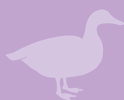
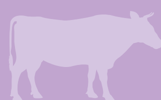
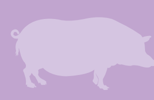
References and appendices

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Appendix A
Specialized procedures for field collection of germplasm[12]

Specific standard operating procedures have been developed for each of the methods and items of equipment used to collect semen and embryos. These standard operating procedures must be followed appropriately.

If samples are to be shipped to the gene bank via courier service, rather than transported by the collection team, contact the nearest courier service company prior to arriving at the collection site and check their latest drop-off times for overnight delivery to the gene bank. These time limits will dictate the working schedule for collection, initial evaluation, packaging and delivery of the collected germplasm.

When arriving at the site of collection, first introduce yourself to your contact person(s) (e.g. owner, breeder or staff member) and discuss clearly the detailed logistics of how the animals will be safely handled and the germplasm collected, handled, analysed and delivered to the gene bank.

Be prepared to answer questions and make sure (verbal agreement) that your contact person is comfortable with the procedures and protocols to be used on the selected animals. Make sure the contact person at the collection site duly completes and signs any written agreements involving ownership of the germplasm (e.g. material transfer agreement or donorship certificate). Sign the agreement (on the behalf of the gene bank) prior to starting collection. Make sure a clean and closed-in environment is available for handling and analysing the collected germplasm.

After the logistics are understood and confirmed, put on the necessary personal protective equipment. Respect the local biosecurity protocols (if applicable).

Unload and arrange the equipment for collection, handling and analysis of germplasm before proceeding to collect the germplasm. Follow the appropriate standard operating procedures for these steps. Make sure the incubator and the necessary media are at the proper temperatures before proceeding.

Use any waiting time (e.g. time required to warm media) to converse with your contact person about the procedures. Continue to encourage questions. Make sure your contact person understands that you value and care about the donor animals, and that all necessary precautions are being taken to minimize their stress and pain. Explain in detail any special procedures, such as electro-ejaculation or embryo flushing, and how the animal may react. Make sure your contact person is comfortable with the procedures.

[12] Adapted from a document provided courtesy of the Canadian Animal Genetic Resources Laboratory for Cryobiology.

When ready to proceed, arrange the collection area. Make sure all necessary precautions are taken to safely constrain the animal and that all persons involved are in a safe working environment.

Proceed with the collection of the germplasm following the appropriate standard operating procedure.

When the germplasm has been collected, immediately transfer and store it in the portable incubator and move it to the clean and closed-in area for subsequent procedures (e.g. evaluation, processing and packing or freezing).

Analyse the quality of the collected germplasm under the microscope. Take this time to show your contact person what the sample looks like under the microscope. The person may never have seen live embryos or motile sperm cells before. Semen concentration can be evaluated using a haemocytometer.

If a problem is encountered, re-collect germplasm from the animal.

If the germplasm cannot be transported or shipped immediately to the gene bank, proceed with the freezing procedures using the appropriate standard operating procedure for semen or embryos. If the collected germplasm can be shipped to the gene bank, proceed with preparing the collected germplasm for shipping to the gene bank.

Accordingly, prepare (freeze or pack) the collected germplasm following the appropriate standard operating procedure.

For frozen germplasm, use a dry-shipper, which should be prepared before visiting the collection site.

For fresh germplasm, transfer the collected material to 15 ml or 50 ml tubes (depending on the volume of the collected material).

Place the tube in a reusable and sealable plastic bag containing shredded absorbent paper and seal the plastic bag.

Place frozen ice packs on the bottom of the shipping container and cover with several layers of absorbent paper (1 cm thick) to avoid direct contact between the germplasm and the ice pack.

Place the sealed plastic bag containing the germplasm on top of the absorbent paper and cover with several more layers of absorbent paper. Secure the shipping container with packaging tape. The shipping container must be spill-proof to be accepted by the courier service company.

Place a shipping label (addressed to the gene bank) on the shipping container and contact the courier service company for pick-up and delivery. Otherwise, deliver the material yourself.

Upon arrival of the collected germplasm at the gene bank, a subsample should be analysed to determine the viability of the material after the cryopreservation process, following the appropriate standard operating procedure. A brief report, including viability, quality and the number of straws processed, should then be prepared and sent to the contact person at the collection site.

Appendix B

List of equipment needed for semen collection and freezing

MAJOR EQUIPMENT AND FACILITIES

- animal handling facilities
- adequate dedicated workspace for semen processing (clean, dry, climate controlled)
- warming cabinet (electric)
- microscope (10X eyepiece with 10X and 43X objectives)
- digital balance (6 kg ± 1 g)
- warm water bath (electric)
- sperm-counting equipment (haemocytometer or spectrophotometer)
- straw filler (preferably with attached label printer)
- semen freezing unit
- semen liquid nitrogen storage tanks (pre-tested)
- source of liquid nitrogen (very important to have a reliable source all year round)

SMALL EQUIPMENT AND CONSUMABLES

- coveralls and boots
- disposable gloves and boot covers
- helmets (head protection), especially for semen collection from stallions
- artificial vaginas, cones and collection tubes
- lubricant
- glass-lined thermos bottles
- thermometers (centigrade)
- semen diluents, extenders and cryoprotectants
- plastic semen straws
- assorted glassware and plasticware
- artificial light

Appendix C

Procedures for cryopreservation and thawing of semen from common livestock species

The following procedures require technical expertise. Before widespread implementation in a national conservation programme, the gene banking team should test the procedures on a small group of animals (preferably of the same breed). The test should include semen collection, freezing and thawing, and insemination to obtain a successful pregnancy.

CRYOPRESERVATION OF CATTLE SEMEN

For a review of bull semen collection, processing and handling, see the classic laboratory manual by Herman *et al.* (1994).

Freezing

1. The collected material should contain approximately 5 to 15 × 10^9 sperm per ejaculate. Avoid temperature changes in the semen during transfer to the processing laboratory. In the laboratory, use a water bath to maintain the temperature at approximately 35 °C.
2. Visually inspect the semen to ensure proper colour (usually milky white) and freedom from abnormalities (e.g. blood, pus). Filter the semen if contaminants such as hair or dung are visible.
3. Microscopically evaluate semen for motility (> 60 percent), and proportions of live sperm (> 70 percent) and abnormal (< 30 percent) sperm (see Appendix D).
4. Evaluate sperm concentration (haemocytometer or spectrophotometer) and determine the final volume needed for a diluted concentration of ≥ 100 × 10^6 sperm/ml.
5. Dilute the semen to the proper volume using the one-step or two-step procedure:

   One-step procedure

   - Add the entire sample to the volume of One-Step Diluent (milk + 20 percent egg yolk, 7 percent glycerol plus antibiotics required to yield a final concentration of 100 µg of tylosin, 500 µg of gentamicin, 300 µg of lincomycin, and 600 µg of spectinomycin in each ml of total volume) required to obtain the desired final sperm concentration.
   - Cool to +5 °C within one hour and maintain for at least two hours.

   Two-step procedure

   - Add Diluent A (milk + 10 percent egg yolk + antibiotics + 3 percent glycerol) to the semen sample in a progressive manner (over 15 minutes at 35 °C) until half the final volume is obtained.

- Cool to +5 °C within one hour.
- Add Diluent B until the final volume is reached (Diluent B consists of Diluent A +11 percent glycerol).
- Keep at +5 °C for two hours.
- For the above methods, protein-free products without milk or egg yolk are commercially available. Using these can reduce sanitation risks.

6. Fill pre-printed 0.25 ml straws with semen (25 to 30 million sperm/straw).
7. Transfer straws to liquid nitrogen vapour at -70 °C to -100 °C for nine minutes.
8. Transfer straws to liquid nitrogen tank and store.

Thawing

1. Before inseminating animals using a given batch of semen, thaw one straw to check for quality.
2. Thaw straws directly in a water bath at +37 °C for 30 seconds.
3. Inseminate cows and heifers transcervically ~12 hours after onset of standing oestrus.

