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SALMONID SPERM CRYOPRESERVATION TECHNIQUES

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This document is a practical guide to be used in conjunction with the manual: Cryopreservation of Salmonid Sperm (Cloud and Osborne 1997), which provides excellent background for the procedures and solutions, and has been used in conjunction with a workshop at the University of Idaho. The purpose of the following document is to provide lists of materials needed including sizes, types, and vendors, plus detailed descriptions of techniques with practical tips often learned only through experience. This guide is particularly useful in the absence of a hands-on practicum.

Contents:

Freezing Sperm Samples:

Materials:

[***NOTE:*** *Compound microscope with 200X objective, pH meter, balance (with 0.01g or 0.001g precision) and Shop-Vac are needed, but these items may be on site or borrowed for the duration of the spawning season. A large centrifuge is helpful in preparation of Freezing Solution, but not essential.*]

Large carboy with distilled or Milli-Q water[1]
Big Tray (2' x 2'), plastic, with cover (Figure 1)
Working platform or "drying tray" (13" x 9" x 2" glass cake pan) (Figure 1)
Test tubes in rack, one test tube per sample
Sealant powder (≥ 1/4'" deep) in small jar (inner diameter ≥4.5 cm)
Metal bubbler trough stand (Figure 2)
Bubbler trough assemblies (Figure 2), one per sample, in small tray [lid from a pipet-tip box]
1-mL disposable pipets, one for each sample
10-mL disposable pipet
Straw clips (each holds 15 straws)
Pipet bulbs/pumps to fit 1-mL and 10-mL pipets
0.5 mL semen straws in units of 15; have several colors ready (sorted in scale envelopes).
Squares of Parafilm, 2" x 2"
Thermometer
Kimwipes
Vacuum pump (or vacuum line) with flexible tube attached to 15-pin filling nozzle
Styrofoam freezing chamber, with metal straw freezing rack. (A permanent black line should be drawn on inside perimeter of this box, at a level 2.5-2.7 cm below the height of the freezing rack, to mark desired level of liquid N_2.)
Liquid nitrogen level measuring stick for dewar (Figure 3)
Liquid nitrogen dipper (Figure 3)
Cryo-gloves
Shop-Vac (to suck up fog when checking liquid N_2 level)
Bags of ice cubes
Fresh chicken eggs
Plastic goblets to hold 5 straws each (Figure 4)
Canes to hold 2 goblets each (Figure 4)
Cane tabs (disposable aluminum labels that fold onto tops of the canes)
Dewar filled with liquid nitrogen[2]
Sharpie marker
Lab timer

Solutions prepared in advance:

Freezing solution (may be prepared evening before, or day of use)
Sperm Activating Solution
Cossun's Solution

[1] Rinse new carboy with acetone. Then clean it (and all glassware) with Micro detergent. Rinse 3x in distilled water.
[2] We arrange for a 160-liter low-pressure liquid nitrogen GasPac (GP) to be delivered to the office, from which we refill our dewars. We transfer the liquid nitrogen to our dewars via a 6-foot flexible stainless steel pigtail hose (which we purchased from the liquid nitrogen company for ~$150) that screws onto the "liquid" port of the GP. The 34-L dewars can be carried to a liquid nitrogen facility for refilling, but the time, gas, and risk involved may be more costly than having the nitrogen delivered.

Daily Procedure:

1) Night before or early morning: make up Freezing Solution (p.13); refrigerate[3] at 4°C.
2) Prepare **Data Sheet** (See Sample Data Sheet, p.19)
3) Label cane tabs & apply to aluminum canes.
4) Transfer liquid N_2 from dewar into freezing chamber using dipper, ~3cm deep; let equilibrate for ~2 hours.
5) Assemble materials in Big Tray (Figure 1):
    a. Glass Working Platform (this tray will act as a chilled platform on which to work)
    b. Test tubes in rack (at least 1 test tube/sperm sample)
    c. jar of Sealant Powder
    d. One bubbler trough assembly/sample, stacked in plastic tray
6) Place in Working Platform (Figure 1):
    a. 1 mL pipets (1/sample) and pipet pump
    b. Metal bubbler trough stand
    c. Clips with 15 straws, pre-sealed ends up[4]
    d. Several squares of Parafilm
    e. Thermometer (should read ~4°C when ready)
7) Assemble filling nozzle & hose & vacuum pump.
8) Collect sperm samples [See "Collecting Sperm", p.8]
9) Place in large cooler on ice:
    a. Goblets (keep dry to prevent straws from sticking in ice)
    b. Labelled canes
10) Empty one bag of ice cubes into Big Tray; keep working platform dry.
11) Get Freezing Solution from refrigerator; place in Big Tray ice.
12) Cover tray. Allow tray to equilibrate to 4°C.

NOTE: *this is very optimistic; the tray never gets this cool in our lab. However, care should be taken at every step of this procedure to avoid warming the sperm.*

13) Check motility of sperm samples [see "Checking Motility of Fresh Sperm", p.9]
14) Assemble bubbler trough in metal support.
15) Measure semen from one male into test tube. [***NOTE:*** *Unless essential, don't bother with samples <1.5mL*]
16) For every **1 mL semen**, add **3mL freezing solution**. Add slowly, agitating to mix. [***NOTE:*** *3 mL of sperm plus 9 mL of freezing solution works well for 20 straws: 2 full canes*.] Cover with Parafilm, place thumb over end, tilt back and forth gently 3 times.
17) Pour semen/freezing solution into bubbler trough.
18) Attach pre-sealed ends of 15 straws to filling nozzle. Suck up solution, place finger over hole in nozzle, and suck solution into plug at top end (because wetting the plug forms a seal). If

[3] The original procedure calls for centrifugation of the Freezing Solution. In the absence of a centrifuge, the solution must have time to settle and separate, which can take somewhere between 2 and 12 hours.

