



**MINIMUM STANDERD PROTOCOL
FOR OVINE/CAPRINE
SEMEN PRODUCTION IN INDIA. (Draft)**

COLLECTION AND PROCESSING OF GOAT/SHEEP SEMEN

Objectives

The purposes of official sanitary control of semen production are to:

- 1) maintain the health of animals on an artificial insemination centre at a level which permits the international distribution of semen with a negligible risk of infecting other animals or humans with pathogenic agents transmissible by semen;
- 2) ensure that semen is hygienically collected, processed and stored.

GENERAL HYGIENE IN SEMEN COLLECTION AND PROCESSING CENTRES

(A) Requirements of Artificial insemination centres

1. The artificial insemination centre is comprised of:

- a. animal accommodation areas (including one isolation facility for sick animals) and a semen collection room, these two premises here on designated as semen collection facilities; accommodation areas should be species specific where relevant;
- b. a semen laboratory and semen storage areas;
- c. administration offices;
- d. a pre-entry isolation facility

2. The centre should be under the direct supervision and control of a centre veterinarian.

3. Only animals associated with semen production should be permitted to enter the centre. Other species of livestock may exceptionally be resident on the centre, provided that they are kept physically apart from these animals.

4. Donors and teasers on the centre should be adequately isolated from farm livestock on adjacent land or buildings for instance by natural or artificial means.

5. The entry of visitors should be strictly controlled. Personnel at a centre should be technically competent and observe high standards of personal hygiene to preclude the introduction of pathogenic organisms. Protective clothing and footwear for use only on the centre should be provided.

6. Individual semen containers and storage rooms should be capable of being disinfected

7. The centre should be officially approved by the Veterinary Authority.

(In this context, Veterinary Authority means the Governmental Authority of an OIE Member, comprising veterinarians, other professionals and para-professionals, having the responsibility

and competence for ensuring or supervising the implementation of animal health and welfare measures, international veterinary certification and other standards and recommendations in the Terrestrial Code in the whole territory.)

8. The centre should be under the supervision and control of the Veterinary Services which will be responsible for regular audits, at an interval of no more than 12 months, of protocols, procedures and records on the health and welfare of the animals in the centre and on the hygienic production, storage and dispatch of semen.

(B) Requirements of Bucks and teaser animals

1. Prior to entering pre-entry isolation facility

The animals should comply with the following requirements prior to entry into isolation at the pre-entry isolation facility where the country or zone of origin is not free from the diseases in question.

- a. Brucellosis – Chapter 8.4.
- b. Ovine epididymitis – Article 14.6.3.
- c. Contagious agalactia – Points 1) and 2) of Article 14.2.1.
- d. Peste des petits ruminants – Points 1), 2 a) or 3) of Article 14.7.10.
- e. Contagious caprine pleuropneumonia – Article 14.3.7., depending on the CCPP status of the country or zone of origin of the animals.
- f. Paratuberculosis – Free from clinical signs for the past two years.
- g. Scrapie – Comply with Article 14.8.8. if the animals do not originate from a scrapie free country or zone as defined in Article 14.8.3.
- h. Maedi-visna – Article 14.5.2.
- i. Caprine arthritis/encephalitis – Article 14.1.2. in the case of goats.
- j. Bluetongue The animals should comply with Articles 8.3.7. or 8.3.8., depending on the bluetongue status of the country or zone of origin of the animals.
- k. Tuberculosis – In the case of goats, a single or comparative tuberculin test, with negative results.

2. Testing in the pre-entry isolation facility prior to entering the semen collection facilities

Prior to entering the semen collection facilities of the artificial insemination centre, rams/bucks and teasers should be kept in a pre-entry isolation facility for at least 28 days. The animals should be tested as described below a minimum of 21 days after entering the pre-entry isolation facility, with negative results.

- a) Brucellosis – Chapter 8.4.
- b) Ovine epididymitis – Point 1 d) of Article 14.6.4.
- c) Maedi-visna and caprine arthritis/encephalitis – Test on animals.
- d) Bluetongue

The animals should comply with the provisions referred to in Articles 8.3.6., 8.3.7. or 8.3.8., depending on the bluetongue status of the country or zone where the pre-entry isolation facility is located.

3. Testing programme for ram/bucks and teasers resident in the semen collection facilities

All rams/bucks and teasers resident in the semen collection facilities should be tested at least annually for the following diseases, with negative results, where the country or zone where the semen collection facilities are located is not free:

- a) Brucellosis;
- b) ovine epididymitis;
- c) Maedi-visna and caprine arthritis/encephalitis;
- d) tuberculosis (for goats only);
- e) bluetongue. The animals should comply with the provisions referred to in Article 8.3.9. or Article 8.3.10.

(C) Conditions applicable to the collection of semen

1. The floor of the mounting area should be clean and provide safe footing. A dusty floor should be avoided.
- 2) The hindquarters of the teaser, whether a dummy or a live teaser animal, should be kept clean. A dummy should be cleaned completely after each period of collection. A teaser animal should have its hindquarters cleaned carefully before each collecting session. The

dummy or hindquarters of the teaser animals should be sanitized after the collection of each ejaculate. Disposable plastic covers may be used.

3) The hand of the person collecting the semen should not come into contact with the animal's penis. Disposable gloves should be worn by the collector and changed for each collection.

4) The artificial vagina should be cleaned completely after each collection where relevant. It should be dismantled, its various parts washed, rinsed and dried, and kept protected from dust. The inside of the body of the device and the cone should be disinfected before re-assembly using approved disinfection techniques such as those involving the use of alcohol, ethylene oxide or steam. Once re-assembled, it should be kept in a cupboard which is regularly cleaned and disinfected.

