as methanol.

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute 1 volume of sperm with 3 volumes of cryoprotectant solution (at 4 °C) and mix gently.

Use 0.5 mL bovine straws (www.imv-technologies.com, Ref: 014650 White), or 0.5 mL CBS straws (ref CBS 014650 from IMV Technologies).

The straws are filled with a P1000 pipette. If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions.

No equilibration time is mandatory.

5.3. Freezing device and container type

The straws layered on a 100 straws rack (IMV Technology ref 007117) are set on a 3-cm thick styrofoam raft floating above 10 cm liquid nitrogen in a closed insulated box (L*l*h= 760*400*350 mm). After a 10-min exposure to liquid nitrogen vapour, the straws are plunged into liquid nitrogen and stored.

5.4. Cooling programme (if available)

Insulated box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

The straws are frozen in a water bath at 37°C for 10 s. These temperature and duration allows that the straw content is rapidly thawed, whereas the inside temperature does not rise above 4°C (MeOH is very toxic molecules at room temperature).

6.2. Sperm washing (if needed)

Procedure written by (name, email)**:** *Name*: **Catherine Labbe, INRA LPGP** *Email:* **catherine.labbe@inra.fr**	**Procedure validated by** (name, email): *Name*:
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Not recommended, as tilapia spermatozoa are very fragile upon thawing. Thank to the high dilution rate upon fertilization, the cryoprotectant toxicity does not alter egg quality.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing.

6.4. Thawed sperm quality assessment

Sperm quality can be assessed by motility percentage (5-60 %) as described in 4.2. Because motility is hardly correlated to fertilization rate, it is recommended to perform a fertility test on a fraction of the straw collection.
Storage duration in liquid nitrogen does not alter sperm quality, provided that care is taken to maintain the straw temperature below -150°C (beware of the straw heating during sorting and manipulation of the collections).

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

Procedure written by (name, email)**:** *Name*: **Catherine Labbe, INRA LPGP** *Email:* **catherine.labbe@inra.fr**	**Procedure validated by** (name, email): *Name*:
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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

Brown trout (Salmo trutta) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of
INRA PEIMA infrastructure and INRA LPGP research unit

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Procedure written by Catherine Labbé, INRA, France, 2018

Version 1_INRA_2018

1. Objectives of the procedure and areas of application

Cryopreservation of valuable genetic material using sperm a genetic support.

2. Bibliographical reference(s) for the described protocol (*if available*)

Procedure published in :
Labbe C, Maisse G: **Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity**. *Aquaculture* 2001, **201**(3-4):287-299.

3. Fish manipulation and sperm collection

3.1. Fish hormonal treatment

Sperm is collected during the spawning season of the brown trout (November-January in Brittany, France). No hormonal treatment is required.

3.2. Fish anaesthesia before manipulation

The males are anaesthetized with tricaine 50 mg/L according to the recommendation of the local animal welfare committee.

3.3. Extender composition for sperm storage (*if needed*)

The 10X StorFish extender can be purchased at the IMV Technologies company, under the ref 018500
https://www.imv-technologies.com/our-solutions/fish/detail/product/storfish-1-litre-qsp-10-litres-.html
Although it is better to cryopreserve sperm the same day as collection, sperm can be diluted (1/2 to 1/10) and stored at 4°C 1-2 days.

3.4. Sperm collection and storage

Sperm is collected by gentle stripping of the males. The urine bladder should be emptied by gentle pressure prior to sperm collection. Use wet cloth to manipulate and hold the fish, to prevent mucus removing.
Sperm can be stored at 4°C (on ice) in its seminal fluid, or be diluted with Storfish. For duration longer than 6-8h, add oxygen to the tube and make sure that the liquid layer is thinner than 1 cm, to allow oxygen diffusion.

4. Sperm quality assessment prior to cryopreservation

The sperm quality parameter the most widely used is the motility percentage. However, when the spermatozoa are collected during the spawning season, a test prior to cryopreservation is not always mandatory (unless sperm is stored for more than 18h).

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4.1. Sperm motility activation solution

The 10X solution is ActiFish, obtained from IMV Technology under the ref 018274
https://www.imv-technologies.com/our-solutions/fish/detail/product/actifish.html

Once diluted 10 times, this 1X solution at 300 mOsm/kg and is devoid of potassium, thereby allowing motility activation in salmonid species.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C).

- Dilute the testicular sperm after filtration 50 times
- On the glass slide, mix 1 µL diluted sperm with 20 µL ActiFish 1X containing 5 mg/mL BSA to prevent spermatozoa from sticking to the glass slide
- Add the coverslip (not mandatory)
- Immediately estimate the percentage of spermatozoa with a rapid and straightforward movement, on several frames at different height of the droplet.