[4] This system is made to fill 15 straws at a time, but multiples of 10 straws use storage space most efficiently because there are 10/cane (Figure 4). The nozzle on the suction pump has 15 holes, so it works best with 15 straws; if fewer than 15 straws are used, extra nozzle holes must be blocked to allow complete filling of straws. Each straw clip should have only one color of straw, and each successive male will have different color straws to help keep them organized (but colors will be repeated). For larger sperm samples, use more than one straw clip of the same color. It is best to fill at least 10 straws per male; 20 if possible, 30 ideally (2 full clips). Use only enough straws to allow complete filling- each straw holds ~0.5ml.

fewer than 15 straws in clip, try to seal off other holes in nozzle (with Parafilm or other material) to create adequate suction.

19) Place straws onto bubbler comb to push out some of the sample and add air-bubble space; wipe ends with Kimwipe.
20) Tamp about 5-10x into sealant powder making ¼" plug. Wipe loose powder off ends with Kimwipe.
21) Hold 3 goblets and straw clip in one hand, release the clip lock with the other hand, and (touching upper tips only) transfer 5 straws into each goblet (sealant powder end up - so sealant won't gum up the goblet) (Figure 5). Place 2 goblets into each numbered cane. Return to cooler. If a single goblet is stored in a cane, it should be positioned at the top of the cane to prevent straws from floating out of the goblet.

When about 3 samples – or 7 canes are prepared (our small freezing chamber will only hold 7 canes diagonally):

22) Recheck level of liquid N_2. Should be up to line drawn 2.5-2.7 cm below surface of rack. [Liquid N_2 should be 2.5-2.7 cm deep in our large chamber, 3.2-3.4 cm deep in small chamber, to accommodate the different height racks] – Use Shop-Vac to suck up fog so level line is visible.
23) Place filled canes onto straw rack in freezing chamber; set timer for 15 minutes.
24) After 15 minutes, using large hemostat, plunge canes into liquid N_2 in chamber; transfer canes to canister in dewar. Note storage canister number on data sheet.

24) When last sample is frozen and stored in dewar, put on cryo-gloves and carefully pour liquid N_2 from freezing chamber back into dewar.

25) Check motility of frozen sperm [see "Checking Motility of Frozen Sperm", p.10].

25) Enter data into computer file; note canister location of each sample within dewar; keep updated worksheet of remaining sperm samples.

NOTE: *Dewars can be stored in walk-in freezers, where the liquid N_2 lasts longer; about 3 months. Check periodically. I use a 24" wooden stick, spray-painted orange, with the "full tank" level marked (Figure 3). Put the stick into the tank, all the way to the bottom, and hold there for several seconds. Withdraw stick and breathe on it – the frosted level will show depth. When the level drops to about 2", refill. Liquid N_2 must <u>always</u> be kept in tank, or samples will be ruined.*

Warning: The 5-wheel roller bases made for 34-L dewars are appropriate for moving the dewars over smooth floors only. The wheels are small and plastic, and do not move well over gravel or other uneven surfaces. Also, the wheels will shatter easily if stored in walk-in freezer. A low cart or dolly arrangement may work better for transporting dewars.

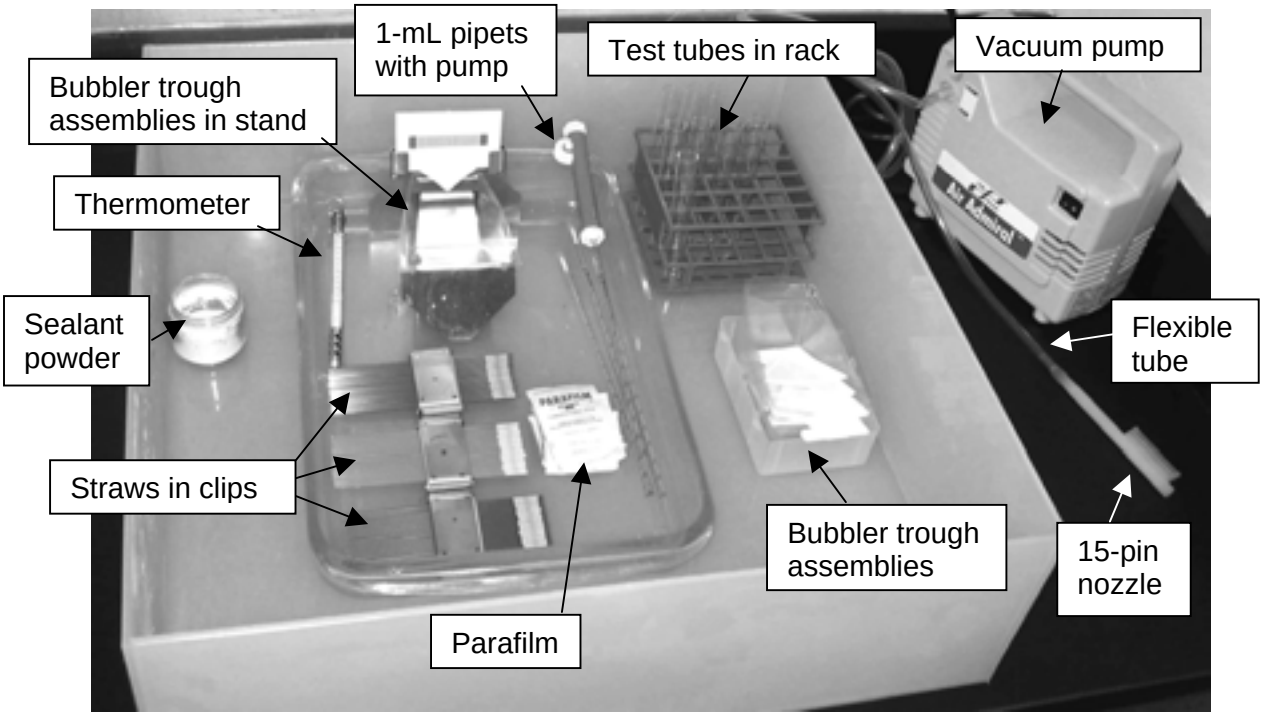


Figure 1.—Big Tray and working platform, with assembled materials.