CRYOPRESERVATION OF BUFFALO SEMEN

Freezing

1. Collect sperm (5×10^9 to 10×10^9 sperm per ejaculate) at 35 °C and transfer to the laboratory.
2. Avoid temperature changes in the semen after collection by placing the sample in a water bath (35 °C).
3. Evaluate sperm visually and microscopically.
4. Measure concentration and determine final volume for a concentration of 100×10^6 sperm/ml.
5. Add Diluent A (milk +10 percent egg yolk + antibiotics + 3 percent glycerol) to the semen sample in a progressive manner (over 15 minutes at 35 °C) until half the final volume is obtained.
6. Cool to +4 °C within 1.5 hours.
7. Add Diluent B up to the final volume (Diluent B consists of Diluent A + 11 percent glycerol).
8. Keep at +4 °C for four hours.
9. Meanwhile, fill pre-printed 0.5 ml straws with semen (about 50–60 million sperm/straw).
10. Cool from +4 °C to -140 °C in five minutes in liquid nitrogen vapour; then plunge into liquid nitrogen.
11. Transfer straws to liquid nitrogen storage.

Thawing

1. Thaw a sample from each batch to check for quality prior to use for AI.
2. Thaw straws directly in a water bath at +35 °C for 30 seconds.
3. Inseminate females transcervically 12 hours after onset of oestrus.

CRYOCONSERVATION OF SHEEP SEMEN

Freezing

1. The collected semen should contain about 4 × 10^9 sperm per ejaculate and should be maintained at 37 °C.
2. Evaluate semen visually and microscopically. Semen should be white and quite viscous.
3. Select only those ejaculates with mass motility above 10 percent and less than 30 percent abnormal sperm.
4. Evaluate sperm concentration and determine final volume for a concentration of 400 × 10^6 sperm/ml.
5. Dilute the semen to the proper volume using a one-step or two-step procedure. For a discussion of semen diluents and extenders for ram semen, see Paulenz *et al*. (2002).
   One-step procedure
   - Add the entire sample the volume of One-Step Diluent (300 mM Tris, 28 mM glucose, 95 mM citric acid, 2 percent [v:v] glycerol, 15 percent egg yolk, 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) required to obtain the desired final sperm concentration.
   - Cool to 4 °C within one hour and maintain for at least 1.5 hours.

   Two-step procedure
   - Add Diluent A to the semen at 30 °C to obtain 60 percent of the final volume (Diluent A consists of 25.75 g of lactose in 250 ml bi-distilled water + 20 percent egg yolk).
   - Cool progressively to +4 °C over two hours (0.2 °C/minute).
   - Prepare Diluent B: Reconstitute milk from a non-fat powder source (4 g into 100 ml bi-distilled water) and adjust pH to 6.6 with a Tris solution (20 g of tri-sodium-citrate-5.5 H_2O into 70 ml H_2O); then mix nine volumes of the resulting solution with one volume of glycerol.
   - Add Diluent B up to final volume (Diluent B consists of Diluent A +11 percent glycerol).
   - Add Diluent B in three equal parts, over 30 minutes, at 4 °C up to the final volume.
   - Keep the semen for 90 minutes at +4 °C.
6. Fill 0.25 ml plastic straws with semen.
7. Place straws horizontally in liquid nitrogen vapour at -75 °C for eight minutes.
8. Transfer directly into liquid nitrogen at -196 °C and store.

Thawing

1. Thaw straws in a water bath at 37 °C for 30 seconds.
2. Assess semen viability: mix one volume of sperm to four volumes of a sodium citrate solution (20 g of tri-sodiumcitrate-2 H_20 in 70 ml bi-distilled water) at 38 °C and estimate the proportion of motile sperm after five minutes and after two hours: only sperm with more than 30 percent of living spermatozoa at two hours should be used for insemination.
3. Proceed to surgical or non-surgical insemination of pre-synchronized recipients.

CRYOCONSERVATION OF GOAT SEMEN

Freezing

1. Collected semen should contain about 4 × 10^9 sperm per ejaculate when sampling occurs in season. Semen should be kept at 32 °C for transfer to the laboratory and processing.
2. Evaluate semen visually for any abnormalities.
3. Wash sperm with a Krebs Ringer Phosphate Glucose Solution (0.9 percent NaCl, 1.15 percent KCl, 1.22 percent $CaCl_2$, 2.11 percent KH_2PO_4, 3.82 percent $MgSO_4$-$7H_2O$, 5.24 percent glucose) by mixing one volume sperm with nine volumes of the washing solution at 28 °C to 32 °C, followed by centrifugation at 500 g for 15 minutes at 20 °C.
4. Discard the supernatant, and evaluate the semen (wave motion, concentration). Select only those ejaculates with a mass motility greater than 60 percent.
5. Calculate the final volume (V). Repeat centrifugation under the same conditions at 20 °C.
6. Dilute the semen to the proper volume using a one-step or a two-step procedure:
   One-step procedure
   - Add the entire sample to the volume of One-Step Diluent (300 mM Tris; 28 mM glucose; 95 mM citric acid; 2 percent [v:v] glycerol; 2.5 percent egg yolk; 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) required to obtain the desired final sperm concentration (≥ 200 million sperm per ml).
   - Cool to 4 °C within one hour and maintain for at least 1.5 hours.

   Two-step procedure
   - Prepare Diluent A: 80 ml of a sodium citrate solution (194 mg glucose +3.52 g sodium citrate +1.05 g streptomycin +50 000 IU penicillin in 100 ml distilled water) supplemented with 20 ml egg yolk.
   - Add V/2 of Diluent A to the pelleted sperm at 20 °C.
   - Cool to +4 °C within 30 minutes (at 0.5 °C/minute).
   - Add V/2 Diluent B (Diluent A + 14 percent v:v glycerol) in three successive steps with ten minute intervals. Diluent should also be at +4 °C.
7. Fill 0.25 ml plastic straws with semen.
8. Freeze straws in liquid nitrogen vapour for five minutes.
9. Plunge directly into liquid nitrogen and store.

Thawing

1. Thaw straws in a water bath at 37 °C for 30 seconds.
2. Assess post-thaw motility.
3. Proceed to insemination of does.

CRYOCONSERVATION OF HORSE SEMEN

Freezing

1. Collect sperm (~8 × 10^9 sperm per ejaculate) and filter on gauze.
2. Dilute semen to an approximate concentration of 50 × 10^6 sperm per ml in 37 °C holding medium. Various suitable media can be used. One example is SMED (100 ml

of nanopure water, NaCl at 37 mM, KCl at 10 mM, KH_2PO_4 at 0.07 mM, $NaHCO_3$ at 35.7 mM, $MgSO_4$ at 2.4 mM, HEPES at 10 mM, $CaCl_2$ at 1.7 mM, fructose at 84.3 mM and glucose at 5.5 mM supplemented with 0.3 g of bovine serum albumen at a pH of 7.2).

3. Centrifuge the extended semen (800 × g for 9 minutes or 400 × g for 13 minutes).
4. Remove the supernatant by aspiration.
5. Suspend the resulting pellets in a small amount of SMED and determine the sperm concentration.
6. Redilute the sample to 400 × 10^6 sperm per ml in skim milk–egg yolk extender (SMEY) (154.8 mM glucose, 4.2 mM lactose, 0.5 mM raffinose, 0.85 mM sodium citrate dihydrate, 1.25 mM potassium citrate, 29.8 mM HEPES, 51.5 mg/ml of skim milk powder, 1 mg/ml of ticarcillin with 2 percent egg yolk).
7. Cool the sample to 15 °C in a shipping container, which will allow it to be held for up to 24 hours.
8. After holding, further cool the sample to 5 °C over two hours.
9. Dilute with SMED-glycerol, so that the final dilution is 4 percent glycerol (up to a 1:1 dilution [v:v]).
10. Load the samples into printed straws.
11. Freeze in liquid nitrogen vapour (4.5 cm above the liquid for ten minutes)
12. Plunge into liquid nitrogen for storage.

Thawing

1. Thaw eight 0.5 ml straws (400 × 10^6 sperm) together in a water bath at +37 °C for 30 seconds.
2. Sperm should be deposited, usually daily, into the uterine body during the oestrous period.

CRYOCONSERVATION OF PIG SEMEN

Freezing

1. When collecting boar semen, it is critical to discard the first emission of sperm and to keep only the second (about 200 ml, rich in sperm, with 40 × 10^9 in total).
2. Filter the semen through gauze to eliminate the bulbo-urethral secretions.
3. Semen should be kept at 37 °C until processing, which should be done as quickly as possible. Ideally, semen will be extended within 15 minutes.
4. Two approaches can be applied, depending on whether collection is in the field or in the laboratory:

   In the field:

   - If semen is collected in the field, the ejaculate should be quickly diluted in 37 °C Beltsville Thawing Solution (BTS) (205 mM of glucose, 20.4 mM of sodium citrate, 14.9 mM of $NaHCO_3$, 3.4 mM of EDTA and 10 mM of potassium chloride), placed in the shipping container and cooled to 15 °C.
   - Upon arrival at the laboratory, centrifuge the samples at 800 × g for ten minutes.
   - Combine the pellets and determine the sperm concentration.