The number of spermatozoa per field of view should range between 100 and 200. Too many cells may induced overestimation of the motility percentage, whereas too less lead to underestimation. Automated motility assessment devices can also be used, but they are not mandatory with regards to the information needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (> 90%) can be reliably used for cryopreservation. When the samples are very precious, lower quality is acceptable, but beware that survival after thawing will be lower.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Most of the collections at INRA were cryopreserved with Cryofish from IMV Technologies, a saline solution to which egg yolk and DMSO are added:
Cryofish 8 volumes, egg yolk 1 volume, DMSO 1 volume.

Since 2017, the new IMV Technology media without animal proteins was used, Freezefish (ref 026520), to which methanol (MeOH) was added:
Freezefish 9 volumes, MeOH 1 volume.

One advantage of using MeOH (over DMSO) is that at thawing, viability of the thawed spermatozoa can last up to 60 min in the cryopreservation medium, thereby allowing some lag time prior to fertilization.

The old Mounib cryoprotectant can also be used with brown trout sperm: 8 volumes Mounib solution (125 mM Sucrose; 6.5 mM Reduced gluthatione; 100 mM KHCO ; Mounib, 1978) 1 volume avian egg yolk emulsion, 1 volume DMSO.

Mounib, M.S., 1978. Cryogenic preservation of fish and mammalian spermatozoa. J. Reprod. Fertil. 53, 13–18.

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute 1 volume of sperm with 3 volumes of cryoprotectant solution (at 4 °C) and mix gently. *Research is in progress to change this ratio and to increase the sperm number in the straws.*

Use 0.5 mL bovine straws (www.imv-technologies.com, Ref: 014650 White), or 0.5 mL CBS straws (ref CBS 014650 from IMV Technologies).

The straws are usually filled with the MRS1 DUAL automatic machine (IMV Technologies ref 022989 230 V). They can also be filled with a P1000 pipette when a small number of straws is to be filled. If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions.

No equilibration time is mandatory, and no damage is induced by a 60 min storage time on ice

5.3. Freezing device and container type

The straws layered on a 100 straws rack (IMV Technology ref 007117) are set on a 3-cm thick styrofoam raft floating above 10 cm liquid nitrogen in a closed insulated box (L*l*h= 760*400*350 mm). After a 10-min exposure to liquid nitrogen vapour, the straws are plunged into liquid nitrogen and stored.

5.4. Cooling programme (if available)

Insulated box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

The straws are frozen in a water bath at 37°C for 10 s. These temperature and duration allows that the straw content is rapidly thawed, whereas the inside temperature does not rise above 4°C (MeOH and DMSO are very toxic molecules at room temperature).

6.2. Sperm washing (if needed)

Not recommended, as trout spermatozoa are very fragile upon thawing. Thank to the high dilution rate upon fertilization, the cryoprotectant toxicity does not alter egg quality.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing when DMSO is used. With methanol, a lag time of 60 min is allowed without impairment of the fertilisation rate (Horvath, A., Labbe, C., Jesensek, D., Hoitsy, G., Bernath, G., Kaczko, D., Bokor, Z., Urbanyi, B., 2015. Post-thaw storage of sperm from various salmonid species. Journal of Applied Ichthyology 31, 119-124.)

6.4. Thawed sperm quality assessment

Procedure written by (name, email)**:**	**Procedure validated by** (name, email):
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Contact information (address, city, country)**: INRA PEIMA, Barrage du Drennec, F-29450 SIZUN**	

Sperm quality can be assessed by motility percentage (5-60 %) as described in 4.2. Because motility is hardly correlated to fertilization rate, it is recommended to perform a fertility test on a fraction of the straw collection.
Storage duration in liquid nitrogen does not alter sperm quality, provided that care is taken to maintain the straw temperature below -150°C (beware of the straw heating during sorting and manipulation of the collections).

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

Procedure written by (name, email)**:** *Name*: **Catherine Labbe, INRA LPGP** *Email:* **catherine.labbe@inra.fr**	**Procedure validated by** (name, email): *Name*: **Lionel Goardon, INRA PEIMA** *Email:* **lionel.goardon@inra.fr**
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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

EUROPEAN CATFISH (*SILURUS GLANIS*) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

University of South Bohemia in Ceske Budejovice
Faculty of Fisheries and Protection of Waters,
Research Institute of fish Culture and Hydrobiology

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Procedure written by Yevhen Horokhovatskyi, JU, Czech Republic, 2018

Version 1_JU_2018

1. Objectives of the procedure and areas of application

Cryopreservation of European catfish sperm for cryobanking of valuable genetic resources, and for laboratory and farm use

