STANDARD OPERATING PROCEDURES FOR GOAT FROZEN SEMEN PRODUCTION

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Updates on this SOP will be made by re-issuing the relevant section of this

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STANDARD OPERATING PROCEDURE FOR GOAT FROZEN SEMEN PRODUCTION

Background

The Standard Operating Procedures (SOP) provide general guidelines for all the activities that are taking place in National Livestock Breeding Offices, Nepal. Several successful attempts to freeze goat semen in recent decades have been reported using various techniques and protocols. NLBO, Pokhara has successfully adopted a new protocol for goat semen freezing since Jan; 2019. The standard operation procedures (SOP) have been documented exclusively based on the adopted protocol and techniques of goat semen collection, processing, and freezing.

Objectives

To produce quality frozen semen of buck for Artificial Insemination in goat

Requirements

- Breeding Bucks for Semen Collection
- Pedigree Records
- Balance Ration
- Housing Requirement
- Health Measures
- Equipment
  - Grooming brush
  - Incubators, Hot air oven, Autoclave, Water Bath
  - Thermometer
  - Semen Straws (0.25ml)
  - Photometer, CASA System
  - Auto dispenser, Tractability Software
  - Semen filling and sealing machines
  - Bio freezer set, Transfer device, Containers, Straw racker
  - Artificial vagina (AV)
  - Microscope (Trinocular)
  - Foot-wears, Napkin, Tissue paper, Towel
  - Stage warmer, LED Display
  - AV Limner, AV cylinders, AV Knot, AV cone, AV Jacket
  - Laminar Air flow
  - Magnetic Stirrer, Magnets
  - Aprons (for collectors and bulls)
  - Annex 2
- Glassware (Conical flask, round bottom flask, measuring cylinders, test tubes, cuvettes, pipette)
- Reagents and Chemicals
  - 70% alcohol, Liquid hand wash
  - Normal saline solution, egg yolk and antibiotics
  - For quality control test (Annex 2)

Procedure

I. Selection Criteria

Breeding Buck for frozen semen production shall be selected based on

- General health conditions
- Pedigree Records
- Physical examination
- Andrological examination (Annex 1)
- Libido Index and Sexual Behavior

II. Management

Buck management comprises of following:

1.Feeding of adequate fodder and feed

- The nutrition of semen donor bucks is the most important factor to be taken into consideration. The bucks should be put on a higher plane of nutrition to have a satisfactory body condition score between three and four on a one to five scale.
- Good nutrition with Zinc, Selenium, Vitamin E, and Vitamin A supplementation is essential during the pre-collection period.
- Legume hay of good quality should be fed up to 500 g per day. The bucks should be exercised daily. The aim is to maintain their body condition score (BCS) between 3 and 4 out of 5 scales.
- Bucks should be fed dry matter at 5-6% of their body weight.
- The dry matter intake of bucks reflects the ability of voluntary feed intake to utilize the feed. While feeding forage and fodder, there should be leftover about 15% of the offered quantity in hay racks that indicate the buck was satisfied and had the chance to choose the fodder
- The donor bucks should be fed 0.2 to 0.4 kg of concentrate ration containing 16-18 percent CP and 65 percent TDN with sufficient minerals and vitamins along with 4 to 5 kg of green fodder preferably wilted or 1 to 2 kg of medium quality hay is sufficient to maintain the normal health and the semen production of buck.
- The body condition score of bucks should be regularly monitored and the amount of feed should be adjusted to maintain the body condition score between 3 and 4.

2.Supply of potable water at all the time

- Adlib fresh water should be given throughout the day.
- The water should be clean, free from organic matter and potable
- If bucks are fed green grass or legumes, a major part of their water requirement can be met by green fodder.
- A buck weighing 50 kg on average will consume 5-6 liters of water per day. However, the daily water intake of bucks will depend on their body weight, age, amount of dry matter intake, and weather conditions.
- A year-round supply of clean and fresh water, treated with Sodium Hypochlorite solution, common salt, and trace minerals is vital to the buck's fertility.
- All essential minerals are of prime importance to bucks for producing good quality semen with higher motility and sperm cell concentration.

3.Housing and floor

- Sheds with elevated flooring with slatted wooden batons or slatted plastic flooring are the best.
- The shed floor must be raised to 1.5 – 2 m off ground.
- Wooden slats must have a separation gap of 1.5-2 cm to allow manure to fall through the ground. Wider slats should be avoided, as they may trap bucks' legs.
- For wooden-baton flooring, the width of each plank shall vary from 7.5 to 10.0 cm and the thickness between 2.5 cm and 4.0 cm.
- A suitable ramp or stairways made of wooden planks or bricks shall be provided to enter the shed.
- Mature bucks should be housed individually in separate pens with an adequate height of the partitions between pens.

- The floor space requirement for mature bucks should be a minimum of 2.25 meters long and 2 meters wide excluding the area covered by feed and water troughs, and hay racks.
- The path in the middle of the shed should be 2 meters wide which can accommodate feed and water troughs.
- An open area attached to each buck pen outside of the shed separately should be built for the exercise of bucks measuring 6 meters long and 2 meters wide for each buck.
- An insulated ceiling and adequate ventilation must be provided in the buck shed. Ridge ventilation on the roof permits hot air/gases to drive out quickly from the shed which has a cooling effect on the shed.
- The opening should be closed during the winter season.