- Dilute samples using BF5 Cooling Extender (CE) (52 mM of TES, 16.5 mM of Tris [hydroxymethyl] aminomethane, 178 mM of glucose with 20 percent egg yolk at 325 mOsm) to 750 × 10^6 sperm per ml.
- Cool semen to 5 °C over 2.5 hours.
- Diluted semen with BF5 freezing extender (91.5 percent of CE, 6 percent glycerol, 2.5 percent Equex Paste [v:v]) to 500 × 10^6 sperm per ml.

At a semen collection and processing centre:

- Dilute one volume of sperm with one volume of Diluent A (anhydrous dextrose, 37 g; tri-sodium-citrate-$2H_2O$, 6 g; $NaHCO_3$, 1.25 g; EDTA diNa, 1.25 g; KCl, 0.75 g in 1 litre of bi-distilled water).
- Cool to 15 °C within two hours.
- Centrifuge at 800 × g for 20 minutes at 15 °C.
- Remove supernatant, which is diluted seminal plasma.
- Resuspend the pellet of sperm with about 10 volumes of Diluent B (8.5 g of fructose, 0.15 g of $NaHCO_3$, 0.015 g of cysteine, 116 ml of bi-distilled water, 34 ml of egg yolk and 1.69 g of equex STM [Nova Chemicals]) to obtain a concentration of 3 × 10^9 sperm per ml.
- Cool the suspension to 5 °C over two hours.
- Add one volume of the diluted sperm solution to one volume of Diluent C (Diluent C consists of Diluent B + 6 percent glycerol); Diluent C must be added in three steps to give a final concentration of 3 percent glycerol and 1.5 × 10^9 sperm per ml.
- Keep at 5 °C for about 90 minutes.

5. Load the semen into 0.5 ml straws.
6. Place straws horizontally at 5 cm above the level of boiling liquid nitrogen for 15 minutes (this will ensure a freezing rate of about 20 °C/minute down to -145 °C.
7. Plunge the straws into liquid nitrogen and store.

Thawing

1. Thaw straws in a 38 °C water bath for 20 seconds.
2. Mix the content of seven straws with 95 ml of Diluent A at 38 °C to obtain one dose for one AI.
3. Inseminate the sow within one hour after this dilution (5.3 × 10^9 sperm per AI).
4. See Pursel *et al.* (1975) and Almlid and Johnson (1987) for more information.

CRYOCONSERVATION OF RABBIT SEMEN

Freezing

1. Collect semen.
2. Prepare Diluent A. In 100 ml bi-distilled water, dissolve 3.028 g of Tris (trishydroxymethylaminomethane), 1.25 g of glucose, 1.67 g of citric acid-H_20, and 5 ml of DMSO (dimethyl-sulfoxide); add one volume of egg yolk to four volumes of solution (Vicente and Viudes-de-Castro, 1996).
3. Add four volumes Diluent A to one volume semen.
4. Progressively cool the diluted semen to +5 °C over one to three hours.

5. Prepare Diluent B. In 100 ml of bi-distilled water, dissolve 8.25 g of lactose and 1.3 ml of glycerol; add 20 percent egg yolk (one volume egg yolk to four volumes of solution).
6. Add one volume of Diluent B pre-cooled at +5 °C to one volume of diluted semen.
7. Fill 0.5 ml straws with semen.
8. Keep for ten minutes at +5 °C.
9. Freeze straws horizontally in liquid nitrogen vapour for three minutes at -120 °C.
10. Plunge the straws directly into liquid nitrogen and store.

Thawing

1. Thaw the straws in a water bath at +37 °C for one minute.
2. Inseminate does intravaginally, followed by an intra-muscular injection of 0.2 ml gonadotropin releasing hormone (GnRH).

CRYOPRESERVATION OF CHICKEN SEMEN

Two methods for cryopreservation of chicken semen are presented below. The primary difference is in the media used.

Method I

With this method the semen can be used directly for insemination after thawing. No need first to wash the semen free of the cryoprotectant.

Freezing

1. Prepare the media.

Table C1. Base medium – Lake's diluent*

	Molecular weight	g/litre	mmol/litre
Sodium-L-glutamate•H_2O	187.13	19.2	102.6
Magnesium acetate•$4H_2O$	214.46	0.7	3.3
Fructose	180.16	8	44.4
Potassium acetate	98.2	5	50.9
Polyvinylpyrrolidone	40 000	3	0.08

*Described in Lake (1968) as "Solution 1".
Note: The final pH of the medium should be 6.9.

The medium can be prepared with or without the cryoprotectant DMA (dimethylacetamide); the two variants are known as Lake-DMA and Lake's diluent, respectively. DMA concentration is 1.8 mol/litre = 157 g/litre = 16.7 volume percentage. To prepare Lake-DMA, take 15.7 g (or 16.7 ml) of DMA and add Lake's diluent to a total volume of 100 ml.

2. Store media in closed vessels to prevent evaporation and place in a temperature-controlled cool box at 5 °C.
3. Collect semen and transport it to the laboratory for further processing and freezing. All further handling is performed at 5 °C (cold room or open top cooler cabinet).

4. Determine the concentration of the non-diluted semen (spectrophotometer or haemocytometer).
5. Diluted semen with Lake's diluent to the desired concentration (e.g. 1.8 × 10^9 sperm/ml).
6. Add half a volume of Lake-DMA to one volume of semen and fill into 0.25 ml straws.
7. Freezing may be performed in a programmable freezer with a constant rate of 50 °C/minute) (maximum rate of most freezers) or in static liquid nitrogen vapour, 1–2 cm above the level of the liquid nitrogen.

Thawing

1. Remove straws from the liquid nitrogen and place in a 5 °C water bath.
2. Move the straws vigorously through the water for 30 seconds. Do not thaw bundles of straws as this will slow the thawing rate. Despite the low temperature of the water bath, the thawing rate will be high enough (average thawing rate from -190 to + 5 °C is 500–600 °C/minute).

Method II

With this method the cryoprotectant must be washed from the thawed semen prior to insemination.

Freezing

1. Collect semen (1.5 × 10^9 sperm per ejaculate).
2. Mix three volumes of semen (an ejaculate is about 300 µl) with four volumes of Diluent A (0.7 g of magnesium acetate [tetra-hydrated] + 19.2 g of sodium glutamate + 5.0 g of sodium acetate + 8.0 g of fructose + 3.0 g P.V.P [MW 10 000 to 15 000] in 1 litre of bi-distilled water).
3. Cool diluted semen immediately over 20–30 minutes to +5 °C (0.5 °C/minute).
4. At +5 °C, add one volume of diluted semen to one volume of Diluent B (Diluent A +11 percent glycerol). This gives a final concentration of 300 × 10^6 sperm/ml.
5. Equilibrate over 30 minutes at +5 °C.
6. Fill 0.25 ml straws with semen.
7. Freeze at a rate of 7 °C/minute from +5 °C to -35 °C, and at a rate of 8 °C/minute from -35 °C to -140 °C.
8. Plunge into liquid nitrogen and store.

Thawing

1. Prepare Diluent C: 0.8 g magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +19.2 g of sodium glutamate +6.0 g of fructose +5.1 g of sodium acetate in 1 litre of bi-distilled water.
2. Prepare Diluent D: 0.8 g of magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +15.2 g of sodium glutamate +6.0 g of glucose +30.5 g of B.E.S (N,N-bis-2 hydroxyethyl-2-amino-ethanesulfonic acid) +58 ml of NaOH (1M/litre) in 1 litre of bi-distilled water.

3. Thaw straws in a water bath at +5 °C for three minutes. Open and transfer semen to a glass beaker. Mix one volume of semen with 20 volumes of Diluent D, still at 5 °C.
4. Remove glycerol by centrifugation at 700 x g at +5 °C for 15 minutes.
5. Discard the supernatant and add one volume of sperm pellet to one volume Diluent D at +5 °C, and proceed to insemination of the hens.

CRYOCONSERVATION OF TURKEY AND DUCK SEMEN

For the present, it is recommended that turkey and duck semen samples be treated in the same way as rooster semen. However, ongoing research is expected soon yield species-specific protocols (Woelders, 2009). For insemination of turkeys, three straws per insemination are recommended.