2. Bibliographical reference(s) for the described protocol (*if available*)

One example of the procedure description and application is presented in the following publication:

Linhart O, Rodina M, Flajshans M, Gela D, Kocour M: **Cryopreservation of European catfish Silurus glanis sperm: Sperm motility, viability, and hatching success of embryos**. *Cryobiology* 2005, **51**(3):250-261.
https://doi.org/10.1016/j.cryobiol.2005.07.005

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (5 and more years old) fish male, during natural spawning season (May - July). The water temperature should be in range 22-23 °C. To induce spermiation, fish should be injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution. The concentration of carp pituitary extract in physiological salt vary in range 1-5 mg/ml depending on fish body weight, while dose for 1 kg of fish is 5 mg. Inject the fish in the muscular tissue along the side of the dorsal fin at a 45-degree angle pointing the needle towards the head of the fish. The maximal volume of suspension that can be injected to one side of the dorsal fin should not exceed 1 ml. Sperm should be collected 24 hours after injection.

3.2. Fish anaesthesia before manipulation

Before each injection and gamete collection, fish should be anaesthetized in a solution containing 2-phenoxyethanol (1:1000).

3.3. Immobilization solutions composition for sperm storage

For 100 ml: add the components to 80 ml of distilled water and adjust the pH to 7.0 with HCl. Thereafter, adjust the volume of the immobilization solutions to 100 ml with distilled water.

Immobilization solutions		For 100 ml solution
NaCl (58.4 g/mol)	200 mM	1169 mg
Tris (121.1 g/mol)	30 mM	363 mg

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pH 7.0

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage into individual plastic (250 ml) containers filled with 10 ml of immobilizing solution (IS). Maximally, 8 ml of sperm should be taken into one container to keep the dilution rate at 1:0.8 (IS/sperm) to prevent spontaneous initiation of motility. After collection, the containers should store under aerobic conditions on ice (4 °C). Usually, the volume collected from 5-12 years old males 7-25 kg males varies (9-15 ml), and the spermatozoa concentration ranges between 0.6×10^9 and 1.6×10^9 spermatozoa/mL semen.

4. Sperm quality assessment

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

To induce sperm motility use freshwater.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C). Activate spermatozoa movement by mixing 1 µl of sperm with 49 µl of swimming medium supplemented with 0.1% BSA on a glass slide prepositioned on the microscope stage. The final dilution should be 1:50. BSA should be added to prevent sperm heads from sticking to the glass slide. The number of spermatozoa per field of view should range between 70 and 100. Moving through different levels of the droplet, estimate sperm motility percentage. The glass coverslip in this case is not needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (>80%) can be used for cryopreservation.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

For 100 ml: take 50 ml of DMSO and 50 ml of 1,2-propanediol.

Cryoprotectant solution		For 100 ml solution
Me2SO (DMSO)	50% (volume)	50 ml

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1,2-propanediol	50% (volume)	50 ml

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute 92% of extended sperm with 8% of cryoprotectant solution at 4 °C, gently mixing. During the 10 min equilibration time, place the obtained suspension into 0.5 mL plastic straws (www.imv-technologies.com, Ref: 014650 White). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing them.

5.3. Freezing device and container type

Whereupon, put filled straws on a 3-cm thick styrofoam raft (dimensions: 40 × 20 × 3 cm) and transfer them to a styrofoam box (dimensions: 52 × 33 × 30 cm), filled to a depth of 10 cm with liquid nitrogen. The detailed illustration is presented in the following publication:
Horokhovatskyi Y, Rodina M, Asyabar HD, Boryshpolets S, Dzyuba B: **Consequences of uncontrolled cooling during sterlet (Acipenser ruthenus) sperm cryopreservation on post-thaw motility and fertilizing ability**. *Theriogenology* 2017, **95**:89-95.
https://doi.org/10.1016/j.theriogenology.2017.03.007.

After a 10-min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen.

5.4. Cooling programme (if available)

Styrofoam box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 40 °C for 6 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary from sample to sample.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.

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- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	**1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;**
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

SENEGALESE SOLE (Solea senegalensis) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

Center for Marine Sciences – CCMAR

Procedure written by Ana Luisa Santos, CCMAR, Portugal, 2018

Version 1_CCMAR_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Senegalese sole sperm for laboratory and farm use.

2. Bibliographical reference(s) for the described protocol

One example of the procedure description and application is presented in the following publication:
M.F. Riesco, C. Oliveira, F. Soares, P.J. Gavaia, M.T. Dinis, E. Cabrita, Solea senegalensis sperm cryopreservation: New insights on sperm quality, Plos One 12 (2017).
https://doi.org/10.1371/journal.pone.0186542

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (2 years and older) fish male, during natural spawning season (15th of March- end of June). Breeders need to be maintained at temperatures of 18±1.2°C. No hormonal treatment is needed although 750 mg/Kg hCG can stimulate sperm production.