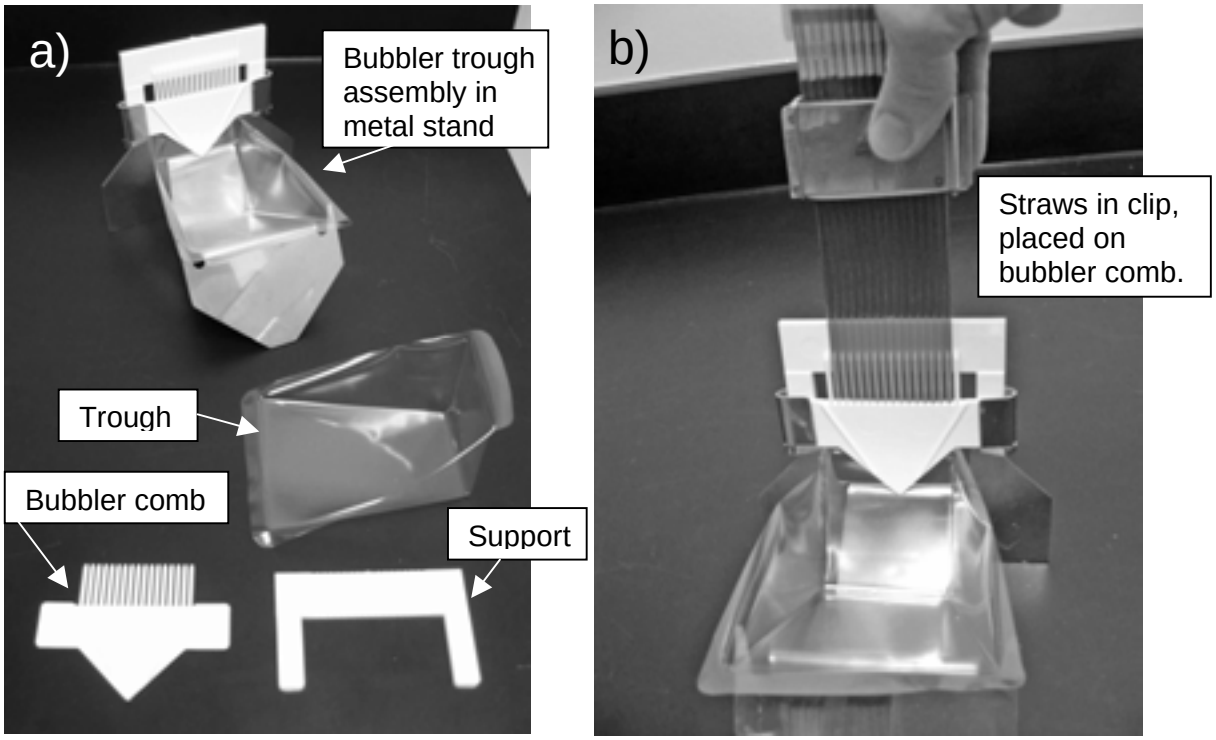


Figure 2.—a) Disposable “Bubbler trough assembly” (including trough, bubbler comb, and support) and metal stand. “Bubbler” refers to the 15-toothed plastic comb that is used to push some of the sperm sample out of each straw so that a bubble is formed, to allow expansion during freezing; b) Demonstration of 15 straws in a clip, fitted onto the straw comb.

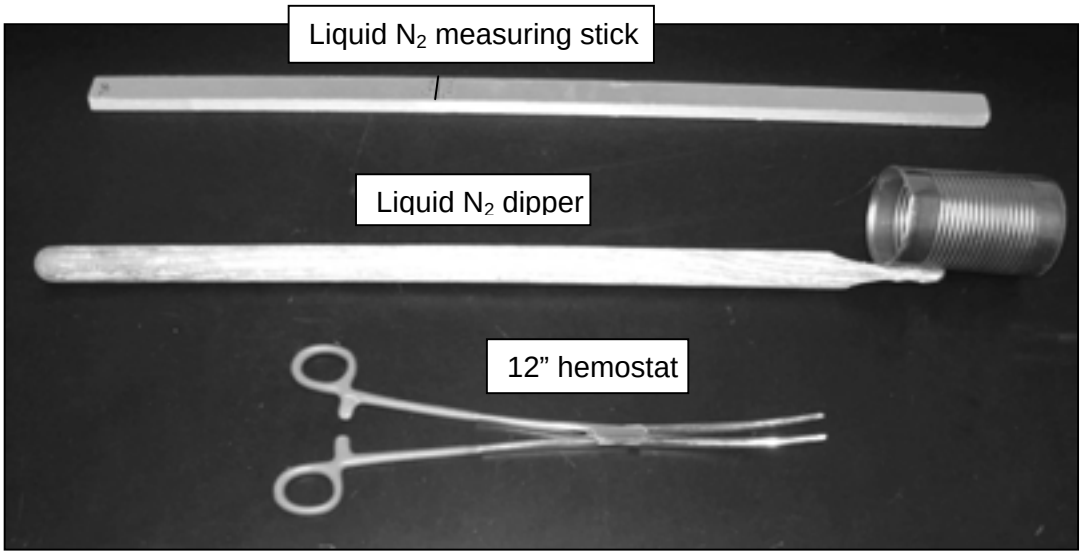


Figure 3.—Non-standard equipment used with liquid N_2 . The liquid N_2 measuring stick is made from a 2-foot stick, spray-painted, with “full” level marked. The liquid N_2 dipper is made from a soup can riveted to wooden net handle. The handle of the 12 inch hemostat has been dipped in Plastic Dip to facilitate handling with gloved fingers.