4.Basic Management of the animals

- Deworming is done once in 6 months or as required. Regular fecal examination will determine the frequency of deworming.
- Hooves of bucks are examined every fortnight and trimmed every three months or as needed. Foot bath is given to all the breeding bulls with 4% formalin or 4% copper sulphate solution.
- Preputial hair clipping of adult bulls should be carried out fortnightly. The length of the hair at the preputial orifice should be cut leaving at about 1 cm to prevent bacterial load in the preputial orifice.
- The body weight at the time of semen collection should have the standard body weight. All bucks should be weighed every month.
- Sick animals are segregated from the normal stock in isolation shed and separate feeding and watering should be provided.
- All newly introduced should be kept on Quarantine for 21 days, during this period collect the sample for disease screening and conduct tests against major contagious diseases.
- Buck should be culled to ensure semen quality and to reduce feed and other expenditures on the following basis
    - The bucks with poor libido
    - Poor serving ability
    - Poor semen quality (Based on Motility, Viability, Concentration etc.)
    - Poor freezability
    - Positive for Brucellosis and Tuberculosis on screening test
    - Old age over 4 years

5.Health Management of Bucks

- The vaccination should be followed to prevent various infectious diseases in goats.
- All the donor bucks and teaser goats should be tested annually for the following diseases and the result must be negative for semen collection.

    a) Brucellosis
    b) Epididymitis
    c) Tuberculosis

6.Recording

All the records regarding the selection and management of breeding Buck should be kept up to date.

III-Training of bucks for semen collection

- Training bucks to give semen in an artificial vagina needs a lot of patience and diligence.
- The training of young bucks for semen collection should be started at the age of 5 to 6 months.
- Mature bucks that have been used for natural breeding are harder to train for semen collection.
- During the initial stages of training a buck, a doe in estrus should be used as a teaser for a few collections. Later the trained buck will mount the teaser doe even if she is not in estrus.
- The quality of semen collected mainly depends on the shape of the artificial vagina (AV), the extent to which it is inflated, and the temperature of the water-filled in it.
- The temperature of the water should be 55º to 60°C (It may vary according to the thickness of the liner). The donor buck should be collected two to three times a week and in two successive collections at 30 minutes intervals.

IV-LABORATORY HYGIENE

- Floor mats (preferably washable) are installed at all laboratory entrances and entry to the laboratory is restricted.
- Windows and doors are kept closed, especially when extender preparation and semen processing procedures are in progress.
- Sink drains are decontaminated routinely with a disinfectant.
- Floors and horizontal surfaces are cleaned and mopped with a disinfectant solution.
- Unnecessary furniture, equipment, and materials are not kept in the laboratory.
- Appropriate numbers of UV lights in respect to the area of the laboratory are fixed with a common operating switch outside the laboratory. These lights are kept '**on**' at least for one hour before the commencement of work in the laboratory.
- Once UV light is fixed; the date is written on it to check the number of hours used.
- The immediate work surface, the parts of equipment, etc. supposed to be handled during the processing of semen are cleaned with 70% alcohol before commencing the work and after completion of work.
- Wearing clean laboratory foot-wears, clean aprons, and hair and mouth masks is insisted upon when entering the laboratory.
- Do not allow dirty glassware to dry.
- Immerse and soak used glassware in water immediately after use.
- Entry of visitors is not allowed during semen collection.
- The exterior surface of all equipment; and furniture is cleaned weekly and all equipment is kept covered by plastic covers when not in use.
- The semen straw filling and sealing machine are thoroughly cleaned immediately after use
- The lens of the microscope is gently cleaned daily with lint-free lens cleaning tissue paper.
- Incubators to maintain the artificial vagina are cleaned and disinfected with 70% alcohol.
- Single distilled water or pure rainwater should be used in Autoclave and thermo-controlled water bath and the water bath is cleaned and filled with fresh water regularly.
- The thermometer kept immersed in the water bath is cleaned daily to have precise temperature reading.
- Hydrogen peroxide or silver-based based fumigation: Hydrogen peroxide/silver oxide is extensively used as a biocide, particularly in applications where its decomposition into non-toxic by-products is important. It is safe to use. Use 150 to 200 ml of appropriate fogging disinfectant for 1000 cubic feet of area fogging.

V-HYGIENE OF SEMEN COLLECTOR

- Before entering the collection arena change civil dress and footwear in the anteroom.
- Wear protective semen collection clothing (pilot suit and cap) and gumboots.
- Dark Blue colored overalls are preferred.
- Before every collection, wash hands with 70% alcohol or use disposable gloves or do both.
- Use a new pair of disposable gloves for each collection. If not, wash and disinfect your hands after each semen collection.
- Use a sterilized napkin to wipe hands and change the napkin after each collection.
- Collector should not enter the AV room during collection.
- Wash the footwear and coat daily immediately after completion of semen collection work.
- The semen collector should not enter the semen laboratory