3.2. Fish anaesthesia before manipulation

Fish need to be anaesthetized with 300 ppm (300 µL/L) 2-phenoxyethanol during 10 min before sperm collection.

3.3. Extender composition for sperm storage (*if needed*)

Extender solution (Mounib solution)		For 100 ml Solution
Sucrose (342.3 g/mol)	125 mM	4.28 g
KHCO3 (100.115 g/mol)	100mM	1 g
Reduced Glutathione (307.32 g/mol)	6.5 mM	199.8 mg

3.4. Sperm collection and storage

Dry out urogenital pore with paper towel and collect the sperm using a syringe (without needle) or a 20 µl micropipette by gently pressing the testes on the fish blind side. Store the samples in eppendorfs and store it on ice in a Styrofoam rack until further analysis. Discard samples contaminated with water, urine or faeces. Alternatively, if samples needed to be transported, sperm centrifugation can be done to eliminate seminal plasma and any urine contamination, substituting the removed volume by Ringer solution.

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Contact information: CCMAR, University of Algarve, Campus of Gambelas, Faro, Portugal	

Sperm can be collected almost all year round even at winter temperatures (10-13ºC). The volume collected from individuals older than 2 years varies between males and type of broodstock. In some stocks, sperm volume collected ranged from 5 to 20 µl in G1 broodstocks and 10–80 µl in wild-captured broodstocks, while in others these values were slightly higher. Cell density and sperm production (total spermatozoa per stripping) ranged from 0.7 to 1.3x10^9 spz/ml and 20x10^6 in G1 males to values of 1–2x10^9 spz/ml and 40–60x10^6 spermatozoa for the wild-captured males.

4. Sperm quality assessment

The sperm quality is assessed using several parameters. Motility and cell viability are the most widely used.

4.1. Sperm motility activation solution

Seawater (SW) at 21°C and 35 ppt salinity.

4.2. Sperm motility assessment

Motility is determined using Computer Assisted Sperm Analysis (CASA system) and ISAS software (ISAS, Proiser, Valencia, Spain). Samples are carried out in a Makler chamber under a 10x negative-phase contrast objective coupled with a digital camera (Basler A312f C-mount, Germany) set for 50 fps.

Prior the analysis, sperm need to be diluted 1:9 in a non-activating medium (Ringer solution). After thawing, activate spermatozoa movement by mixing 1 µl sperm and 5 µl activation solution (SW) and assess motility.

Non-activating medium (Ringer solution)		For 100 ml Solution
HEPES (238.3012 g/mol)	20 mM	476.6 mg
KH_2PO_4 (74.5513 g/mol)	5 mM	37.3 mg
$MgSO_4$ (120.366 g/mol)	1 mM	12 mg
$CaCl_2$ (110.98 g/mol)	1 mM	11.1 mg
NaCl (58.4 g/mol)	136 mM	794 mg
KCl (74.5513 g/mol)	4.7 mM	35 mg
pH 7.5 300 mOsm/Kg		

4.3. Sperm quality threshold

Only sperm samples that display total motility higher than 75% can be used for cryopreservation.

4.4. Sperm Viability assessment

Dilute 5 µl of sperm in 500 µl of 1% NaCl buffer and add 2.5 µl of propidium iodide PI at 1

µl/ml at final concentration. Analyse in a flow cytometer after 5 min incubation in the dark.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Cryoprotectant solution		For 100 ml solution
Extender solution		80 ml
DMSO (78.13 g/mol) (Volume)	10%	10 ml
Egg Yolk (volume)	10%	10 ml

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute one volume of sperm with two volumes of cryoprotectant solution. During 2 min equilibration time, load sperm with cryoprotectant solution into 0.25 ml plastic straws (https://www.imv-technologies.com, Ref. 005565). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing.

5.3. Freezing device and container type

Whereupon, put filled straws on a 2 cm horizontal rack above liquid nitrogen in a Styrofoam box. After a 10 min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen and store it in a nitrogen container.

5.4. Cooling programme (if available)

No available

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 25°C for 10 s.

6.2. Sperm washing (if needed)

Not needed

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary with time.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.

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- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

Procedure written by (name, email)**:**	**Procedure validated by** (name, email):
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Work package number	**WP3**	**Mains actors**	**1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;**
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

GILTHEAD SEABREAM (Sparus aurata) SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

Center for Marine Sciences – CCMAR

Procedure written by Ana Luisa Santos, CCMAR, Portugal, 2018

Version 1_CCMAR_2018

1. Objectives of the procedure and areas of application

Cryopreservation of gilthead seabream sperm for cryobanking of valuable genetic resources, and for laboratory and farm use.