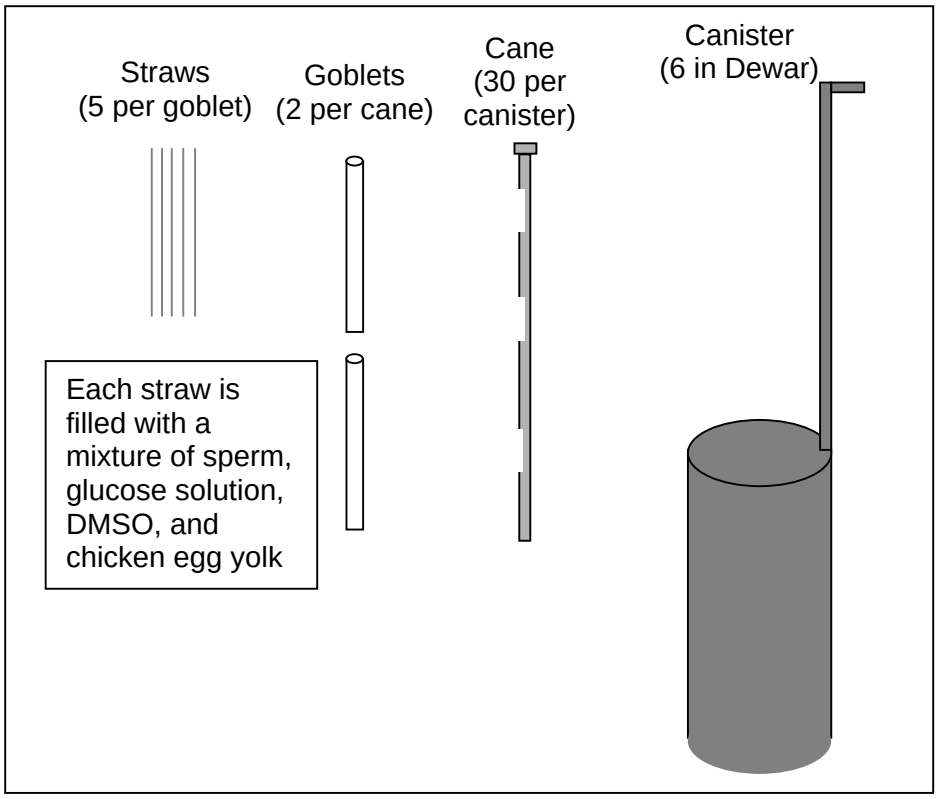


Figure 4.—Storage containers for cryopreserved sperm. Canisters are suspended in the liquid N_2 dewars. Ideally, straws are prepared in multiples of 10 from each sperm sample, filling 2 goblets on each cane, to use space efficiently. If a single goblet is stored in a cane, it should be positioned at the top of the cane to prevent straws from floating out of the goblet.

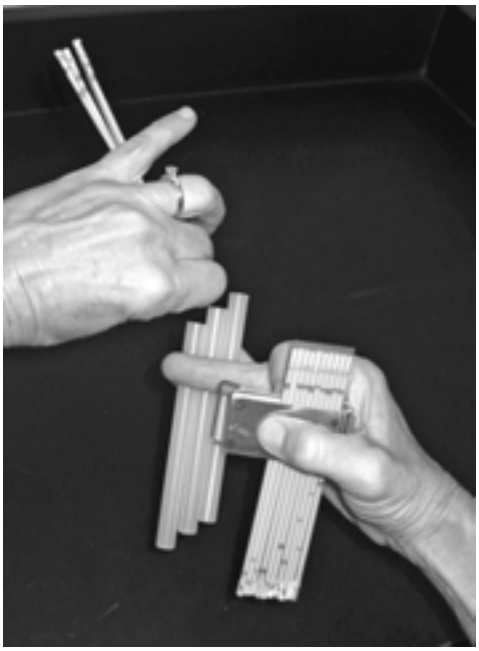


Figure 5.—Transferring 5 filled straws to each goblet, touching tips of straws only to avoid unnecessary warming. Pre-sealed ends of the straws must be inserted into the goblets, to avoid gumming up the goblets with loose sealant powder on the other end of the straws.

Collecting Sperm:

Materials:

Large (~40 qt) and small (lunch size) coolers, with ~1/2" layer of ice in bottom, stored in walk-in freezer.
3-oz Dixie cups
3" x 6" liquid-tight ziploc baggies
Terrycloth towel
Sharpie marker
Pencil
Clipboard/Data Sheet (see Sample Data Sheet, p.19)
Newspaper

Procedure:

1) Take one or both coolers from freezer, and place several layers of newspaper in each, cut to fit. The newspaper insulates sperm samples from ice, to prevent immediate freezing of unprocessed samples.

2) Place Dixie cups, baggies, Sharpie marker in cooler. Bring towel, clipboard, pencil.

3) When fish is ready, wipe belly, and hold Dixie cup in stream of milt. Avoid collecting any water or urine. Discard if feces fall into cup. Ideally, collect at least 3 mL of milt.

4) Pour into ziploc baggie labeled with sample number. Blow air into bag, seal, and place horizontally on newspaper in cooler, to allow maximum aeration. As long as sperm is not in danger of freezing, place samples in single layer on newspaper and jiggle occasionally to promote maximum aeration.

NOTE: *When cooler is still very cold or air temperature is near freezing, take great care <u>not</u> to freeze unprocessed samples in the cooler. Freezing sperm in this manner will ruin it. Leave lid off, or place samples on lid if necessary, until ice in cooler warms.*

5) Note sample number, fish tag number, etc. on data sheet.

6) Back in lab, check sperm motility (see Procedure, p.9); discard sample if <50% motile.

Checking Motility of Fresh Sperm:

Materials:

Compound microscope; 200X objective
Smaller cooler with ice
Small amount of Sperm Activating Solution in vial, placed in corner of cooler.
200 μL pipette
10 μL pipette
Pipette tips
Water rinse bottle
Beaker for rinse water
Kimwipes
Glass microscope slide
Wastebasket
Data sheet (see Sample Data Sheet, p.19)

Procedure:

1) Place glass slide under 200X microscope objective, and focus just above slide surface. Move stage toward you to give access to slide without changing focus.

2) Place small cooler with ice, a few sperm samples, and the vial of Sperm Activating Solution within reach, along with pipettes and tips. Place all materials within easy reach, because speed is essential.

3) Pipette 200 μL of sperm activating solution onto slide. Do not discard pipette tip.