Precautions to be taken by Semen Collector

i) Hygiene and Cleaning for Collection Arena/Yard

- Collection area can be used 2 to 3 semen collection crates so that the mounting buck will get sufficient choice of stimulus animal. The arena should provide good flooring for the buck and the teaser.
- Ensure that there are no slippery surfaces in the collection area.
- All cracks and crevices in the ceiling, walls, and yard are sealed through appropriate materials to control pests and insects.
- Collection yard should be decontaminated routinely with a disinfectant before and just after semen collection
- Wearing of clean aprons, hair, and mouth masks is insisted upon when entering the semen collection yard
- Buck apron is to be tied for all bucks under semen collection and entry of visitors is not allowed during semen collection.

ii) Collect semen from only a clean and groomed buck

- Check the coat and underline of the buck to be collected for any dung or dirt
- If soiled, clean carefully with soap or mild detergent long before collection.
- Rinse then with clean water and dry with a clean towel.
- Towel used on one animal should not be reused on another.
- Ensure that the animal is dry at collection.
- The preputial hair of the buck to be collected should be short (less than 2 cm) and hooves trimmed.

iii) Provide adequate sexual preparation for the Buck

- Adequate sexual preparation eliminates the need for intensive collection.
- Select an appropriate teaser goat and present it in an area affording the buck good footing.
- Novel stimulus elicits a sexual response in buck and hence the collector should attempt novelty through the following approaches:
- Presentation of the same stimulus animal in a new location,
- Presentation of a new stimulus animal or
- Combination of animals in the original location or presentation of new stimulus animals in a new location.
- Buck should be allowed at least one false mount before taking collection.
- During the false mount the buck should be encouraged to mount directly from the rear.

- The semen collector should ensure that whenever a buck mounts every effort must be made to assure that the teaser animal does not move.
- Use of buck aprons during false mount will avoid the penis touching the hindquarters of the stimulus animal.
- Tie the buck aprons just before starting the false mount. Use separate buck aprons for each ejaculation and each buck.

iv) Personal Hygiene

- Keep fingernails trimmed and clean, wash hands, and wear a clean lab coat and caps.
- Do not eat, drink or smoke in a lab.
- Wash hands with soap and water and rinse with 70% alcohol before commencing work in the laboratory.
- Dry hands with a clean towel.
- Never touch semen bottles with wet hands
- Mobile phones should be prohibited in the laboratory

VI-CLEANING AND STERILIZATION OF EQUIPMENT

For all disinfection or sterilization methods, cleaning is critical. Cleaning can also remove a large number of organisms. Proper cleaning can be achieved by physical scrubbing. This should be done with detergent and warm water to get the best results.

1) Cleaning glassware and plastic ware:

- Washing to be done using only phosphate-free detergent. NOT any other soap.
- Wash the used glassware under running water.
- Add about 10ml of Phosphate-free detergent to 10 liters of water in a tub.
- Soak all the soiled lab wares in it for at least an hour.
- With a bottle brush wash each item of glassware.
- Transfer all these into another tub containing fresh tap water and wash thoroughly under tap water.
- Rinse all the washed items with distilled water three times.
- Drain and allow them to air dry.
- For the microscopic glass slides, they are immersed in water immediately after use and boil slides for about five minutes in hot water and detergent solution. After cooling, brush both sides of the slides many times in running water and immerse in distilled water. Wipe water with a clean cloth and immerse in a mixture of ethanol and chloroform (8:2). Wipe with a clean cloth and keep for next use.

2) Sterilization in Hot Air Oven

All the washed glassware such as beakers, flasks, measuring cylinders, glass Pasteur pipettes, slides, glass test tubes and centrifuge tubes, collection cups, tubes, filling needles, and filling nozzles (metal only) are sterilized in Hot Air Oven.

Hot Air Oven:

- Cover or wrap each washed and dried item of glassware with aluminum foil. Ensure that the openings are well covered.
- Put all the glass items in the Hot Air Oven.
- Switch on and set to 160^0C for 60 minutes. Keep for an hour and switch off the oven.
- Let the items be inside and allow cooling.
- Remove them from the oven and keep them in the storage cupboard or incubator.

3) Sterilization in Autoclave

All plastic (Poly-propylene materials) such as all bottles, membrane filter assembly, rubber caps, tips and tip boxes, the needle used in filling and sealing machine, water, glycerol, artificial vagina (only in low-pressure autoclave), and buffer are sterilized in Autoclave.

Autoclaving Procedure

- Use autoclavable plastic bags to put in items to be autoclaved.
- When water, buffer, or glycerol is to be autoclaved fill $3/4^{th}$ bottle. It is important to leave some air space. Do not tighten the screw caps completely leave one thread loose. This is done to ensure proper autoclaving and also to retain the shape of the bottle intact otherwise due to pressure it may lose shape. Keep the liquid items vertical in the plastic bags and see that remain so when put in the autoclave drum.
- With a rubber band close the mouth of each bag tightly which contains the materials.
- To any one of the bags put a small piece of sterilization (chemical) tape to ensure that the autoclaving is done properly.
- Open the lid of the autoclave and pure clean tap water up to the level mark. Then place inside the autoclave, the drum which contains all the bags of materials to be autoclaved.
- Fasten all the knobs of the autoclave. It is easier to tighten the knobs which are oppositive to each other.
- Loosen the safety valve knot (two-three threads) to remove the residual air.
- Switch on the autoclave. After about 15-20 minutes, steam will be passed continuously from the safety valve, then tighten it.
- The digital display will show the pressure and temperature. Autoclaving is done at 121^0C under 15 psi pressure for 15 minutes. But for the buffer, it should be autoclaved at 5 psi pressure for 15 minutes. The autoclave will beep and will auto stop showing the display as 'Sterilized".
- All the thermo-resistant rubber wares are sterilized by autoclaving at 5 psi pressure for 10 minutes.
- Switch off the autoclave and wait for a while to cool. Then loosen the valve to let escape the steam and then the knobs are unscrewed.
- Open the lid and remove the autoclaved materials. The indicator tape will show a STERILIZED mark.