5) The lubricant used should be clean. The rod used to spread the lubricant should be clean and should not be exposed to dust between successive collections.

6) The artificial vagina should not be shaken after ejaculation, otherwise lubricant and debris may pass down the cone to join the contents of the collecting tube.

7) When successive ejaculates are being collected, a new artificial vagina should be used for each mounting. The vagina should also be changed when the animal has inserted its penis without ejaculating.

8) The collecting tubes should be sterile, and either disposable or sterilized by autoclaving or heating in an oven at 180°C for at least 30 minutes. They should be kept sealed to prevent exposure to the environment while awaiting use.

9) After semen collection, the tube should be left attached to the cone and within its sleeve until it has been removed from the collection room for transfer to the laboratory

(A list of equipment needed for semen collection and freezing, is attached as **annexure 1**)

(D) Conditions applicable to the handling of semen and preparation of semen samples in the laboratory

1. Diluents

a) All receptacles used should have been sterilized.

b) Buffer solutions employed in diluents prepared on the premises should be sterilised by filtration (0.22 µm) or by autoclaving (121°C for 30 minutes) or be prepared using sterile water before adding egg yolk (if applicable) or equivalent additive and antibiotics.

c) If the constituents of a diluent are supplied in commercially available powder form, the water used should have been distilled or demineralised, sterilised (121°C for 30 minutes or equivalent), stored correctly and allowed to cool before use.

d) Whenever milk, egg yolk or any other animal protein is used in preparing the semen diluent, the product should be free from pathogenic agents or sterilised; milk heat-treated at 92°C for 3–5 minutes, eggs from SPF flocks when available. When egg yolk is used, it should be separated from eggs using aseptic techniques. Alternatively, commercial egg yolk prepared for human consumption or egg yolk treated by, for example, pasteurisation or irradiation to reduce bacterial contamination, may be used. Other additives should also be sterilised before use.

e) Diluent should not be stored for more than 72 hours at +5°C before use. A longer storage period is permissible for storage at -20°C. Storage vessels should be stoppered.

f) A mixture of antibiotics should be included with a bactericidal activity at least equivalent to that of the following mixtures in each ml of frozen semen: gentamicin (250 µg), tylosin (50 µg), lincomycin–spectinomycin (150/300 µg); penicillin (500 IU), streptomycin (500 µg), lincomycin–spectinomycin (150/300 µg); or amikacin (75 µg), divexacin (25 µg). The names of the antibiotics added and their concentration should be stated in the international veterinary certificate.

2. Procedure for dilution and packing

a) The tube containing freshly collected semen should be sealed as soon as possible after collection, and kept sealed until processed.

b) After dilution and during refrigeration, the semen should also be kept in a stoppered container.

c) During the course of filling receptacles for dispatch (such as insemination straws), the receptacles and other disposable items should be used immediately after being unpacked. Materials for repeated use should be disinfected with alcohol, ethylene oxide, steam or other approved disinfection techniques.

d) If sealing powder is used, care should be taken to avoid its being contaminated.

3. Conditions applicable to the storage and identification of frozen semen

Semen for export should be stored in straws separately from other genetic material not meeting the requirements of this chapter with fresh liquid nitrogen in sterilised/sanitised flasks before being exported.

Semen straws should be sealed and code marked in line with the international standards of the International Committee for Animal Recording (ICAR).

Prior to export, semen straws should clearly and permanently be identified and placed into new liquid nitrogen in a new or sterilised flask or container under the supervision of an Official Veterinarian. The contents of the container or flask should be verified by the Official Veterinarian prior to sealing with an official numbered seal before export and accompanied by an international veterinary certificate listing the contents and the number of the official seal.

(Cryopreservation of Sheep/Goat semen are attached as **annexure 11&111**)

(Guidelines for basic semen analysis is attached as **annexure V1**)

Annexure (1) List of equipment needed for semen collection and freezing

Major equipment and facilities

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

Small equipment and consumables

1. coveralls and boots
2. disposable gloves and boot covers
3. helmets (head protection), especially for semen collection from stallions
4. artificial vaginas, cones and collection tubes
5. lubricant
6. glass-lined thermos bottles
7. thermometers (centigrade)
8. semen diluents, extenders and cryoprotectants
9. plastic semen straws
10. assorted glassware and plasticware
11. artificial light

Annexure (11) Cryo-preservation 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. (Paulenz *et al.* (2002))

One-step procedure

- a. 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, 1mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) required to obtain the desired final sperm concentration.
- b. Cool to 4 °C within one hour and maintain for at least 1.5 hours.

Two-step procedure

- a. 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).
 - b. Cool progressively to +4 °C over two hours (0.2 °C/minute).
 - c. 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 trisodium- citrate-5.5 H₂O into 70 ml H₂O); then mix nine volumes of the resulting solution with one volume of glycerol.
 - d. Add Diluent B up to final volume (Diluent B consists of Diluent A +11 percent glycerol).
 - e. Add Diluent B in three equal parts, over 30 minutes, at 4 °C up to the final volume.
 - f. 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 H₂O 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.

Annexure (111) Cryo-preservation 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 CaCl₂, 2.11 percent KH₂PO₄, 3.82 percent MgSO₄·7H₂O, 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

- a. Add the entire sample to the volume of One-Step Diluent (300 mM Tris; 28mM 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).
- b. Cool to 4 °C within one hour and maintain for at least 1.5 hours.

Two-step procedure

- a. 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.
 - b. Add V/2 of Diluent A to the pelleted sperm at 20 °C.
 - c. Cool to +4 °C within 30 minutes (at 0.5 °C/minute).
 - d. 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.
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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

Annexure (V1) Guidelines for basic semen analysis

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.

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 $\geq 40X$ 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 1000X 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 perc