Appendix D
Guidelines for basic semen analysis

Cryopreservation of semen can be a valuable step in ensuring the long-term survival of a given AnGR. However, these efforts will be futile and a waste of resources if the sperm is not fertile. Therefore, collected semen should be evaluated for quality and viability prior to processing and freezing (Jeyendran, 2000).

Semen should be evaluated as soon after collection as possible. Exposure to temperature changes, light and contaminants are generally detrimental to semen quality. Specific equipment, such as computer-aided semen analysis (CASA) systems can be used for automated evaluation of motility parameters; however, subjective evaluation under a microscope is also done, and technicians should be trained in these techniques.

Three basic characteristics should be addressed when evaluating semen and estimating sperm viability:

1. sperm concentration;
2. motility; and
3. morphology.

SPERM CONCENTRATION

Concentration is most accurately estimated with specialized equipment, such as a spectrophotometer. Counting can also be done manually, under the microscope, using a haemocytometer. A haemocytometer is a thick glass slide with two vessels serving as counting chambers. Each chamber is marked with a grid pattern etched into the glass, creating a background of squares. Diluted semen (usually 1:100) is pipetted into the chambers and the haemocytometer is viewed under a microscope. Because counting is easier and more accurate when the sperm are immobile, sperm are usually killed by including a small quantity of formaldehyde in the diluent. By counting the number of sperm within a sample of squares in the grid and considering the size of the squares of the grid and the dilution rate, the concentration and number of sperm in the original sample can be estimated. This information can then be used to determine the proper quantities of semen extender needed to obtain the desired concentration of sperm in the semen to be packed and cryopreserved. Sperm concentration can also be used as an indicator of the health of the semen donor (low concentration may indicate a health problem).

MOTILITY

The movement of the sperm should be checked: first, because movement indicates that the sperm are alive; and second, because motility is correlated with fertility. Two types of motility are usually evaluated – gross motility and individual motility.

Gross motility

1. Place a drop of diluted semen on a pre-warmed slide (37 °C) and examine sperm at 10X under a standard or phase-contrast microscope.
2. Look for general movement of the sperm with rapidly moving waves and individual swirls of sperm within the waves.

Individual motility

1. Place, on a pre-warmed slide, a drop of semen diluted (1:10) in saline solution, citrate or extender. When CASA equipment is used, chambers of a special design are needed (Makler chambers).
2. Position a cover slip over the mixture and examine under ≥40X magnification.
3. Estimate the proportion of individual sperm that are moving progressively forward (so-called "progressive forward motility"). This can be done by randomly picking ten or more sperm in different areas of the slide, counting those with forward motility and dividing by the total.
4. Although motility and its correlation with fertility may vary by species, the following figures can be used as a general guideline:
   - > 70 percent = very good
   - 50 to 60 percent = good
   - 40 to 50 percent = satisfactory
   - 30 to 40 percent = acceptable, but undesirable
   - < 30 percent = unsatisfactory

MORPHOLOGY

Abnormally shaped or damaged sperm are less likely to be capable of fertilization than normal sperm are (Berndtson *et al.,* 1981). Mixing the semen with a stain (e.g. eosin-nigrosin) highlights the sperm so that abnormalities can be readily identified under a microscope. Two kinds of abnormalities can be defined: primary abnormalities, which are assumed to have occurred in the testes; and secondary abnormalities, which arise in the epididymis or ejaculate. The proportion of normal sperm should be > 70 percent.

1. Place a drop or stripe of stain on a warmed microscope slide.
2. Add a small amount of semen.
3. Mix the semen and the stain with another slide and then use the narrow edge of the second slide to smear the mixture across the first slide.
4. Cover the mixture with a cover slip and examine under 1 000X magnification (oil immersion).
5. Examine the sperm for abnormalities, including the following:
   - abnormally shaped (tapered or pear-shaped) or sized (too large or small) heads;
   - missing or stump tails;
   - coiled or bent tails;
   - detached or creased (folded-over) acrosome;
   - clumping of multiple sperm; and
   - plasma droplets on tails.
6. Count at least 100 sperm and calculate the proportion (%) of abnormalities.
7. Discard semen if the proportion of abnormalities is too high (more than 30 percent).

Appendix E
Commonly used superovulation schemes for donor cattle embryo recovery

Figures E1 and E2 show two commonly used superovulation schemes.

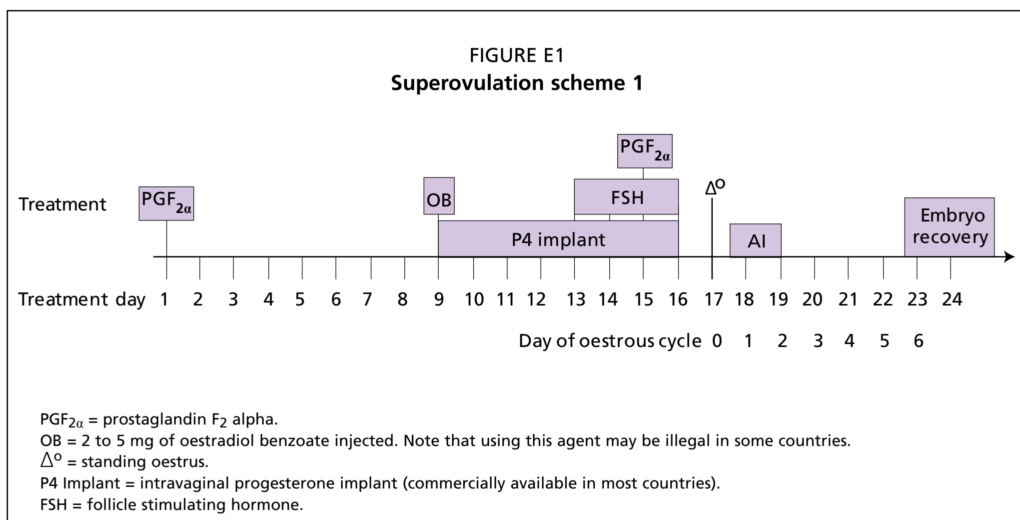


FIGURE E1
Superovulation scheme 1

$PGF_{2\alpha}$ = prostaglandin F_2 alpha.
OB = 2 to 5 mg of oestradiol benzoate injected. Note that using this agent may be illegal in some countries.
Δ^o = standing oestrus.
P4 Implant = intravaginal progesterone implant (commercially available in most countries).
FSH = follicle stimulating hormone.

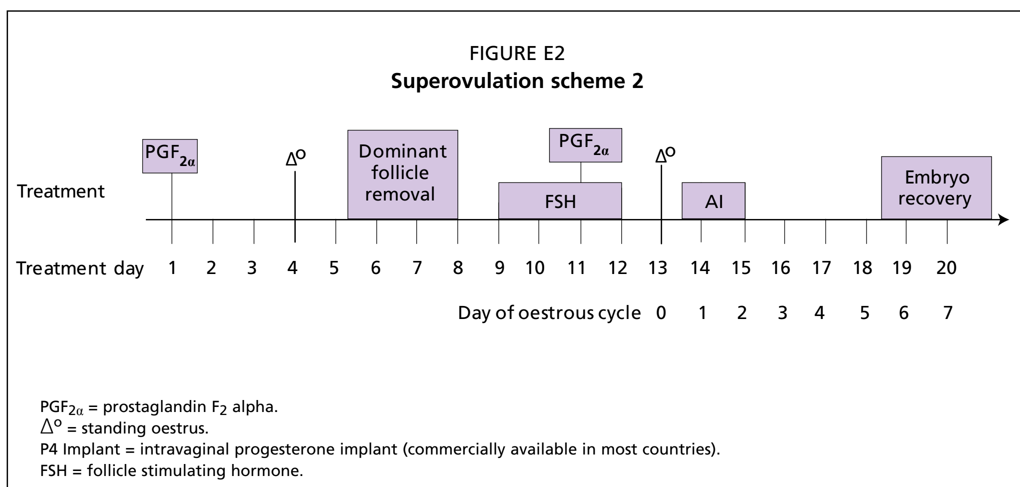


FIGURE E2
Superovulation scheme 2

$PGF_{2\alpha}$ = prostaglandin F_2 alpha.
Δ^o = standing oestrus.
P4 Implant = intravaginal progesterone implant (commercially available in most countries).
FSH = follicle stimulating hormone.