4) Sterilization in UV Light

All the thermos-sensitive materials such as all rubber wares, semen straws, filter papers, rubber tubes, filling tubes, filling cones, coverslips, microscopic slides, etc. are sterilized under the UV chamber.

UV light sterilization

- A bunch of such filter papers is thrashed to remove dirt, if any, and sterilized under UV lamp exposure for 30 minutes.
- Semen straws/cones/tubes/micropipette tips etc. are Sterilized in UV light for 30 minutes.
- The sterilized/autoclaved rubber wares are kept in a UV chamber after drying.
- With a rubber band close the mouth of each bag tightly which contains the materials.

5) Cleaning and Sterilization of Artificial vagina (AV)

- Remove water from the used AV jacket before washing.
- Clean the AV thoroughly with a soft sponge brush under running tap water and then soak in a warm neutral cleanser for about 30 minutes.
- Then rinse in warm and clean water and finally soak in distilled water for about 20 minutes.

- For sterilization, the fully assembled AV is autoclaved at 5 psi pressure for 15 minutes in a low-pressure AV sterilizer.
- The sterilized AV is stored in the incubator at 45^0C after filling with clean water for the next day's use.

VII-SEMEN COLLECTION, PROCESSING, EVALUATION, AND FREEZING

1. Preparation of buck for semen collection

- The grooming of bucks under semen collection and wiping is done with sterilized napkins.
- Prepuce is cleaned with normal saline solution at the time of semen collection and dried with clean sterilized napkins.
- Sterilized buck aprons to be tied for all bucks under semen collection.

2. Preparations in the laboratory

- Clean slides and cover slips are placed on a stage warmer set at 37^0C before commencing the semen collection.
- Adequate amount of normal saline is filled in cuvettes for use in Photometer.
- All equipment (Microscope, Water bath, Auto dispenser, Tractability Software, and all other necessary items) should be ready ON position before collection.

3. Preparation of Artificial Vagina (AV)

- The water jacket of AV is filled up to 0.5 to 0.75 levels with 50 and 55^0C water in summer and winter respectively.
- Air is blown into the jacket through the air valve to get additional pressure.
- Temperature of the semen collection cups is maintained at around 37^0C.
- To maintain the temperature and to protect the ejaculated semen from ambient temperature, an insulation bag is attached to the collection cups after collection.

4. Buffer preparation

Is to be done in a separate media preparation room which has been sterilized regularly by formalin/commercially available disinfectant or fumigation. The buffer should be prepared by a competent person in the laboratory (lab technician/lab assistant) in the worktable/laminar airflow unit (LAFU) after being sterilized by the use of 70% alcohol or UV light.

a. Chemicals used for buffer preparation namely:
   - Tris Buffer (hydroxy methyl amino methane)
   - Citric acid (Citric acid monohydrate)
   - D-Fructose or D-Glucose

Should be either of Analytical Grade (AR) or Graded Reagent (GR). Laboratory Grade (LR) of chemicals should not be used due to low purity. Chemicals should be weighed by an analytical electronic balance for high accuracy to arrive at the desired pH and osmolarity.

b. Preferably ultra-pure water (of 16-18 mega ohms) should be used for the preparation of the buffer. Alternatively, freshly prepared distilled and autoclaved water shall be used.
c. The buffer should be prepared by a competent person in the laboratory (lab technician/lab assistant) and all receptacles used should have been sterilized. The lab technician should have a change of clothing (apron, cap, and mask) and wear laboratory footwear before entering the diluter preparation room.
d. The hands of the technician and the worktable / laminar airflow unit (LAFU) should be sterilized by using 70% alcohol.

e. Preferably the technician may wear surgical disposable gloves. The buffer preparation should be done under a LAFU.

f. The composition of the Tris-buffer (for 1000ml) is as follows

- Tris - 32.2 grams
- Citric acid - 18.2 grams
- Fructose/Glucose- 10.0 grams

g. Ultra-pure water / autoclaved distilled water is added to make 1000 ml and the same is mixed well.

h. The buffer is then autoclaved at 5 psi for 15 minutes. If an antibiotic is used for Tris-buffer, a 0.22-micron membrane filter should be used instead of autoclaving.

i. The pH of the buffer is an essential factor for the survival of sperm cells and the quality of semen, after cooling the pH of the buffer is checked using a pH meter which has been standardized and has been put in at least 20 minutes before the estimation of the pH. The pH of the buffer should be between 6.7 and 6.9.