2. Bibliographical reference(s) for the described protocol

One example of the procedure description and application is presented in the following publication:

E. Cabrita, V. Robles, S. Cunado, J.C. Wallace, C. Sarasquete, M.P. Herraez, Evaluation of gilthead sea bream, Sparus aurata, sperm quality after cryopreservation in 5ml macrotubes, Cryobiology 50 (2005) 273-284.
https://doi.org/10.1016/j.cryobiol.2005.02.005

S.M. Guerra, D.G. Valcarce, E. Cabrita, V. Robles, Analysis of transcripts in gilthead seabream sperm and zebrafish testicular cells: mRNA profile as a predictor of gamete quality, Aquaculture 406 (2013) 28-33.
https://doi.org/10.1016/j.aquaculture.2013.04.032

3. Fish manipulation

3.1. Fish hormonal treatment

No hormonal treatment is required to induce spermiation. Sperm is collected from mature fish males, during reproductive season, which may vary according to temperature/photoperiod control. In broodstocks maintained in natural conditions, sperm can be collected from November to February.

3.2. Fish anaesthesia before manipulation

Gilthead seabream males are anaesthetised with 2-phenoxyethanol in the broodstock tanks at a 100 ppm (100 µL/L) concentration. Transport them to a small tank containing 125 mg/l MS-222 or 300 ppm (µL/L) 2-phenoxyethanol and wait 5 to 10 minutes. Thereafter collect the fish from the anaesthesia tank and wash it with seawater to remove anaesthesia from skin.

3.3. Extender composition for sperm storage

The extender used for this species is a 1% NaCl solution (~300 mOsm/Kg).

Extender solution		For 100 ml solution
NaCl (58.44 g/mol)	1%	1 g

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Contact information: CCMAR, University of Algarve, Campus of Gambelas, Faro, Portugal	

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage with a 1 ml syringe without a needle and repeat this procedure until all the sperm is collected avoiding contamination of sperm with water, mucus, urine or faeces. Store sperm in polystyrene tubes (Falcon tubes) at approximately 7 °C until further use. Protect the tubes in a styrofoam rack to avoid the contact between the tubes and the ice.
Usually, the volume collected from 1-2 years old males varies between males and breeding season (2-7 ml).

4. Sperm quality assessment

The sperm quality is assessed using several parameters. Motility and cell viability are the most widely used.

4.1. Sperm motility activation solution

Sperm motility is activated using seawater.

4.2. Sperm motility assessment

Motility is determined using Computer Assisted Sperm Analysis (CASA) under a phase-contrast light microscopy equipped with 10x magnification. Predilute fresh sperm (100-500X) with 1% NaCl. To activate motility use 1 µl of sperm to 10 µl of seawater in a Makler chamber.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (>80%), and densities higher than 2.5 x 10^9 should be used for cryopreservation.

4.4. Sperm Viability assessment

Add 2.5 µl propidium iodide (PI) at a concentration of 2.4 mM and 0.5 µl SYBR-14 at a concentration of 0.1 mM to 30 µl of sperm and 500 µl of extender (NaCl 300 mOsmol/Kg). After 5 min incubation in the dark, analyse in a flow cytometer.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

Cryoprotectant solution		For 100 ml solution
Extender solution		95 ml
DMSO (78.13 g/mol)	5%	5 ml
Optional*: BSA (66430.3 g/mol)	10 mg/ml	1 g

*BSA may improve post-thaw quality in bad quality samples.

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5.2. Sperm preparation for cryopreservation

Dilute sperm in cryoprotectant solution 1:6 (sperm:cryoprotectant, v/v). Load the sperm into 0.5 ml straws (www.minitube.com, Ref. 13408) for 4 min (equilibration time) and place it in a horizontal rack.

5.3. Freezing device and container type

Place the rack in the Styrofoam box containing liquid nitrogen and perform sperm freezing at 2 cm above the surface of liquid nitrogen in vapour phase for 10 min. After 10 min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen and store the samples in a nitrogen container.

