This practical demonstration will briefly demonstrate a protocol used to freeze bovine semen for private use by the owner. It is important that practitioners familiarise themselves with the legal aspects around semen freezing, as set out in the Animal Improvement Act, Act 62 of 1998, as well as the regulations R1682 of 21 November 2003. Due to time constraints, this will not form part of the demonstration.

The protocol will be demonstrated in five parts, as follows:

Preparation:

1. Prior to attempting cryopreservation of semen from the bull, the bull must be examined for breeding soundness (in other words clinically healthy, acceptable semen quality, negative for TB, BM, BVD, BLV, Trichomonas and Vibrio).
2. Equipment required include:
   - Record book or sheet
   - 2 hot water flasks
   - 2-3 bottles (50ml) Phosphate Buffered Saline (PBS),
   - one 60ml catheter-tip disposable syringe (sterile),
   - scissors,
   - disinfectant such as F10SC®,
   - electro-ejaculator (El Toro®) or artificial vagina,
   - clean collection cones and sterile semen tubes (5 x 15ml and 2 x 50ml),
   - sheath wash or sheath scraping apparatus and Steve’s Transport Medium®,
   - 3 to 8 x 15ml semen extender such as Triladyl® (frozen),
   - 3 water jackets for 15ml semen tubes (can be made by drilling a 12mm hole into the cap of a 50ml semen tube,
   - microscope equipped with heated stage and phase contrast
   - slides, cover slips, glass pipettes, eosin nigrosin stain,
   - haemocytometer,
   - sheep counter or differential cell counter
   - 10ml upright test tube,
   - 100ul calibrated pipette,
   - a walk-in fridge or cool-room is required during warm weather,
   - semen straws printed or hand-written with all the relevant information
   - thick latex gloves, preferably sterile
   - plastic comb to create air bubbles in semen straws
   - coloured methyl cellulose powder
   - aluminium semen freezing rack
   - large polystyrene box
   - forceps to handle frozen straws
   - liquid nitrogen and a liquid nitrogen flask
   - goblets to store straws in
Semen Collection from the Bull:

1. Prepare two hot water flasks: one with water at 20°C and the other with water at 35°C.
2. Thaw 15ml of the semen extender to 35°C.
3. Prepare a clean semen collection cone and tube with a water jacket attached, at 35°C. Fill the jacket with 35ml water at 35°C.
4. Put the bull in a safe crush with a pole behind his hind legs to prevent slipping, immobilise the bull.
5. Carefully place the El Toro electro-ejaculator probe.
6. Cut the hair around the sheath opening approximately 1cm, and remove any dirt.
7. Wash the external part of the sheath opening well with a disinfectant solution. It is critical to make sure that no dirt is present during semen collection since it will contaminate the frozen semen sample which could lead to infections in the cow's uterus after Artificial Insemination.
8. Wash the inside of the sheath with 50ml PBS and 20ml air put into a 60ml catheter-tip syringe (alternatively use normal method for sheath wash). Massage the fluid back into the fornix of the sheath vigorously for 50 strokes.
9. Repeat no 7 and rinse out completely by massaging the fluid out of the sheath.
10. The first sample can be submitted for Trichomonas and Vibrio culture.
11. Collect semen as per breeding soundness examination, taking care not to contaminate the sample. Ideally a full erection should be achieved. Do not collect unnecessary seminal fluid (pre-sperm and post-sperm fractions).
12. Using a sterile glass pipette, observe the mass motility (hanging droplet) of the fresh semen sample, it should be at least 3 to continue.
13. If more than 7ml semen was collected, prepare another water jacket at 35°C and divide the sample into two equal parts after inverting it a few times to ensure that the sample is homogenous throughout.
14. Dilute the semen 1:1 with semen extender (both still in water jackets at 35°C) by adding 1ml semen extender at a time (to prevent osmotic shock to the sperm cells during glycerol/water exchange) and inverting the tube in between. Ensure that you keep the caps clean (do not place it on any surface) before inverting every time.
15. Replace the water in the water jackets with water at 20°C. During very cold weather this step can be left out, as the water jacket would almost have reached that temperature by now.
16. Place the semen and semen extender mixture inside the 20°C water jackets on top of ice packs or ice cubes inside a small polystyrene container (not inside ice water).
17. The sample and water jacket should now equilibrate gradually to 4°C in no less than 2 ½ hours. It is important to slowly decrease the temperature from 20°C to 4°C to prevent osmotic shock to the sperm cells during the transfer process of glycerol and water over the sperm membrane.