j. Whenever chemicals of a new batch/company are introduced, care should be taken to test its quality before use. (By splitting the sample-check the freezability)

5. Diluter preparation

In deep freezing buck semen, freezing media contains a buffer to adjust pH, egg yolk as cell membrane protectant, D-Glucose and citric acid as nutrients, and Glycerol as a cryo-protectant. And Benzylpenicillin and streptomycin sulfate are used as antibiotics

- The autoclaved buffer is cooled. If not used immediately it should be stored in a refrigerator at 4-8^{0}C Celsius.
- The diluter can be prepared fresh on the day of collection early in the morning or on the previous day in the evening. If prepared on the previous day it may be noted that antibiotics should be added only in the morning before use.
- All hygienic measures as mentioned in the buffer preparation should also be employed during diluter preparation by the lab technician and should be carried out under a LAFU.
- Egg yolk is separated in the sterile zone, under euro air, or on a laminar airflow table and 20% of egg yolk is taken in a one-liter graduated measuring cylinder
- The buffer prepared the previous day is added, and to this 8% of Glycerol (AR grade from a reputed company) is added.
- Glycerol can be warmed at 60°C temperature after measuring to increase its miscibility or added to the buffer before sterilization/autoclaving the previous day.
- Eggs used for the preparation of diluter should be fresh and purchased from a known source to avoid Mycoplasma / Salmonella infections.
- The eggs should be stored in a refrigerator after wiping with dry cotton for not more than 2-3 days.
- Just before diluter preparation eggs shall be wiped with 70% alcohol.
- Egg yolk shall be separated from albumin using an egg separator onto autoclaved filter paper (What man filter paper. No. 1 or filter paper of equivalent quality) and rolled over to remove the entire albumin before adding to the buffer.
- A mixture of antibiotics is added with a bactericidal activity to the diluter: Benzylpenicillin @ 1000 IU/ml and Streptomycin sulfate @ 1mg/ml or GLTS combination.
- Animal origin free semen extender (Soya Based with phospholipid) having GLTS antibiotics combination in active units per 100 ml of extender (Gentamicin 25mg/100 ml

of extender, Lincomycin 50 mg/100 ml of extender, Tylosin 5mg/100 ml of extender and spectinomycin 30mg/100 ml of extender) can be used instead of egg yolk-based extender.
- The pH of the final diluent should be tested daily and recorded
- The glass stopper is put on the measuring cylinder and diluents are mixed gently and transferred to a conical flask.
- The work area should again be sterilized by the use of 70% alcohol.

Composition of Diluent A and B (Diluent "A" contains Tris and egg yolk and Diluent "B" contains Tris, egg yolk, and glycerol.

Components	**Diluent A**	**Diluent B**
Tris Buffer (Autoclaved)	80%	72%
Egg Yolk (Fresh)	20%	20%
Glycerol (Autoclaved)	-	8%
Benzyl penicillin	@ 1000 IU/ml	@ 1000 IU/ml
Streptomycin sulphate	@ 1mg/ml	@ 1mg/ml

6. Semen Collection

- The artificial vagina (AV) method of semen collection is painless, quicker, and does not stress the buck.
- The AV has an inner rubber liner (containing water at a temperature of 55-60^{0}C) placed between the liner and the outer wall.
- Check the temperature of the artificial vagina before collection.
- The Ideal temperature of AV is 42ºC for an adult buck and between 40-42ºC for young bucks. (Pressure- 45 to 55 mm of Hg. Younger bucks require higher pressure.)
- A graduated semen collection cup should be connected with AV and should be held tightly by one hand.
- Semen is collected in AV while the buck mounts a doe in heat or a teaser doe. This can be a natural heat or induced by prostaglandin or estrogen.
- A doe in heat usually stands better for a buck than weather or another buck. The doe in heat emits a smell that causes the buck to give a better ejaculate.
- The doe is usually tied or held for a buck. After a false mount, the AV is placed over the penis as it is extended while mounting.
- The stimulation provided by the AV's warm water liner, lubrication, pressure, and a thrusting movement of the buck produce an ejaculation.
- The artificial vagina should not be shaken after ejaculation to avoid lubricant and debris passing down to the collection cup. Never use chipped or defective collection tubes.
- The AV should be changed when the buck has failed to ejaculate during the first mount with insertion of the penis.
- The collecting cups should be sterilized by autoclaving or heating in an oven at 160°C for at least 30 minutes. Sterilized cups should be sealed and kept in incubators until the next use.
- After semen is collected, the collection cup should be left within its jacket or kept in a container having warm water at 37^{0}C while transported to the laboratory window. The insulated jacket protects sperm cells from direct sunlight and cold temperatures.
- Avoid contamination of the semen by water, lubricating jelly, or other harmful substances.
- Be certain to identify the semen collection tube with the correct buck number.

- Report any abnormal events to laboratory personnel (for example bloody semen, leak in inner liner, loss of ejaculate). Immediately after collection dip the AVs in a container with a neutral detergent solution for cleaning.
- Teamwork between buck handler and semen collector is essential to assure correct buck identification, use of correct sexual preparation procedure, and safety of employees. At the time of collection, both the collector and herdsman should avoid sudden moves. Avoid distractions during semen collection. The entry of visitors shall be prohibited.
- The semen collector should understand the individual buck's sexual behavior and physiology. The semen collector and buck attendants should not be changed frequently. Never mistreat or abuse a buck at the time of semen collection. The too high temperature of AV, forcing of the AV on the penis, and grasping of the penis instead of the sheath are painful conditions leading to sexual suppression.