5.4. Cooling programme (if available)

No available

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 25°C for 30 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa may change with time.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

Procedure written by (name, email):	**Procedure validated by** (name, email):
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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

TENCH (*TINCA TINCA)* SPERM CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of

University of South Bohemia in Ceske Budejovice
Faculty of Fisheries and Protection of Waters,
Research Institute of fish Culture and Hydrobiology

Fakulta rybářství
a ochrany vod
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Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Procedure written by Yevhen Horokhovatskyi, JU, Czech Republic, 2018

Version 1_JU_2018

1. Objectives of the procedure and areas of application

Cryopreservation of Tench sperm for cryobanking of valuable genetic resources, and for laboratory and farm use

2. Bibliographical reference(s) for the described protocol (*if available*)

One example of the procedure description and application is presented in the following publication:
Rodina M, Gela D, Kocour M, Alavi SMH, Hulak M, Linhart O: **Cryopreservation of tench, Tinca tinca, sperm: Sperm motility and hatching success of embryos**. *Theriogenology* 2007, **67**(5):931-940.
https://doi.org/10.1016/j.theriogenology.2006.11.007

3. Fish manipulation

3.1. Fish hormonal treatment

Usually, sperm is collected from mature (4 and more years) fish male, during natural spawning season (June - July). The water temperature should be in range 18-22 °C. To induce spermiation, fish should be injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution. The concentration of carp pituitary extract in physiological salt vary and can be within 1-5 mg/ml depending on fish body weight, while dose for 1 kg of fish is 1 mg. Inject the fish in the muscular tissue along the side of the dorsal fin at a 45-degree angle pointing the needle towards the head of the fish. The maximal volume of suspension that can be injected to one side of the dorsal fin should not exceed 1 ml. Thirty hours after injection collect the sperm.

3.2. Fish anaesthesia before manipulation

Before each injection and gamete collection, anaesthetize the fish in a solution containing 2-phenoxyethanol (1:1000).

3.3. Immobilization solution for sperm storage

Immobilization solution		For 100 ml solution
NaCl (58.44 g/mol)	180 mM	1052 mg
KCl 74.55 (g/mol)	2.7 mM	20.1 mg
$CaCl_2$* $2H_2O$ (147.02 g/mol)	1.4 mM	20.6 mg
$NaHCO_3$ (84.00 g/mol)	2.4 mM	20.2 mg

3.4. Sperm collection and storage

Dry out urogenital area with paper towel and collect the sperm by abdominal massage into individual plastic (5 ml) syringes containing 2 ml of immobilization solution leaving 2 ml of

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the volume for air inside and store on ice at 4 °C. To prevent spontaneous initiation of motility, keep the dilution rate 2:1 (immobilization solutions : sperm). Avoid contamination of sperm with water or faeces. Usually, the volume of sperm collected from fish with 1 kg of body weight is up to 1 ml, while the spermatozoa concentration ranges between 1×10^9 and 3×10^9 spermatozoa/mL semen.

4. Sperm quality assessment

The sperm quality parameter the most widely used is the motility percentage.

4.1. Sperm motility activation solution

To induce sperm motility use freshwater.

4.2. Sperm motility assessment

Motility is subjectively estimated under a dark-field microscope equipped with ×10 binocular and ×20 objective, at a room temperature (18-20 °C). Activate spermatozoa movement by mixing sperm and activation solution at an approximate ratio of 1:5000. For fresh sperm, place 50 µl of freshwater on a glass slide under the microscope and add sperm using the tip of dissecting needle by mixing thoroughly for 2 s. The number of spermatozoa per field of view should range between 70 and 100. Moving through different levels of the droplet, estimate sperm motility percentage. The glass coverslip in this case is not needed.

4.3. Sperm quality threshold

Only sperm samples that display high and progressive motility (>80%) can be used for cryopreservation.

5. Sperm cryopreservation procedure

5.1. Composition of the cryoprotectant solution

For 100 ml: take 50 ml of DMSO and 50 ml of 1,2-propanediol.

Cryoprotectant solution		For 100 ml solution
Me2SO (DMSO)	50% (volume)	50 ml
1,2-propanediol	50% (volume)	50 ml

5.2. Sperm manipulation before cryopreservation

Before freezing, dilute 90% of extended sperm with 10% of cryoprotectant solution (at 4 °C) gently mixing. During the 10 min equilibration time, place the obtained suspension into 0.5 mL plastic straws (www.imv-technologies.com, Ref: 014650 White). If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions. For laboratory experiments, they can be used without sealing them.

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5.3. Freezing device and container type

Whereupon, put filled straws on a 3-cm thick styrofoam raft (dimensions: 40 × 20 × 3 cm) and transfer them to a styrofoam box (dimensions: 52 × 33 × 30 cm), filled to a depth of 10 cm with liquid nitrogen. The detailed illustration is presented in the following publication Horokhovatskyi Y, Rodina M, Asyabar HD, Boryshpolets S, Dzyuba B: **Consequences of uncontrolled cooling during sterlet (Acipenser ruthenus) sperm cryopreservation on post-thaw motility and fertilizing ability**. *Theriogenology* 2017, **95**:89-95. https://doi.org/10.1016/j.theriogenology.2017.03.007.