4) Pipette 10 μL of sperm sample into activating solution, and quickly smear back and forth horizontally using side of pipette tip. Lift pipette tip from middle of slide to minimize flow across slide.

5) Quickly move slide under objective, then focus through depth of sample to evaluate motility within ~15 seconds. Good sperm should have frenzied activity everywhere. Dilute or slower activity is not as good. If < 50% motility, discard sample. It is common to see some sperm apparently caught in the surface tension without moving; if the rest looks good, ignore it. Speed is essential; motility declines quickly. About 95-98% motility is common for good sperm.
6) Rinse slide into waste beaker, wipe dry with Kimwipe, replace on microscope. Discard and replace tip on 10 μL pipette.

7) Note results on data sheet.

Checking Motility of Frozen Sperm:

The motility of one straw from each sperm sample should be checked after freezing in liquid nitrogen. This can be done on the same day as the samples are frozen, and results should remain the same indefinitely, as long as the samples remain in liquid nitrogen. Due to the extremely brief time that thawed sperm remain active, straws used to check motility cannot then be used to fertilize eggs.

Materials:

SAME AS MATERIALS LISTED FOR CHECKING FRESH SPERM, P.9, plus:
Thermometer
Cryo-gloves
Large hemostat
2 one-L beakers (one for thawing straw, one for "waste")
Scissors
Glass petri dish
Data sheet (see Sample Data Sheet, p.19)

Procedure:

1) Pour about 500 mL water into beaker; adjust temperature to 10-11°C (50-52°F).
   NOTE: *water from our drinking fountain is just below this temperature.*
2) Place all above materials within easy reach. Speed is essential, especially with thawed sperm!
3) Select sample number on Sperm List; locate in dewar.
4) Place glass slide under 200X microscope objective, and focus just above slide surface. Move stage toward you to give access to slide without changing focus.
5) Place cryo-glove on one hand, and hold large hemostat with other hand. Raise canister part-way out of the dewar with gloved hand, pull out desired cane using hemostat, and grab cane also with gloved hand. Pull out one straw using large hemostat (keeping goblet in cane), and drop the straw into beaker of 10-11°C water. Replace items in dewar, then close dewar. As soon as the straw hits the water, start counting seconds "1 one-thousand, 2 one-thousand…." up to 30 one-thousand. Remove cryo-glove. While counting:
6) Pipette 200 μL of Sperm Activating Solution onto slide. Do not discard pipette tip.
7) After 30 seconds of counting, pull the straw from the water, wipe with a Kimwipe, hold over wastebasket and snip off lower end. Hold straw over glass petri dish (holding straw and upper end in one hand), and snip off top between fingers so that sample falls into dish. (*If sample is somewhat frozen, that's OK. If too frozen to come out, check water temperature, or wait a little longer.*)
8) Quickly pipette 10 μL of sperm sample into activating solution on slide and smear back and forth horizontally using side of pipette tip. Lift tip in middle of slide to minimize flow across slide.
9) Quickly move slide under objective, and focus through depth of sample to evaluate motility AS QUICKLY AS POSSIBLE, within ~15 seconds of thawing. Thawed sperm wears out extremely quickly. The best sperm will have about 40% motility. If motility is < 5%, discard sample. Motility of about 25% is common.
10) Rinse slide into waste beaker, wipe dry with Kimwipe, replace on microscope. Replace tip on 10 μL pipette.
11) Note results on data sheet.

Using Cryopreserved Sperm to Fertilize Eggs:

Materials:

- Plastic dishpan to hold all materials
- Sperm sample list & Pencil
- Thermometer
- 2 one-liter beakers (one for thawing straws, one for waste)
- 5 or 10 ml pipette
- Pipette bulb
- Large hemostat
- Small forceps
- Straw clamp with band & scissors OR vise grips/wooden tray/Fiskars clippers
- Cotton gloves
- Latex gloves – several, large
- Cryo-gloves
- Bottle of warm water (to adjust water for thawing)
- Cossun's solution

NOTE: *Large batches of eggs should be divided into ≥ 2 pans for fertilization with thawed sperm.* ***Speed is essential****, as the sperm becomes inactive less than ~30 seconds after thawing.*

Procedure:

1) Pour about 1 L of water into beaker; adjust temperature to 10-11°C (50-52°F).
2) Select sample number on Sperm List; locate in dewar.
3) Place pipette with bulb into jar of Cossun's solution; draw up 5 ml (and leave there).
4) Wear thin cotton gloves covered by latex gloves (for protection with dexterity.) Replace latex gloves if torn.
5) About 40 seconds before sperm is needed (while eggs are being taken or immediately after), pull sample cane from dewar with large hemostat.
6) Using small forceps, pull white goblets from cane and dump up to 10 straws into water[5].
   - Immediately begin counting "1-one-thousand…..up to 30-one-thousand", and separate straws in the water using gloved fingers during this time.

7) Collect the straws, line up in hand, clamp top end, loop band on pinkie[6] (Figure 6) **OR** lay straws in wooden tray, clamp with modified vise grips (Figure 7).
8) Hold straws over waste beaker; clip bottom of straws using scissors ***or*** clippers.
9) Hold straws over eggs; clip straws just below cotton plugs to dump sperm onto eggs.
10) Pipette 5 ml Cossun's solution (or a little more) onto eggs & sperm; swirl pan.

} About 10 seconds, or ASAP!

11) Water can be added after about 1 minute.
12) Mark off samples used on Sample list (check numbered canes).

[5] Only 10 straws can be handled at a time in most hands. Also, manipulating more than one cane would be difficult.
[6] The two techniques described here for holding the straws are designed to facilitate holding all straws over the pan of eggs and clipping the tops off to release sperm without dropping all the snipped tops into the pan of eggs. See descriptions of the "Clip-&-Band" technique (Figure 6) and the "Modified Vice Grips" technique (Figure 7) on the following page.