Appendix F

List of equipment and supplies needed for non-surgical embryo collection and transfer in cattle

EQUIPMENT

- animal holding chute
- temperature-controlled water bath
- liquid nitrogen tank
- stereo microscope with a heated stage
- Cassou gun and sheaths

RENEWABLE SUPPLIES

- boots (washable)
- coveralls (washable or disposable)
- plastic gloves (disposable)
- plastic foot covers
- paper towels
- disinfectant liquid
- liquid soap
- Petri dishes
- small sterile plastic embryo dishes
- scissors
- lidocane
- donor semen (2 to 4 units)
- plastic syringes 10 or 12 ml and needles for tailblock procedures
- flushing medium (Delbecco's phosphate-buffered saline)
- holding medium (e.g. TCM-199, a commercially available medium)
- foetal calf serum (commercially available)
- plastic straws for the embryos (e.g. 0.25 ml)
- straw labelling equipment (preferably with label printer)
- liquid nitrogen
- record book (very important!)

Appendix G
Technical procedures for cryopreservation and thawing of livestock embryos

It is anticipated that the team responsible for the cryopreservation and/or thawing of embryos will have demonstrated their technical expertise before implementing an embryo recovery and banking programme. Freezing cattle, goat and sheep embryos is now a common practice for in-field use. Freezing early-stage horse embryos is practised commercially, but freezing later-stage equine embryos has still has not been widely mastered. Freezing pig embryos is more difficult, although there have been some recent advances in this area. For these reasons, only procedures for ruminant and horse embryos are outlined here.

CRYOPRESERVATION OF BOVINE EMBRYOS
Freezing

1. Collect embryos non-surgically from a superovulated donor female at day seven of the oestrous cycle, evaluate them for morphological development, and assign them an embryo-quality grade. The embryos should be at the compact morula and blastocyst stages if the procedure is progressing correctly.
2. Maintain the embryos in a clean environment that is free from possible contaminants and maintained at around room temperature (20 to 30 °C). Freezing should be done as soon as possible after collection (i.e. within four to six hours).
3. While grading quality, check that the zona pellucida is intact on all embryos (under 50X light microscope) and that embryos are free from adherent material.
4. Wash the embryos from one donor (no more than ten embryos) in five consecutive baths of phosphate-buffered saline (PBS) solution containing broad-spectrum antibiotic and 0.4 percent bovine serum albumin. Use different glass and plastic ware for each donor and new micropipettes for each subsequent wash.
5. Treat the embryos to two washes with trypsin (60 to 90 seconds in total) to remove or deactivate any viruses. Trypsin wash is sterile porcine-origin trypsin (1:250) in Hank's balanced salt solution at a concentration of 0.25 percent.
6. Wash the embryos an additional five times in PBS – antibiotic solution with 2 percent bovine serum albumin.
7. Equilibrate the embryos at room temperature for ten minutes in PBS with 10 percent foetal calf serum and 10 percent glycerol.
8. Place the embryos between two or four air bubbles in 0.25 ml sterile, pre-labelled plastic straws. Most often, one embryo is cryopreserved per straw.

9. Place straws horizontally in a freezing unit and cool from room temperature to -7 °C at a rate of 5 °C/minute.
10. Induce seeding at -7 °C by contact at the extreme end of the straw with liquid nitrogen-cooled tweezers, and freeze the embryo to -35 °C at a rate of 0.5 °C/minute.
11. Plunge the straws directly into liquid nitrogen and then store them in liquid nitrogen at -196 °C.

Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. Important! Do not bring the straws up above the frost line of the liquid nitrogen tank (the neck of the tank) until the correct straw is identified for embryo transfer.
2. Thaw the straw rapidly in a water bath at 20 °C for 30 seconds or 39 °C for 8 to 25 seconds, depending on the initial embryo-freezing rate. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 1M (molar) sucrose solution for ten minutes; then reduce the sucrose concentration in a stepwise procedure.
3. Prepare the Cassou gun and clean the perineal region of the recipient. Transfer the contents of one straw (one embryo) into the uterine horn corresponding to the corpus luteum of a day-seven recipient female.

CRYOPRESERVATION OF GOAT AND SHEEP EMBRYOS

Freezing

1. Follow steps 1 to 6 of the bovine protocol.
2. Equilibrate embryos collected from one donor female at room temperature for ten minutes in PBS with 10 percent foetal calf serum and 10 percent cryoprotective agent. Ethylene glycol can be used as a cryoprotectant for sheep, whereas glycerol is to be used for goats.
3. Place one or two embryos between two or four air bubbles in a 0.25 ml sterile pre-labelled plastic straw.
4. Place the plastic straws horizontally in the freezing unit and cool from room temperature to -7 °C at the rate of 5 °C/minute.
5. Induce seeding at -7 °C and freeze embryos to -30 °C at a rate of 0.3 °C/minute.
6. Plunge straws directly into liquid nitrogen. Store the straws in liquid nitrogen at -196 °C.

Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. Important! Do not bring the straws up above the frost line of the liquid nitrogen tank (neck of the tank) until the correct straw is identified for embryo transfer. Thaw one straw at a time and transfer the embryo(s) before thawing the next straw.
2. Thaw the straw rapidly in a water bath at 20 °C for 30 seconds. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 0.5M sucrose solution for ten minutes then reduce the sucrose concentration in a stepwise procedure.
3. Proceed to transfer the embryo into a prepared female.

CRYOPRESERVATION OF HORSE EMBRYOS

Freezing

The following procedure is the slow-freezing approach of Czlonkowska *et al*. (1985). Commercial vitrification kits are also an option.

1. Collect embryos approximately six to seven days after insemination. Transport them to the processing laboratory and wash them in PBS solution.
2. Equilibrate embryos collected from one donor female at room temperature by washing four separate times (for ten minutes each wash) in solutions of 2.5, 5, 7.5 and 10 percent glycerol in PBS.
3. Load each equilibrated embryo into a 0.25 ml straw in a drop of 10 percent glycerol solution between two air bubbles.
4. Seal the straw and place vertically in a freezing rack.
5. Cool the straw from room temperature to -6 °C at a rate of 1 °C /minute.
6. Hold the straw at -6 °C for five minutes and then seed with forceps cooled in liquid nitrogen.
7. Freeze the straw by reducing temperature to -33 °C at a rate of 0.3 °C /minute.
8. Hold the straws at -33 °C before plunging into liquid nitrogen.
9. Store the straws in liquid nitrogen.

Thawing

1. Remove the straw(s) from the tank and expose them to air for 10 seconds.
2. Submerge the straws for one minute in a 35 °C water bath.
3. Expel the embryos into a PBS solution containing 10 percent glycerol and 0.25M sucrose.
4. Wash out the cryoprotectant by incubating the embryos for ten minutes each in solutions of 0.25M sucrose in PBS with progressively decreasing concentrations of glycerol (7.5, 5, 2.5 to 0 percent).
5. Proceed to embryo transfer.

Appendix H

Bovine oocyte collection procedures

TECHNIQUE I – COLLECTION FROM DISSECTED OVARIES VIA "SLASHING"

Materials

- oocyte collection medium (OCM) – see Table H1
- L-glutamine
- BSS + heparin
- Pen/Strep
- 1 × saline solution (0.9 percent) – see Tables H3 and H4
- Petri dishes
- bench top paper
- 400 ml beaker
- scalpel handle
- scalpel blades (sizes #11 and #20)
- haemostat
- mouth pipette (optional)

Preparation for oocyte collection

1. Prepare Oocyte Collection Medium + supplements (OCM+ – see Table H2).
   a. Add the following to 1 litre of Oocyte Collection Medium (OCM – see Table H1)
      - BSS + heparin (stock) 20 ml
      - Pen/Strep (e.g. Gibco 15140-122) 10 ml
      - L-glutamine (e.g. Gibco 25030-081) 10 ml
   b. Place OCM+ at room temperature at least two hours before the arrival of the ovaries.
2. Set up working area for collection (one station per person).
   a. Cover bench top with paper.
   b. Place the following on the bench top:
      - 400 ml beaker
      - scalpel handles
      - scalpel blades (sizes #11 and #20)
      - haemostats
      - 1 × saline solution brought to room temperature to wash ovaries