After a 10-min exposure to liquid nitrogen vapour, plunge the straws directly into liquid nitrogen.

5.4. Cooling programme (if available)

Styrofoam box gives fast but uncontrolled cooling rate.

6. Sperm thawing procedure

6.1. Thawing device and programme

Thaw the frozen straws in a water bath at 40 °C for 6 s.

6.2. Sperm washing (if needed)

Not needed.

6.3. Time of sperm viable state after thawing

Use the sperm for fertilization or analysis immediately after thawing as the viability of spermatozoa vary from sample to sample.

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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Work package number	**WP3**	**Mains actors**	1 - INRA ; 3 – UoS ; 6 – NAIK ; 7 – IFREMER ;9 – JU ; 20 – CCMAR ;
Work package title	NA3 – Common standards and tools		
Task	Task 3.3: Cryobanking and cryopreservation of genetic resources		

RAINBOW TROUT (Oncorhynchus mykiss) spermatogonial stem cell (SSCs) CRYOPRESERVATION

This procedure was written during the AQUAEXCEL^{2020} project, thanks to the contribution of **INRA LPGP research unit**

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The procedure was established from projects funded by AQUAEXCEL^{2020} and by CRB Anim project 2013-2019_ ANR-11-INBS-0003

Procedure written by Catherine Labbé, INRA, France, 2018

Version 1_INRA_2018

1. Objectives of the procedure and areas of application

Spermatogonial stem cells (SSCs) are diploid cells found in immature and mature testis of juvenile and adult trout. When isolated and grafted into a recipient fry, these cells have the ability to colonize the embryonic gonad and to ultimately differentiate into spermatozoa or ova, depending on the fry sex. This bipotency allows that functional eggs are produced from cryopreserved SSCs.

Cryopreservation of the SSCs allows the cryobanking of these valuable stem cells. Indeed, not eggs and embryos can be cryopreserved in fish. The SSCs thereby allow that eggs are produced after transplantation of the thawed SSCs into recipient fries, maturation and reproduction. The eggs can be fertilized by cryopreserved sperm from the same strain or from another genetic background.

2. Bibliographical reference(s) for the described protocol (*if available*)

References for the use of SSCs is found in Goro Yoshizaki's work:

- Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., Yoshizaki, G., 2007. Production of trout offspring from triploid salmon parents. Science 317, 1517.
- Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proceedings of the National Academy of Sciences of the United States of America 103, 2725-2729.
- Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y., 2011. Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 6, 55-61.

Purification and characterization of SSCs is found in Florence Le Gac's work:

- Bellaiche, J., Lareyre, J.J., Cauty, C., Yano, A., Allemand, I., Le Gac, F., 2014. Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. Biol Reprod 90, 79.

The publication of the work leading to this cryopreservation procedure is not achieved yet.

3. Fish manipulation and SSCs collection

3.1. Fish anaesthesia before manipulation

In the best condition, the SSCs are collected on 9 months old males, before spermatozoa are formed in the testes.
Fish are anaesthetized with Tricaine or phenoxy-ethanol (3 mL/10 L water) and euthanasia is performed by a blow on the head, according to the recommendation of the local animal welfare committee.

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3.2. SSCs collection and storage

The whole procedure is described in Bellaiche et al (2014). After careful removal from the fish, the testes are minced, dissociated in trypsin and filtered (30 µm mesh). SSCs are pre-purified on percoll gradient (red cells removal). The day after, they are further enriched by centrifugal elutriation.

After the enrichment and concentration steps, SSCs are diluted (about 0.5 10^6 cells/mL) with L15-SpgA solution (composition below) with 0.5 % BSA (w/v), and kept at 4°C or on ice prior to cell counting and concentration.

L15-SpgA composition	SIGMA reference	MW (g/mol)	Final concentration	Quantity per L
L15 powder	L4386		12.18 g/L	12.18 g
Hepes	H3375	238.30	20 mM	4.76 g
Na bicarbonate	S6297	84.01	5 mM	0.42 g
Lactic acid	L1375	112.06	110 mg/L	110 mg
Reduced gluthation	G6013	307.32	16.10^{-3} mM	5 mg
CuCl 500 µg/L	C3279	170.48	2.10^{-6} mM	2.7 mg
Se($NaSeO_3$) 50mg/L	S5261	172.94	6.10^{-3} mM	0.8 mg
$Mn(SO_4)_2$ 0.3 mg/l	M8179	169.02	1.10^{-6} mM	169 mg
Vitamine E 20 mg/ml	T3001	472.74	30 mM	14.2 g

4. SSCs quality assessment and concentration prior to cryopreservation

4.1. SSCs counting and quality assessment

The cell suspension is counted on Malassez cell after trypan blue staining and analysis on a conventional microscope (x200). The cells with altered plasma membranes will be blue. At least 200 cells per slide should be counted. A faster way is to use a flow cytometer. The altered population will have a lower light scattering signal (SSC) and be displayed as a specific sub-population (this population is usually stained with the membrane-impermeant dye propidium iodide). At least 2000 cells should be counted.