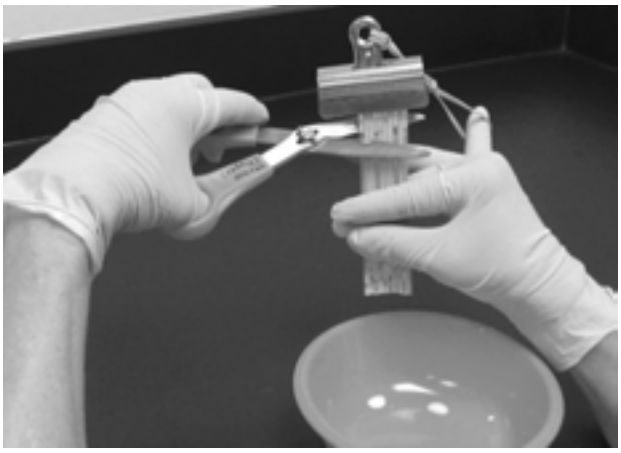
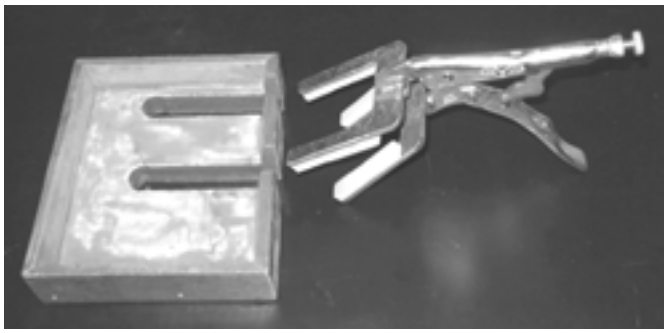


Figure 6.—The “Clip-&-Band” technique for holding 10 straws of thawed sperm over a pan of eggs. This technique was designed to permit snipping off the tops of the straws and releasing sperm into the pan without dropping all the straw tops into the pan as well. The straws are clamped on top using a large paper clamp or bag clamp, and a rubber band through the clamp handle is grasped by one finger or wrapped around the wrist of the hand holding the straws. Care must be taken not to cut the rubber band along with the straws!

a)



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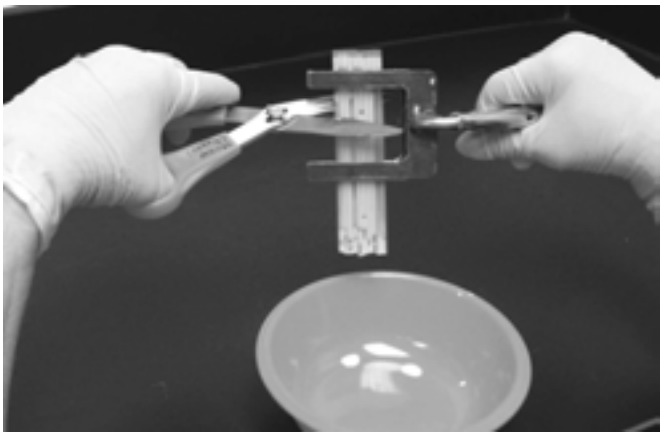

Figure 7.—The “Modified Vice Grips” technique for holding 10 straws of thawed sperm over a pan of eggs. This technique was designed to permit snipping off the tops of the straws and releasing sperm into the pan without dropping all the straw tops into the pan as well. A wooden tray was designed to hold the straws parallel, with slots to permit grasping the straws with modified vice grips. U-shaped metal extensions were welded onto the jaws of a small vice grips, and self-adhesive weather-stripping inside the extensions created soft pads to hold the straws, so the straw tops could be cut without dropping pieces.

Solution Preparations

<u>Freezing Solution (2X recipe)</u>: make fresh freezing solution daily - or evening before.
[*fill and keep many vials containing 5.4 g glucose on hand for quick start; use 2 for this recipe*]

1) Place ~ 150 ml distilled water in 200 ml volumetric flask with stir bar.
2) Add 10.8 g glucose (dextrose); rinse all powder into flask and stir until dissolved.
3) Using a small magnet, remove stir bar from flask (and put it into the 8-oz. Freezing Solution jar); bring flask to 200 mL with distilled water.

4) Separate egg white from yolks of 3 eggs. Collect whole egg yolks in Dixie cup, egg white in extra jar or Ziploc bag. *[**NOTE:** egg white can be discarded or saved for cooking.]*
5) Place yolks on one side of 24 cm filter paper.
6) Rupture yolk membrane with wooden applicator, on side toward center of filter paper, fold edge of filter paper over yolk to grab membrane, pour loose yolk across paper into clean Dixie cup. Fold up sides of filter paper as this is done to form a trough, then squeeze to empty.

7) Into 250 mL graduated cylinder, pour 150 mL of glucose solution.
8) Add 20 mL DMSO, bringing total solution up to 170mL mark [***NOTE:*** *<u>DO NOT</u> get this on skin. It can carry chemicals through skin – you can <u>taste</u> this as soon as you touch it!*]
9) Add egg yolk to bring solution up to 196.6 mL mark (that is, 26.6 mL yolk – but don't try to measure this in separate container!); add more glucose solution drop-wise up to 200 mL mark.
10) Pour into 8-oz. jar with stir bar; mix well on stir plate.
11) Chill to 4°C and allow to settle. [***NOTE:*** *This solution needs to settle and separate into two parts. Speed of separation is inconsistent (~2-12 hrs?), despite trials comparing fresh or store-bought eggs, little stirring or much stirring. Shaking is NOT a recommended alternative to stirring, as this appeared to delay separation.* ***Prepare solution the night before*** *if possible, for better separation by morning. A centrifuge can be used if available, for rapid preparation.*]
12) After chilled and settled, pipet from supernatant.