Procedures

1. Clean the ovaries with 1 × saline solution.
2. Slash the ovaries to release oocytes.

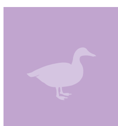
   a. Add 150 ml OCM+ to each beaker.
   b. Attach a haemostat to the base of the ovary. Cut the excess tissue away from the ovarian stalk by using a #20 scalpel blade and blot off blood with absorbent tissue.
   c. Slash follicles in the size range 2–8 mm. Hold the ovary above the beaker and make several small incisions to each follicle using a #11 scalpel blade. Follicular fluid and blood in the collection medium may result in clotting of the medium, thereby rendering it impossible to retrieve oocytes. To prevent clotting, avoid slashing large follicles (> 10 mm) and corpora lutea. Once all the follicles on the ovary have been slashed in one direction, go back over it and slash each in the opposite direction, making an X through each follicle. This effectively opens the follicles and allows the oocytes to be washed out.
   d. Submerge the ovary in OCM and swirl it several times. Repeat this process until all ovaries have been processed, with a maximum of ten ovaries per person.
3. Identify and isolate oocytes
   a. Once a group of ovaries has been processed, fill the beaker with OCM+ and incubate it at room temperature for five minutes to allow oocytes to settle.
   b. Bathe the outside of the beaker with ethanol and place the beaker on a stable surface and allow oocytes to settle again for a few minutes.
   c. Using aseptic technique, slowly aspirate OCM from the top of the beaker down to 50 ml. Be careful to not disturb the oocytes on the bottom of the beaker. Stop immediately if this occurs and allow the oocytes to settle again. Fill the beaker again with OCM+ and let it settle for another five minutes. Then slowly aspirate down to 50 ml.
   d. Transfer the remaining media, with oocytes, to two grid plates. Wash the beaker with about 20 ml of OCM and add to the grid plates.
   e. Collect cumulus oocyte complexes as fast as possible using a mouth pipette. Place the retrieved cumulus oocyte complexes into the first Petri dish containing OCM+ for further washing.
   f. Transfer the cumulus oocyte complexes from the first dish to the next, leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

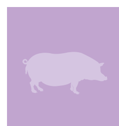
Video demonstrations of the above techniques can be found at http://www.animal.ufl.edu/hansen/ivf/Videos.htm courtesy of Dr P. Hansen, University of Florida.

TECHNIQUE II – COLLECTION VIA ASPIRATION

Materials

- holding medium (HM) – see Table H5
- 1 × saline solution (0.9 percent)
- Petri dishes
- bench top paper
- 50 ml conical tube
- 10 ml plastic syringe
- 20–22 gauge needle
- mouth pipette (optional)





Preparation

1. Set up working area for collection (one station per person) by covering bench to with paper.
2. Place the following materials on the bench top:
   - 50 ml conical tube
   - tube holder
   - 10 ml air syringe
   - 20–22 gauge needle
   - saline brought to room temperature to wash ovaries

Procedures

1. Clean the ovaries with 1 × saline solution.
2. Extract the oocytes from the ovaries via aspiration.
   a. Use the syringe to aspirate every follicle in the size range 2–8 mm.
   b. Deposit the follicular fluid slowly in the 50 ml conical tube.
   c. After aspirating all the ovaries, allow the oocytes to settle.
   d. Using a Pasteur pipette, slowly aspirate the oocytes from the bottom of the 50 ml tube. Be careful not disturb the oocytes.
   e. Place the oocytes in a grid dish containing enough HM to cover the dish.
   f. Wash the oocytes with HM three times.
   g. Collect cumulus oocyte complexes as fast as possible using a mouth pipette. Place the retrieved cumulus oocyte complexes into the first Petri dish containing OCM+ for further washing.
   h. Transfer the cumulus oocyte complexes from the first dish to the next, leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

PREPARATION OF DIFFERENT MEDIA FOR OOCYTE COLLECTION

Table H1. Oocyte collection medium (OCM) (without supplements)

Ingredient	Quantity/litre	Location
Medium 199 with Hank's Salts (e.g. Sigma M-0393)	1 bottle = 10.6 g	Refrigerator
$NaHCO_3$ (e.g. Sigma S-5761)	0.35 g	TC cabinet*
HEPES (e.g. Sigma H-3375)	5.95 g	TC cabinet

*TC cabinet = temperature controlled cabinet.

1. Mix Medium 199, HEPES, and $NaHCO_3$ with 0.95 litres MilliQ $H_2$0.
2. Using 10M NaOH, adjust pH to ~7.4 and bring the volume to 1 litre.
3. Sterile-filter the medium into bottles.
4. Store at 4 °C for up to three months.
5. Label as "OCM" or "OCM - supplements", including date of preparation.

Table H2. Oocyte collection medium (OCM) (with supplements)

Ingredient	Amount	Location
BSS + heparin (stock)	20 ml	Freezer
Pen/Strep (e.g. Gibco 15140-122)	10 ml	Freezer
L-glutamine (Stock)	10 ml	Freezer
OCM - supplements (Stock)	1 litre	Refrigerator

1. On the day of use, add BSS + heparin, Pen/Strep and L-glutamine to the OCM.
2. Date and label "OCM + Supplements".
3. Make 500ml if only one person is slashing – half of Pen/Strep and L-glutamine aliquots can be refrozen.

Table H3. 10X saline stock solution

Ingredient	Amount	Location
NaCl	90 g	TC cabinet*
MilliQ H_2O	1 000 ml	

*TC cabinet = temperature-controlled cabinet.

1. Combine ingredients and mix thoroughly.
2. Sterile-filter the solution.
3. Date, label "10 × saline", and store at 4 ºC.

Table H4. 1X transport saline 0.9 percent (prepare from 10 × solution)

Ingredient	Amount	Location
10 × saline	100 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	10 ml	Freezer

1. Mix ingredients and fill with MilliQ H_2O to 1 litre.
2. Date, label and store at 4 °C.

Table H5. Holding medium (HM) – HEPES Talp

Ingredient	Amount	Location
BSA, fraction V (Sigma A-3311)	120 mg	Refrigerator
HEPES-TL	39.2 ml	Refrigerator
Na pyruvate (Sigma P-5280)	0.4 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	0.4 ml	Freezer

1. Combine and mix ingredients, ensure that the final pH is approximately 7.4.
2. Sterile-filter the medium.
3. Date, label "HEPES-Talp" and store at 4 ºC for one week.

Appendix I
In vitro maturation of bovine oocytes

MATERIALS

- Petri dishes
- mouth pipette (optional)
- heat-pulled Pasteur pipette
- oocyte maturation medium (OMM – See steps 1 and 2 under “Preparation” below)
- medical grade mineral oil
- warming plate
- incubator
- laminar flow hood

PREPARATION

1. Add 8.835 ml of Medium 199, 20 µl of FSH stock, 125 µl of LH stock, 1 ml of foetal bovine serum (FBS) and 100 µl of Pen/Strep to a 15 ml tube. Filter the medium through a 0.4 µm membrane (see Zhang *et al.,* 1992).
2. Add 10 µl oestradiol stock.
3. Prepare 25-µl maturation droplets of filtered OMM on a Petri dish (five to nine droplets per dish), cover with mineral oil and equilibrate in an incubator (5 percent CO_2) for at least three hours.
4. Prepare two to four 75 µl wash droplets of OMM on a separate Petri dish.

PROCEDURE

1. Rinse the cumulus oocyte complexes at least twice in the 75 µl droplets containing OMM.
2. Transfer ten cumulus oocyte complexes to each 25 µl droplet of OMM under oil.
3. Incubate for 22 hours at 39 °C and 5 percent CO_2.

It is essential that the oocytes be collected, washed and incubated in OMM as quickly as possible to ensure maximum developmental rates. This entire process should never exceed two hours.

Blood is toxic to oocytes and embryos, so it is imperative that they be washed thoroughly to remove blood prior to transfer to OMM.

Appendix J
Bovine *in vitro* fertilization protocol

MATERIALS

- centrifuge carriers
- Percoll (ENHANCE-S Plus)
- sperm-TL (Table J1)
- IVF Talp (Table J4)
- HEPES Talp (Table J5)
- SP Talp (Table J6)
- penicillamine, hypotaurine and epinephrine (PHE) medium
- heparin
- CR1aa medium
- 15 ml conical tubes
- Petri dishes
- mouth pipette (optional)
- heat-pulled Pasteur pipettes
- sterile pipette tips and pipettor
- microcentrifuge tubes (1.5 ml)
- medical grade mineral oil
- laboratory tissues (e.g. Kimwipes®)
- standard haemocytometer
- water bath
- incubator
- centrifuge
- stereomicroscope
- laminar flow hood

PREPARATION

1. Prepare PHE medium.
   a. Prepare primary stocks of 2 mM penicillamine (3 mg/10 ml saline), 1 mM hypotaurine (1.09 mg/10 ml saline) and 250 mM epinephrine (1.83 mg dissolved in 40 ml of the following solution: 165 mg 60 percent Na lactate syrup, 50 mg Na metabisulfate and 50 ml H_2O).
   b. Combine 5 ml of 1 mM hypotaurine, 5 ml of 2 mM penicillamine, 2 ml 250 mM epinephrine and 8 ml saline and sterile filter.
   c. Aliquot PHE medium into sterile microfuge tubes and store in a light-resistant container at -20 °C indefinitely.