7. Extension and Evaluation of semen

Macroscopic examination: Immediately after semen collection, it can be evaluated grossly for volume, color, and contamination. Normal semen has a uniform opaque appearance free from hair, dirt, pus, urine, and other contaminants.

Color: Freshly collected buck semen is white to yellow. Milky white, Creamy white, and slightly yellow color are considered normal for further processing. The Pink color of semen indicates the presence of blood and the grey and brown color indicates semen contamination or some kind of infection. The freshly collected semen should be milky in color and should be free of contaminants, such as urine, blood, pus, and dirt.

Volume: Generally, the volume of ejaculate ranges between 0.5 to 2.5 ml. Semen volume can be assessed either using a calibrated collection cup or in a pasture pipette attached to a syringe. The skill of the semen collector is also important in getting good ejaculate volumes.

Presence of foreign matter: Foreign materials, if any, the semen is discarded.

Consistency: The consistency of semen is a direct measure of sperm concentration. Consistency is graded as a thick, medium, thin and watery. Semen samples with thick and medium consistency have higher sperm concentration and thin and watery consistency have low sperm concentration.

Score	Consistency	No. of spermatozoa (Thousand million)
5	Thick creamy	4.0 – 5.0
4	Creamy	3.0 – 4.0
3	Thin creamy	2.0 – 3.0
2	Milky	1.0 – 2.0
1	Cloudy	0.3 – 1.0
0	Watery	Insufficient

Gross motility: This shall be seen in freshly collected semen samples when held against diffused light and closely observed. Wave-like movements can be seen with naked eyes.

Microscopic Evaluation of semen: A sample of semen should be examined under the microscope to determine sperm motility and morphology, and the presence of WBC. The presence of WBC indicates an infection, often by *Brucella ovis*, which causes epididymitis. Good quality semen is the keystone of successful A.I. To determine the quality of semen, it is necessary to examine it under a microscope

Mass activity/wave motion: Mass activity is observed by placing a 5 mm diameter drop of neat semen on a warm slide without a cover slip and observed for the swirls/waves. Factors that affect the mass movement of the spermatozoa include concentration, percentage of progressively motile cells, and the speed/vigor of sperm motion. The higher and more concentrated the wave movements, the better the quality of the semen. This is assessed with motility scores ranging from 0 to 4. Semen samples with the motility scores 3 and 4 should only be used for freezing. Semen samples having 0 to 2 motility scores should be discarded.

Assessment of Motility of spermatozoa

Score	Class	Aspects of wave motion
0	Dead	Total immobility
1	Poor	little or no individual cell motion
2	Fair	No swirls, but prominent individual cell motion
3	Good	Slower swirls and eddies
4	Very good	Rapid swirls motion and eddies

Initial motility: The initial motility of the semen sample is done by taking a 3-4mm diameter drop of diluted semen (A drop of semen is diluted in 2 ml of the extender by use of a micropipette tip or sterilized semen straw) onto a warm slide (maintained on a slide warmer at 37^0C) and a cover slip (18 mm square or round) is put on the drop. On a phase-contrast microscope at a magnification of 100/200 times (10X eyepiece and 10/20X objective). Descriptive assessment of individual motility

- Very good 80-100% motile
- Good 70-80% motile
- Fair 50-70% motile
- Poor 50% motile

Visual microscopic analysis of individual progressive motility is somewhat subjective even when performed by very skilled people and becomes tedious when large numbers of the sample must be analyzed. Computer Assisted Semen Analysis (CASA) systems have the potential to increase the objectivity of analysis and reduce worker fatigue.

- Semen samples selected for freezing should have a minimum of 70% progressive initial motility.
- Percentage of live and dead sperm: The percentage of dead sperm is estimated during motility evaluation and semen with less than 80% live sperm is discarded.
- Percentage of abnormal sperms: Similarly, the percentage of abnormal sperms also is estimated and semen with more than 20% abnormal sperms also is discarded.
- After macroscopic and microscopic evaluation, the semen, if found good for further processing is extended with a calculated quantity of extender (the quantity calculated by Photometer based on inputs we supplied) in a sterilized conical flask so as to pack 100 million sperms/Straw.

8. The Sperm concentration Measurement

- The sperm concentration is assessed using a spectrophotometer which has been standardized for the buck and validated regularly at least once in six months period.
- A fresh cuvette is used for each sample/ejaculate.
- Sampling tubes should also be changed in the diluter unit for each ejaculate and never reused.
- Initial dilution of semen with diluent's maintained at 37^0C in a thermo-controlled water bath. The good quality buck semen contains or should contain a minimum of 1500

million spermatozoa/ml of semen. For a minimum concentration of **100 million per dose** should be allocated.

9. Removing the enzyme phospholipase A:

For removing the enzyme;

- Add tris buffer twice the semen volume.
- Place semen sample in 15 ml centrifuge tube and centrifuge for 7 minutes at the speed of 3000 rpm.
- Decant supernatant and discard.