<u>Sperm Activating Solution</u>: (store up to several months in the refrigerator)

1) Measure into 1,000 mL beaker:
    a. 4.5 g NaCl
    b. 0.605 g TRIS
    c. 0.75 g Glycine

2) Add 500 mL distilled or deionized water
3) Stir to dissolve.
4) Measure with pH meter while stirring; adjust to pH 9.0 using NaOH solution.
5) Pour into 1 L jar; mark with date; refrigerate at 4°C

Cossun's Solution: (store up to several months in the refrigerator.)
(NaCl – 125mM; $CaCl_2$ – 0.1 mM; Tris-HCl/pH9.2 – 30mM)

1) Measure into 1000 mL beaker:
   a. 3.65 g NaCl
   b. 0.0016 g $CaCl_2$
   c. 1.85 g Trizma pre-set crystals
2) Add 500 mL distilled or deionized water; stir to dissolve.
3) Measure with pH meter while stirring; adjust to pH 9.2 using NaOH solution.
4) Pour into 1 L jar; mark with date; refrigerate at 4°C

Pen-Strep Solution: (store up to several months in the refrigerator, or freeze in aliquots)
[*NOTE: This is only used for storage of unfrozen milt; I don't use it*]

If you don't have a balance that weighs the small amounts of streptomycin and penicillin needed for the 100 mL solution, use the procedure starting with 400 mL water.

To 100 mL distilled water, add:
   a. 0.602 g NaCl
   b. 0.298 g KCl
   c. 0.477 g HEPES
   d. 0.0125 g streptomycin
   e. 0.0079 g penicillin

OR

to 400 mL distilled water, add:
   a. 0.05 g streptomycin
   b. 0.03 g penicillin

Mix, discard 200 mL; to remaining 200 mL add:
   c. 1.20 g NaCl
   d. 0.60 g KCl
   e. 0.95 g HEPES

(This solution will dilute the milt 1:1, so add 1 mL solution for every mL milt)

Table 1.—Equipment to be ordered once. This list does not include the compound microscope with 200X objective, pH meter, and Shop-Vac, that will need to be acquired or borrowed for these procedures.

Procedure	Item	Description	Vendor	Catalog #	Units	Cost
Chemicals: Cleaning	Acetone[a]		Sigma	179124	500 mL	$ 24.10
	Micro-90 cleaner		VWR	21830-416	1 qt	9.44
Motility testing	Pipettor, 200 µL	Fixed volume, w/o ejector tip	VWR	40000-224	Each	57.53
	Pipettor, 10 µL	Fixed volume, w/o ejector tip	VWR	40000-214	Each	57.53
	Hemostat	12", curved	Widget Supply	BBB70	Pair	5.97
	Small forceps	Straight or curved, 4.5"	VWR	82027-388 82027-392	Pair	6.66
	Plastic dip	For 12" hemostat handle	Hardware store		1 can	9.00
	Thermometer	-5° to 45°C	VWR	61019-272	Each	8.39
	Spatulas	For weighing chemicals	VWR	57952-005	Pk of 3	29.29
Sperm preparation	Carboy, 20L	for distilled water	VWR	16334-220	Each	155.80
	Stir plate	Small	VWR	33994-356	Each	120.00
	Big Tray, plastic	Autoclave tray, 20" x 20"	VWR	32800-070	Each	136.20
	Glass cake pan	9" x 13"	grocery store		Each	5.00
	Pipette pump, 2 mL		VWR	53502-222	Each	11.92
	Pipette pump, 10 mL		VWR	53502-233	Each	19.39
Sperm loading	Vacuum pump	110V/60HZ	IMV Internat. Corp.	B005-60Hz	Each	393.25
	Connecting tube	For vacuum pump	IMV Internat. Corp.	007825	Each	11.80
	Filling nozzle	15 pins, medium straws	IMV Internat. Corp.	007297	Each	108.90
	Straw clips	holds 15 straws (get ~6)	IMV Internat. Corp.	B104	6 x Each	152.70
	Bubbler stand	metal	IMV Internat. Corp.	007116	Each	119.20
	Test tube rack	Half-rack	VWR	66023-845	Each	11.24
Freezing	Straw freezing rack	Holds 58 medium (or other metal rack, ~ 2 inches high)	IMV Internat. Corp.	007118	Each	102.30
	Canes, 10mm	Holds 2 goblet, ~180 for 6 canisters in 34L dewar	IMV Internat. Corp.	XC052	2 x Pkg of 100	32.00
	Goblets, 10mm	White, holds 5 straws, ~360/dewar	IMV Internat. Corp.	PA015-005561	360 x Ea. ($0.09 ea)	32.40
	Liquid Nitrogen Dewar	34 L, Taylor-Wharton	VWR	55708-488	Each	1,472.67
	Cryo gloves	Water-resistant, 14" (check size?)	VWR	32885-735	Pair	76.19
	Freezing chamber (extra-thick Styrofoam), ≥11"x11" inner dimensions[b]	Thick-walled container used to ship dry ice or frozen material; cardboard outside. Must have lid.	Find, or purchase from dry ice shipper			
	Alarm timer	4-channels, countdown, clock, etc.	VWR	62344-641	Each	22.72
TOTAL						$3,191.59

[a] Use acetone to clean large carboy, and other plastic materials before first use. Clean all glassware with Micro cleaner, rinse in hot water, then rinse 3 times in small amount of Milli-Q water. Use about 1 drop of Micro-90 cleaner at a time; it lasts practically forever.

[b] Inner dimensions that will accommodate the length of aluminum canes (11.5') is handy, but bigger boxes use more liquid N_2. An 11" width will work with canes on a slant. In a 10"x10" box (minimum size) only 7 canes fit diagonally.

Table 2. ── Glassware or plastic-ware, minimum one-time purchase. Many items may be already stocked in a laboratory.