2. Move PHE medium (400 µl) and heparin (200 µl) from freezer to oven (39 °C). PHE medium should be covered with aluminium foil (light sensitive).
3. Make fertilization microdrops.
   a. Make five 44 µl drops of fertilization media (IVF-Talp) in each 35 mm dish. Cover with pre-warmed and pre-gassed mineral oil (ten oocytes per drop).
   b. Make four 70 µl drops of fertilization media in a 35 mm dish (washing medium). Cover with mineral oil.
   c. Equilibrate in CO_2 incubator (39 °C) for at least two hours.
4. Fill one conical tube with ~10 ml HEPES-Talp. Label the tube.
5. Fill one conical tube with ~5 ml of IVF-Talp. Label the tube.
6. Fill one conical tube with 5 ml SP-Talp. Label the tube.
7. Also prepare one conical tube with 8 ml HEPES-Talp.
8. Transfer tubes of HEPES-Talp (cap tightly) and SP-Talp (cap tightly) to the water bath (39 °C).
9. Transfer IVF-Talp (cap loosely) to CO_2 incubator.
10. Prepare Percoll Gradient: Label one conical tube "Percoll Gradient" and fill the tube with 100 µl of sperm TL and 900 µl of ENHANCE-S Plus.
11. Carefully transfer Percoll Gradient to the water bath.

PROCEDURES

1. At 22 to 24 hours post-maturation, thaw one straw of semen in water at 39 °C for 30 seconds. When getting semen straws out of the liquid nitrogen tank, be sure not to raise any of the other straws above the frost line. Use special semen forceps.
2. Dry a straw, hold it in a laboratory tissue to keep it warm and prevent exposure to light! Cut off the sealed ends and slowly layer thawed semen on top of the Percoll Gradient. Centrifuge at 1 200 rpm for 20 minutes.
3. Check the viability of the thawed semen. Dilute one drop of semen with SP-Talp and place 5 µl of the suspension on a slide. View at 40X magnification to assure that motile sperm are present.
4. While the centrifuge is running, pour 1 ml of HEPES-Talp (from conical tubes in CO_2 incubator) into a Petri dish (35 mm). Remove oocytes from each well of OMM plate and transfer to the dish containing HEPES-Talp.
5. Transfer the oocytes to the washing medium (from step 3b in Preparation).
6. Transfer up to ten oocytes into each 44 µl fertilization microdrop. Return the IVF plate with the microdrops to the incubator when finished. You only have 15 minutes to wash and transfer all oocytes to the microdrops. Set a timer and ask for help if necessary.
7. After the centrifuge stops, carefully remove the carrier with the Percoll Gradient from the centrifuge. There should now be a sperm pellet, if not you must completely start again with new gradient and semen.
8. Within the laminar flow hood, aspirate the Percoll down to the sperm pellet. Slowly add the 5 ml of pre-warmed SP-Talp to the conical tube containing the sperm pellet. Transfer the tube to the second pre-warmed centrifuge carrier and centrifuge at 1200 rpm for an additional ten minutes.

9. After the centrifuge stops, aspirate the SP-Talp down to the sperm pellet. Return the conical tube with the sperm pellet to the water bath.
10. Determine sperm pellet concentration (see standard haemocytometer procedure).
    - Gently swirl the sperm pellet to mix the sperm with any remaining medium. Use a clean pipette tip to transfer 5 µl of sperm into 95 µl of water; pipette gently to mix.
    - Clean the haemocytometer and coverslip by washing with water followed by 70 percent ethanol; dry with a tissue.
    - Using a new pipette tip, transfer 10 µl of diluted sperm into each chamber (each side) of the haemocytometer.
    - Use 40X magnification to count sperm cells in the five squares arranged diagonally across the central square on one side of the haemocytometer. Use an event counter to keep track of how many cells are counted. Record the count.

    a. Continue counting on the second side of the haemocytometer, counting the five diagonally arranged squares to obtain the total haemocytometer count. If the count of one side varies more than 10 percent from that on the other side, then the diluted sample was not properly mixed. Repeat the procedure starting at step a. When the count is consistent, record the total count and continue the procedure.

    b. Clean the haemocytometer and coverslip with water followed by ethanol.
11. Prepare sperm suspension for insemination.

    Note that the final sperm suspension used for IVF is composed of fertilization medium and sperm pellet produced by Percoll separation. The following instructions summarize and simplify the determination of quantities of pelleted sperm and medium required:

    a. Use the following formula to calculate the volume of sperm pellet needed per 300 µl of final sperm suspension, assuming that 1×10^6 sperm/ml is the desired concentration in the final fertilization medium, using the formula:
    - 7 500/x = µl of sperm pellet to make 300 µl of final sperm suspension when inseminating with 1×10^6 sperm/ml; where x is the average haemocytometer count (total haemocytometer count divided by two).

    b. If a concentration other than 1×10^6 sperm/ml is desired, then the volume of the sperm pellet must be adjusted to accommodate the difference. To adjust this volume perform the following calculation:
    - Sperm concentration desired/1×10^6 (sperm/ml) = sperm concentration adjustment factor.
    - Multiply the volume of the sperm pellet by this adjustment factor to yield the volume of sperm pellet needed to prepare 300 µl of final sperm suspension at the desired concentration.
    - Example: if a bull requires 2×10^6 sperm/ml rather than 1×10^6 sperm/ml, 2×10^6 sperm/ml/1×10^6 sperm/ml = adjustment factor of 2.
    - (adjustment factor) × (µl of pellet needed for 1×10^6 sperm/ml) = µl of sperm pellet needed to yield 2×10^6 sperm/ml in 300 µl of final sperm suspension providing 2×10^6 sperm/ml in the fertilization microdrop.

    c. Calculate the volume of fertilization medium needed in the final sperm suspension (i.e. by subtracting the volume of the sperm pellet from 300 µl).

d. Place the calculated amount of fertilization medium into an Eppendorf microcentrifuge tube. Then add the calculated amount of sperm pellet to the tube. Sperm stick to plastic, so add the fertilization medium to the tube first. Mix gently by pipetting up and down several times within the tube. Immediately begin fertilizing the oocytes in the microdrops, as the pH of this solution will change rapidly.

12. Fertilization
    a. Add 2 µl heparin (for a final concentration of 2 µg/ml of heparin in the fertilization medium), 2 µl of PHE medium and 2 µl of final sperm suspension to each fertilization microdrop.
    b. Record the time and the date on each IVF microdrop plate.
    c. Incubate for 18 hours at 39 °C in a humidified atmosphere of 5 percent CO_2 in air.
13. Embryo culture
    a. Make five 20 µl microdrops of CR1aa in a 35 mm dish. Cover the drops with oil. Incubate for at least 20 minutes to the allow medium to equilibrate.
    b. Thaw one vial of hyaluronidase (1 mg/ml). Place the solution in a 1.5 ml conical tube. Incubate the tube in the water bath for about two minutes.
    c. Place the oocytes (which are hopefully zygotes/embryos) in the tube containing the hyaluronidase solution. Each tube can accommodate up to 300 embryos.
    d. Vortex the oocytes at maximum speed for three minutes.
    e. Transfer the oocytes to one 35 mm dish containing HEPES-Talp. Rinse the tube with HEPES-Talp to capture all embryos.
    f. Wash the oocytes four times in CR1aa.
    g. Place ten oocytes in each microdrop of CR1aa.
    h. At day three, transfer the oocytes into new microdrops of CR1aa.
    i. Check development at day seven.

MEDIA PREPARATION

Table J1. Sperm-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	100	582	TC cabinet*
KCl (Sigma P-5405)	3.1	23	TC cabinet
$NaHCO_3$ (Sigma S-5761)	25	209	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.29	3.48	TC cabinet
HEPES (Sigma H-3375)	10	238	TC cabinet
Lactic acid (Sigma L-7900)	21.6	183.4 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2 \bullet 2H_20$ (Sigma C-7902)	2.1	29	Desiccator
$MgCl_2 \bullet 6H_20$ (Sigma M-2393)	1.5	31	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, HEPES, lactic acid, and phenol red to a beaker. Bring volume to 80 ml with double distilled water (ddH2O) and dissolve completely.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to other ingredients.