Procedures in the lab:

18. Thaw the rest of the semen extender and then cool it down to 4°C again.
19. Invert the semen and semen extender mixture a few times and filter through a small gauze swab into a pre-cooled 50ml semen tube. Leave the semen tube in ice water permanently from now on.
20. Put exactly 7.9ml tap water into the 10ml upright test tube and add 100ul of the semen and semen extender mixture using the calibrated pipette.
21. Using a glass pipette, place a drop of the water-semen mixture next to the cover slip of the haemocytometer, just enough to completely fill (but not over-fill) the chamber of the haemocytometer. Leave it for 5 minutes in order for the dead sperm cells to sediment to the bottom of the chamber.
22. Using a pipette, place a drop of the semen-extender sample on a cover slip next to a drop of eosin-nigrosin stain and mix gently. Place a drop of this mixture on a clean microscope slide and make a smear for morphology purposes. Label the slide with the bull's number and date for future reference and put aside to dry.
23. In the meantime, determine the individual motility of sperm cells in the semen and semen extender mixture as per breeding soundness examination (% linearly motile:% aberrantly motile:% immotile). There should be at least 65% motile sperm at this point to continue.

24. Now count all the sperm cells in 20 of the large blocks of the haemocytometer, this should give the concentration of the semen and semen extender mixture in sperm cells x 10^6/ml.

25. Evaluate the morphology smear by using the 100X objective and immersion oil. In order to continue with the freezing process, the sample needs to have a 75% or higher normal morphology score.

26. Calculate the total number of motile sperm cells as follows:
   Total number of motile sperm cells (x10^6) = concentration (x 10^6/ml) x % motile sperm cells x volume of semen and semen extender mixture (ml).

27. Calculate the number of straws that can be filled as follows:
   Number of straws to be filled = Total number of motile sperm cells ÷ number of motile sperm cells required per straw (eg 10 x 10^6). Keep in mind that approximately 50% of the motile sperm cells will become immotile after freeze-thawing. It is wise to leave an extra safety margin at this point.

28. Next calculate the final volume required to fill all the straws as follows:
   Final volume (ml) = Number of straws to be filled x volume of the straws (eg 0.25ml).

29. Extend the semen mixture to the final volume by adding more semen extender (both at 4° C). Never add more than the original volume of semen at one time, and always homogenise the sample between fillings by inverting the tube.

In the cold room:

30. Wear thick latex gloves to minimise heat transfer from your hands to the straws.

31. Place a small amount of methyl cellulose powder on a clean, smooth and flat surface such as a stainless steel table, and prepare a small dish of cold water.

32. Grab about 8 to 9 straws near the plunger end, and fill them by submerging the open end under the semen mixture and sucking on the cotton plunger end until you can feel that the straw is full.

33. Put the straws into the plastic comb to create an air bubble adequate in size to prevent the straw from bursting or the plug from being pushed out when the contents become frozen.

34. Repeat steps 29 and 30 until the comb is full (25 straws).

35. Do not loose the run-off from the comb.

36. Grab about 8 to 9 straws from the comb, and create plugs by pressing the open ends of the straws (where the air bubbles are) into the methyl cellulose. The plugs should be 1-2mm in size.

37. Rinse the straws in the cold water to remove excess methyl cellulose powder and to ensure that the plugs become hard.

38. Wipe the excess fluid gently off the straws taking care not to heat the straws.

39. Place the straws on a semen rack, 1cm apart, and repeat steps 29 to 35 until the rack is full.

40. When the rack is full, place the straws in nitrogen vapour, 4cm above the surface of the liquid nitrogen, and equilibrate for 20 minutes.

41. Submerge the straws under the liquid nitrogen, and pack them into the goblet.

42. Transfer the goblet to the nitrogen flask for storage.

Quality control:

Perform and record the following:

Post-thaw motility: Minimum requirement 35% linearly motile.
Concentration: Minimum requirement 5 x 10^6 motile sperm cells per straw.
Morphology: Minimum requirement 75% normal sperm cells.
Culture (colony count): Minimum requirement

It is wise to have quality control checked and reported by a third party to prevent bias.
References:


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9) Heriberto Rodríguez-Martínez, State of the art in farm animal sperm evaluation. Reproduction, Fertility and Development, 2007 19,91 -101, Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Ullsväg 14C, Clinical Centre, PO Box 7054, SE-750 07 Uppsala, Sweden.