Item	Minimum #	Description	Vendor	Catalog #	Units	Cost each
1 L beakers	2	Polypropylene	VWR	13890-148	Pk of 3	$ 34.17
100 mL graduated cylinder	1	PMP	VWR	83008-888	Pk of 2	24.67
250 mL graduated cylinder	1	PMP	VWR	83008-898	Pk of 2	35.01
100 mL Volumetric flask	1	Nalgene	VWR	29615-007	Each	30.30
200 mL Volumetric flask	1	Nalgene	VWR	29615-030	Each	34.12
1 L jars	3	Glass	VWR	EP323-32A	Case of 12	29.15
500 ml - 1 L bottle	1	Whatever is available				
8-oz. Jars	4	Glass	VWR	89043-556	Case of 24	24.41
Glass petri dish		Bottom only, 100 x 15mm	VWR	89000-322	Case of 12	18.62
Small plastic vials	≥10	Polyethylene vials with caps, about 7 mL	VWR	66022-398	Case of 1,000	94.13
Wash bottle	1	Nalgene	VWR	16651-595	Pk of 4	27.35
Scissors	1	Paper scissors, 6”	VWR	82027-596	Each	12.02
Scissors, heavy-duty	1				Each	20.00
Chip bag clamp[a]	1	3” wide, with hole in handle	Grocery store		Each	2.00
Stir bars	5	1” x 3/8”; get 4 or 5	VWR	58948-983	5 x each	10.60
Plastic dish pan	1	About 12” x 14”	Grocery store		Each	5.00
TOTAL						$401.55

[a] Or modified vice grips holder.

Table 3. ──Consumables; supplies that will need to be reordered periodically.

Procedure	Item	Description	Vendor	Catalog #	Units	Cost
Solutions: Sperm Activator	Distilled (Milli-Q) water	Fill large carboy	University of MN, Dep't of Biology			free
	NaCl	Minimum 99.5%	Sigma	S 9625	500 g	$23.60
	TRIS – TRIZMA base	Cell culture tested	Sigma	T 6066	100 g	$22.10
	Glycine	>99.5%, SigmaUltra	Sigma	G 7403	100 g	$26.90
	NaOH, 1 N	(pH adjust.; used rarely)	Sigma	319511	500 mL	$12.20
Freezing solution	Glucose (dextrose)	Minimum 99.5%	Sigma	G 8270	100 g	$17.50
	DMSO	Plant cell culture tested	Sigma	D 4540	1 Liter	$70.60
	Chicken eggs	3-6 eggs/day	grocery store		1 dozen	$1.00
	Bags of ice cubes	1-2 bags /day	grocery store		1 bag	$1.00
Cossun's solution	$CaCl_2$	Plant cell culture tested	Sigma	C 2536	500 g	$41.70
	Trizma pre-set crystals	PH 9.1	Sigma	T 9818	100 g	$66.70
Pen-Strep sol'n	KCl	>99%, powder	Sigma	P 5405	250 g	$17.20
	HEPES	>99.5%, powder	Sigma	H 6147	25 g	$37.90
	Streptomycin	-sulfate, embryo tested	Sigma	S 1277	5 g	$16.20
	Penicillin	potassium salt; embryo tested	Sigma	P 4687	1M units	$13.60
Sperm collecting	3-oz Dixie cups	Paper	grocery store		Box of 200	$3.00
	Specimen bags	3" x 6", Ziploc, water tight	VWR	11217-102	Pkg of 250	$60.64
Motility testing	Pipette tips	Fits 1-200uL	VWR	53508-794	Pack of 960	$21.00
	Microscope slides	Plain slide, 1"x 3"	VWR	48300-036	Gross of 144	$33.55
Freezing solution	Filter paper	24cm diameter, medium	VWR	28450-182	Pkg of 100	$36.79
	Weighing boats	6 x 4.1 x 0.8 cm	VWR	12577-053	Pkg of 250	$17.72
	Wood Applicator	148 mm x 2 mm	VWR	10805-018	Pkg of 1,000	$4.65
Sperm loading	Test tubes, 19 mL	16 mm x 125 mm	VWR	47729-578	Pkg of 1,000	$56.96
	1 mL pipets	Disposable, 1 per sample	VWR	53300-240	Pkg of 200	$40.21
	10 mL pipets	Disposable, 1 per day	VWR	20171-042	Pkg of 200	$49.78
	Kimwipes EX-L	4.5" x 5"; get several	VWR	21905-026	Box of 280	$2.05
	Parafilm	2" x 250'	VWR	52858-076	Each	$11.64
	Bovine medium straws[a]	0.5 mL, red	IMV Internat. Corp.	AAA434-005709	100 straws	$6.50
		0.5 mL, blue	IMV Internat. Corp.	5697	100 straws	$6.50
		0.5 mL, green	IMV Internat. Corp.	AAA435-005710	100 straws	$6.50
		0.5 mL, yellow	IMV Internat. Corp.	AAA439-005590	100 straws	$6.50
	Bubbler trough assemblies	One per sample	IMV Internat. Corp.	006935	Pkg of 25	$19.15
	Sealant powder, white	white	IMV Internat. Corp.	10338	100 g bag	$13.30
	Cane tabs, white	tabs that fold onto cane tops	IMV Internat. Corp.	XC053	Bag of 100	$6.50
	Latex gloves	Large size (order one size up)	VWR	32916-556	Pkg of 100	$6.39
TOTAL						$777.53

[a] Several other straw colors are also available. Order the transparent colors so that sperm can be seen filling the straws.

Vendors:

IMV International Corporation
11725 95th Avenue North
Maple Grove, MN 55369
800-342-5468 phone
763-488-1888 FAX
www.IMVUSA.com

Sigma-Aldrich
3050 Spruce Street
St. Louis, MO 63103
800-521-8956
www.sigmaaldrich.com

VWR International, Inc.
Goshen Corporate Park West
1310 Goshen Parkway
West Chester, PA 19380
800-932-5000
www.vwr.com

Widget Supply
www.widgetsupply.com

Sperm Cryopreservation Data

Date:_____

River:_____

Species/strain:_____

Sample #	Fish tag #	Straw color	Initial motility	Thawed motility	# straws frozen	# straws remaining	Comments

Reference

Cloud, J. G., and C. Osborne. 1997. Cryopreservation of Salmonid Sperm. Department of Biological Sciences, University of Idaho, Moscow, Idaho.

Copies of Cloud and Osborne's (1997) manual can be purchased for $7.50 (includes handling and mailing) by check or money order made out to the Department of Biological Sciences, University of Idaho. Address orders to:

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