3. Check to ensure that pH = ~7.4 and then adjust volume to 100 ml with ddH2O.
4. Sterile-filter the resulting solution into a bottle.
5. Date, label "SP-TL", and store at 4 oC for up to two weeks.

Table J2. IVF-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	114	666	TC cabinet*
KCl (Sigma P-5405)	3.2	23.5	TC cabinet
$NaHCO_3$ (Sigma S-5761)	25	210.4	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	4.08	TC cabinet
Lactic acid (Sigma L-7900)	10	84.92 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2$•$2H_20$ (Sigma C-7902)	2	30	Desiccator
$MgCl_2$•$6H_20$ (Sigma M-2393)	0.5	10	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, lactic acid, and phenol red to a beaker.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to the other ingredients.
3. Bring the volume to 80 ml with ddH2O, and dissolve completely.
4. Check for pH ~7.4 and then adjust the volume to 100 ml with ddH2O.
5. Sterile-filter into a bottle.
6. Date, label "IVF-TL", and store at 4 oC for two weeks.

Table J3. HEPES-TL

Ingredient	Final (mM)	mg/500 ml	Location
NaCl (Sigma S-5886)	114	3330	TC cabinet*
KCl (Sigma P-5405)	3.2	120	TC cabinet
$NaHCO_3$ (Sigma S-5761)	2	84	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	20.4	TC cabinet
HEPES (Sigma H-4034)	10	1,200	TC cabinet
Lactic acid (Sigma L-7900)	10	424.6 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	500 µl	TC cabinet
$CaCl_2$•$2H_20$ (Sigma C-7902)	2	150	Desiccator
$MgCl_2$•$6H_20$ (Sigma M-2393)	0.5	50	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, HEPES, lactic acid, and phenol red to a beaker.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to the other ingredients.
3. Bring the volume to 450 ml with ddH2O and dissolve completely.
4. Check for pH ~7.4 and then adjust volume to 500 ml with ddH2O.
5. Sterile-filter into a bottle.
6. Date, label "HEPES-TL", and store at 4 oC for two weeks.

Table J4. IVF-Talp

Ingredient	Amount	Location
BSA, EFAF (Sigma A-6003)	60 mg	Refrigerator
IVF-TL	9.8 ml	Refrigerator
Na pyruvate (Sigma P-5280)	100 µl	Refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	Freezer

1. Combine and mix all ingredients; pH should be ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "IVF-Talp", and store at 4 oC for up to one week.

Table J5. HEPES-Talp

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	Refrigerator
HEPES-TL	20 ml	Refrigerator
Na pyruvate (Sigma P-4562)	0.2 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	0.2 ml	Freezer

1. Combine and mix all ingredients; pH should be ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "HEPES-Talp", and store at 4 oC for up to one week.

Table J6. Sperm–Talp (SP-Talp)

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	Refrigerator
SP-TL(Specialty Medium BSS-009-D)	9.5 ml	Refrigerator
Na pyruvate (20mM Stock)	0.5 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	Freezer

1. Combine and mix all ingredients; adjust pH to ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "Sperm-Talp", and store at 4 oC for up to one week.

Table J7. 10X SP-TL (for Percoll Gradient)

Ingredient	g/100 ml	Location
NaCl (Sigma P-5886)	4.675	TC cabinet*
KCl (Sigma P-5405)	0.23	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.348	TC cabinet
HEPES (Sigma H-4034	2.38	TC cabinet

*TC cabinet = temperature-controlled cabinet.

1. Combine and mix ingredients. Do not adjust pH!
2. Bring volume to 100 ml with double distilled H2O.
3. Sterile filter.
4. Date, label "10X SP-TL", and store at 4 oC for up to one month.

Appendix K
Harvesting tissue samples for cryoconservation of somatic cells

Collection of somatic cells for gene banking can be done via two approaches: 1) collection and cryopreservation in the field; and 2) field collection with processing and freezing in the laboratory. The choice will depend on the situation in the given country. Protocols for both approaches are described below.

IN THE FIELD (SAME FOR BOTH PROTOCOLS)

1. To prevent the infection of tissue samples, ensure that the conditions for sampling are as clean as possible, especially the working area for placing the sampling equipment.
2. Identify the animals to be sampled.
3. Restrain each animal and record as much of the following information as possible (see Section 9 for more details about data collection):
   - site-specific data, including GPS coordinates;
   - animal-specific information (Section 10), including a digital photo; and
   - sample-specific information (e.g. vial and animal numbers).
4. Clean and disinfect the area of skin from where the sample is to be taken.

PROTOCOL 1 – ONE-STEP COLLECTION AND FREEZING IN THE FIELD[12]

In the laboratory prior to field collection

1. Prepare a cryoprotectant medium by mixing 70 percent PBS and 30 percent of 87 percent glycerol, yielding a final concentration of 26.1 percent glycerol.
2. Store aliquots of the mixture in small, tightly capped vials (2–3 ml: e.g. centrifuge tubes) sufficient for five or six tissue samples (0.3 ml per sample).

Required equipment

- specialized ear tagger with tissue biopsy punch (see example in Photo K1)
- combination ear tag/sample cap (see example in Photo K2)
- portable cryotank with liquid nitrogen
- micropipette and tips
- plastic storage vials with cryoprotectant (prepared as described above)
- latex gloves and disinfectant spray (e.g. ethanol 70 percent)
- animal restraining equipment

[12] Groeneveld *et al.* (2008).

PHOTO CREDIT: CAISLEY INTERNATIONAL GMBH

PHOTO K1
Ear tagger

PHOTO CREDIT: CAISLEY INTERNATIONAL GMBH

PHOTO K2
Ear tag / sample vials

Collection procedure

1. Fill the sample cap of the ear tag with 0.3ml of the cryoprotective medium and secure it in position in the ear tagger.
2. Place the ear tagger[13] in the proper position on the ear of the animal and collect the sample by squeezing firmly the handles of the ear tagger. This will push the ear biopsy into the sample cap.
3. Remove the sample cap containing the ear biopsy from the tagger and shake it gently to ensure the cryoprotectant medium fully covers the ear biopsy stored inside.
4. Place the sample cap directly into the portable cryotank.
5. The samples may be moved to a larger tank for long-term storage upon return to the gene bank. This must be done as quickly as possible to avoid the risk of thawing.

PROTOCOL TWO – FIELD COLLECTION AND LABORATORY PROCESSING

Required equipment

- sampling tool (e.g. surgery kit, scalpel, hole punch or ear notcher/tagger)
- tweezers
- sample vials
- laboratory gloves and disinfectant spray (e.g. ethanol 70 percent)

[13] Ear taggers and similar sampling tools can be purchased commercially from various sources, including the following: www.prionics.com, www.caisley.de, www.biopsytec.com and www.prosampler.com

- animal restraining equipment
- transport container with ice (e.g. Styrofoam box)

Collection procedure

1. Use the chosen tool to obtain a skin sample (e.g. ear notch, skin biopsy).
2. Insert the sample into a sterilized, labelled storage vial.
3. Place the vial into the transport container.
4. Return to the laboratory for further processing and long-term storage.

Processing procedure

1. Slice the tissue sample into small pieces preferably 0.25 to 1.0 cubic mm.
2. Submerge in phosphate-buffered saline (PBS) supplemented with 80 mg/ml of streptomycin sulfate, 60 000 units/ml of benzylpenicillin and 20 000 IU/ml of potassium penicillin G.
3. Wash the samples (four to five pieces) in 20 percent foetal calf serum-PBS (FPBS).
4. Place pieces in vitrification solution (20 percent [v:v] ethylene glycol, 20 percent [v:v] dimethylsufoxide in FPBS).
5. Load the mixture into 0.25 ml French straws (1.5 cm of FPBS, air bubble, samples in 4 cm of vitrification solution, air bubble and 1.5 cm of FPBS).
6. Seal the straws.
7. Plunge the straws vertically into liquid nitrogen.

THAWING SAMPLES (BOTH PROTOCOLS)

Samples are thawed by holding the straw or sample cap in liquid nitrogen vapour for ten seconds and then submerging them in a 23 °C water bath for five seconds. The sample is then expelled into a dish, diluted with 0.25 M sucrose in FPBS for five minutes and then placed in FPBS (without sucrose) for five minutes. Samples can then be cultured for SCNT or used for DNA extraction.