To concentrate the cells at 20.10^6 cells/mL:

- Centrifuge the SSCs 150 g 15 min 4°C.
- Resuspend the pellet with the L15-SpgA solution without BSA so that the final concentration is 20.10^6 cells/mL.
- Store on ice at 4°C

4.2. SSCs quality threshold

The cell quality is usually good (> 90-95 % cells with intact plasma membrane), as the dead cells will burst and will not be seen during the counting. There is no specific threshold when considering the

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preciousness of these cells. Beware that the grafting efficiency of these SSCs may be reduced if too many altered cells are cryopreserved (and the losses at thawing may be high).

5. SSCs cryopreservation procedure

5.1. Composition of the cryoprotectant solution

The permeating cryoprotectant is propane, 1-2 diol. No animal product is added to the cryoprotectant solution, and the Cryo3 from the STEM ALPHA Company replaces the usual animal serum or egg yolk or BSA or milk powder.

For 10 mL Cryoprotectant solution with the cells:

- L15-SpgA solution		5 mL
- PVP-40 (polyvinylpyrrolidone 40 000 MW)	1 % (w/v)	0.1 g
- Sucrose (MW 342.3 g/mol)	50 mM	171 mg
- Cryo3 from STEM ALPHA (France)	30 % (v/v)	3 mL
- Propane, 1-2 diol	10 % (v/v)	1 mL
Mix well		
- Cell suspension at 20.10^6/mL in L15-SpgA solution		1 mL
Mix gently		

The final cell concentration in the cryoprotectant solution is 2.10^6/mL

5.2. SSCs manipulation before cryopreservation

SSCs in the cryoprotectant should be kept at 4°C or on ice. No specific equilibration time is mandatory

Use 0.5 mL bovine straws (www.imv-technologies.com, Ref: 014650 White), or 0.5 mL CBS straws (ref CBS 014650 from IMV Technologies).

The straws can be filled with a P1000 pipette. If the straws are to be used for cryobanking purposes, they should be sealed according to the manufacturer's instructions.

5.3. Freezing device and container type

The SSCs are to be cryopreserved in a programmable freezer; any type with 500 µL straws holder is suitable (Kryo 360 from Planer, Mini Digitcool from IMV Technologies etc.)

5.4. Cooling program

Loading at 0°C
-1°C/min up to -7.4°C
Hold 10 min at -7.4°C
-0.3 °C/min up to -40 °C
-2,5 °C/min up to -80 °C
Plunging into liquid nitrogen and storage into liquid nitrogen

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6. SSCs thawing procedure

6.1. Thawing device and programme

The straws are thawed in a water bath at 10°C for about 20 s. This is very slow, the straws are removed from water once all ice crystals are melted (seen by the straw content becoming transparent).

6.2. SSCs washing

- Open several straws in the same 15 mL falcon tube
- Measure the recovered volume (for SSCs dilution volume and concentration estimation)
- Dilute 10 times the SCCs with L15-SpgA solution in 5 steps with 60 s equilibration between steps.

The total recovery time prior to centrifugation should be about 20 min.

- Centrifuge 15 min, 150g, 4°C
- Resuspend the pellet in the same initial volume as the one measured after straw opening

6.3. Time of SSCs viable state after thawing

The cell suspension at about 2.10^6 cells/mL can be stored up to 3 days at 4°C. We believe that transplantation should yield better grafting rates when the cells are used the same day as thawing than later (but not tested after the first day).

6.4. Thawed SSCs quality assessment

Same as prior to cryopreservation. By flow cytometry, the population with a lower SSC value should not be above 25-30 % of the total population.

The grafting success is about 80 % of the transplanted fries (same as with fresh SSCs).

7. Safety

- Beware of local burn with liquid nitrogen, and work in a well ventilated room to prevent asphyxia.
- Trypan blue is suspected to be a carcinogenic molecule:
  - H351 suspected of causing cancer
  - H361 suspected of damaging fertility or the unborn child
- Work with gloves and lab coat. Get the security datasheet for all the products used and behave accordingly.

8. Sample traceability

This is still to be determined in the AQUAEXCEL^{2020} consortium.
Each facility has its own traceability process, but the straws should bear the date of cryopreservation, the species, and the cell type.

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