Extension of buck semen

Two-step dilution should be followed for buck semen and the extended semen is immediately transferred to the filling, sealing, and printing machine for further processing of semen at room temperature. For extension;

- Add diluent A which is half the volume of the total calculated diluent.
- Once temperature reaches 20^0C add a half volume of diluent B.
- Wait for 30 minutes and add the remaining half the volume of diluent B after 30 minutes.

Pre-filling motility test: Pre-filing motility of the extended semen should be examined and semen with less than **60%** motility should be discarded and no further processing is done.

10. Printing, filling, and sealing of straws

- Sterilized mini straws (0.25 cc) of reputed companies should be used.
- Printing of straws is done either before or after filling by the use of an automatic straw printing machine/Jet printer and the straws are imprinted with the details of buck number /name /breed /name of the organization /batch number/year etc.
- All semen stations shall follow the color codes assigned by the Government for filling of semen in straws:
- The clarity of printing on all straws should be checked.
- When the straw filling is done at 4°C, a spacious cold handling cabinet is used to maintain semen equilibration temperature while filling; and sealing the straws
- Air space of 1 cm and the quality of sealing of straws at laboratory end should be checked at random
- After filling and sealing of straws the unit is cleaned with 70% alcohol
- All glass wares are immersed in neutral detergent in a basin for washing and sterilization.

11. Semen racking, equilibration, and freezing

- Racking: Once filling and sealing of straws is done, sealed semen straws are immediately counted in the counting chamber and racked on racks by use of the ramp and racks.
- Equilibration: Equilibrate at 4^0C in a cold handling cabinet or refrigerator for **2 hours.** This pre-freeze storage period is termed as equilibration period. During this period the sperm cells become permeated with glycerol and an ionic and osmotic equilibrium is established with the media. This period also helps the spermatozoa to gain resistance prior to freezing stress.
- Freezing: After equilibration, straws can be frozen in a biological freezer (Forced Vapor Freezing Method-Annex). Alternatively, it can be done custom frozen 4 or 10 cm above liquid nitrogen vapor for 10 minutes and then immersed in liquid nitrogen.
- Post Thaw evaluation: Evaluate post-thaw motility after 24 hours and store straws.

VIII. QUALITY CONTROL OF FROZEN SEMEN

- Only samples that show a Post Thaw Evaluation (PTM) of 45% and above should be preserved, and those below 45% PTM should be discarded. (Annex 3)
- Bulls having more than Acrosome Integrity (15% disintegrity) are re-examined for three consecutive tests and are suggested for culling. Frozen semen samples should contain at least 70% sperm with intact acrosome. (Annex 4)
- Frozen semen samples should contain at least 40% hypo-osmotic reacted spermatozoa. Good semen sample may contain 40% to 50% of spermatozoa with Hypo-Osmotic Swelling (Tail curled). Bucks having less than 40% Hypo-Osmotic Swelling sperms are advised for sexual rest and correcting the causative factors. (Annex 5)
- Semen having higher percentage of motile spermatozoa for longer periods of incubation at 37^0C are considered as better samples in terms of viability of spermatozoa. Bulls having low percentage of viability are advised for sexual rest and correcting the causative factors. *(Annex 6)*
- It is recommended that the potential sire may contain 20-30% dead spermatozoa and about 15-20% abnormal spermatozoa in the first ejaculate. The numbers decrease with the increasing number of collections. Bulls having more than 20% dead sperms in *Live and Dead Sperm Count Test* in neat semen are advised for sexual rest and correction causative factors. *(Annex 7)*

IX. DISTRIBUTION OF FROZEN SEMEN STRAW

- The distribution of frozen semen straws to the field has to be done after completion of minimum 28 days on demand.

ANNEXES

Annex 1

. The scrotal circumference of a yearling buck should at least be 25 cm. and this increases with the advancement of age and the onset of the breeding season. Bucks can be classified as excellent, satisfactory, or questionable based on the results of their breeding soundness examination (table1).

Reference Chart for Bucks

Class	Scrotal Circumference (<14 months)	Scrotal Circumference (>14 months)	Motility	Morphology	Debris
Excellent		> 25 cm	> 50 %	> 90%	no white blood cells
Satisfactory	Information not available		> 30 %	> 70 %	no white blood cells
Questionable			< 30 %	< 70 %	may have white blood cells

Annex 2

List of chemicals and stains required

For Hypo-Osmotic Swelling Test (HOST) 1) Tri-Sodium citrate 2) D fructose 3) Double distilled water 4) 3% Rose Bengal stain	**For Assessment of Acrosome Integrity** 1) 1% formaldehyde **2)** Giemsa stain
For live dead count 1) Eosin (water soluble) **2)** Nigrosin (water soluble) **Sperm concentration-Photometer** **1)** Normal Saline (0.9%)	**Media preparation** 1) Tris (Hydroxymethyl methylamine) 2) D glucose (dextrose)/D Fructose 3) Citric acid 4) Egg yolk 5) Glycerol 6) Benzyl penicillin/streptomycin **7)** Autoclaved distilled water

Annex 3

Forced Vapor Freezing Method

This is carried out using a programmable bio-freezer. The rate of freezing is highly precise and the program is fed into the computerized freezer. The rate of fall in temperature is as follows:

- The machine is put on at 20^0C (room temperature) and brought down at 10^0C per minute till 4^0C.
- Here it is paused automatically for the transfer of straws from the Cold handling unit.
- Each batch can freeze 42 racks of 175 straws or 7350 straws. (Digit cool model of IMV)
- A straw is cut and placed on the thermocouple to record graphically the fall of temperature within the straw. The machine is then closed, and once the temperature is stabilized at 4^0C it is started again.
- The rate of fall of temperature is @ 4^0C from 4^0C to minus 5^0C.
- From minus 5^0C the rate of fall has been programmed at @ 25^0C per minute up to minus 110^0C.
- Finally, from minus 110^0C to minus 140^0C at @ 35^0C per minute.
- After minus 140^0C the freezer is opened and the straws are removed from the racks and placed into pre-cooled goblets and then plunged into LN at minus 196^0C.

Samples as per requirement are drawn after freezing to assess post-thaw motility evaluation.

Annex 4
Post thaw evaluation

- Post-thaw motility (PTM) of semen should be checked immediately after freezing (0 hour) by thawing 2 to 3 random sample of frozen semen straws per bulls / batch at 37^0C for 30 seconds in water bath. After 48 hours period in LN_2, the frozen semen straws are re-evaluated.
- The frozen semen straws are transferred to semen store for storage and distribution after re-evaluation in 48 hours after freezing.
- For thawing, each straw should be removed from the container by using a forceps and should jerk the straw once to remove any LN2 in the factory seal end.
- Forward motility under the phase contrast microscope (200X) with a warm stage / biotherm is observed
- Only samples that show a PTM of 45% and above should be preserved, and those below 45% PTM should be discarded.
- Spermatozoa should show progressive motility meaning headfirst straightforward travelling a distance. Samples showing higher percentage of progressively motile spermatozoa for longer duration of incubation at 37^0 C are considered better samples in terms of viability.

Annex 4
Acrosome Integrity

Acrosome, a cap like structure on the head of the spermatozoa covers 60% of the anterior portion of the nucleus. The morphology of the acrosome should be maintained for the sperm to undergo capacitation and acrosome reaction in the female reproductive tract for attaining the fertilizing ability.

- A thin smear is made on a clean grease-free pre-warmed slide.
- The slide is air dried
- Immersed in 5% formaldehyde for 30 min. at 37^0C for fixing.
- Wash the slide in running water and air dry
- Prepare Giemsa stain working solution and pour on slide for 3 hours at 37^0C. After staining, the slides are washed in water and dried.
- Observe the morphology.
- A total of 100 sperms are counted and the acrosome integrity is classified as intact, altered and completely lost.
- Calculate the percentage of acrosome alterations.
- The maximum permissible level of acrosome disintegrity is 15%
- Bulls having more than 15% disintegrity are re-examined for three consecutive tests and are suggested for culling
- Frozen semen samples should contain at least 70% sperm with intact acrosome.

Annex 5
Hypo-Osmotic Swelling Test (HOST)

Fluid transport occurs in an intact sperm cell membrane under hypo-osmotic conditions until equilibrium is reached. Due to influx of fluid there will be bulging of plasma membrane resulting in ballooning. The tail fibre curls or bends when plasma membrane "balloons". This phenomenon is known as "tail curling" or swollen sperm. Spermatozoa with chemically and physically intact membrane will show tail curling under hypo-osmotic conditions whereas spermatozoa with an inactive membrane will not. During cryopreservation, spermatozoa are subjected to stress that can alter the membrane integrity so HOST is found useful.

- Measure 0.367 g of sodium citrate and dissolve in 50 ml double distilled water (DDW). Weigh 0.675 g of D-fructose and dissolve in 50 ml DDW. Mix equal volume of these solutions. This is known as HOS medium.
- Take 0.1ml frozen thawed semen. Mix it with 1ml HOS medium
- Incubate the mixture at 37^0C for 30 minutes.
- Place a drop and examine under the microscope for tail curling

- Make a smear out of another drop.
- Alternatively, can make smear stain with 3% Rose Bengal stain for 10 minutes.
- Wash the excess stain.
- Calculate the percentage of spermatozoa showing tail curling.
- Samples showing higher percentage of spermatozoa with tail curling indicate good samples.
- Frozen semen samples should contain at least 40% hypo-osmotic reacted spermatozoa. Good semen sample may contain 40% to 50% of spermatozoa with Hypo-Osmotic Swelling (Tail curled). Bucks having less than 40% Hypo-Osmotic Swelling sperms are advised for sexual rest and correcting the causative factors.

Annex 6
Post-Thaw Viability Test

Viability of Spermatozoa in female reproductive tract before it meets an ovum is a pre-requisite quality for successful fertilization. Post thaw incubation of frozen semen at 37^0C is a good indicator of in-vitro viability of spermatozoa.

- Take frozen semen straw and thaw the sample, adopting the standard procedures.
- Estimate the percentage of progressively motile spermatozoa immediately after thawing ('0' hours).
- Incubate the thawed sample (collected in a small test tube) in water bath at 37^0C.
- Evaluate the motility of the sample at 1, 2 and 3 hours of incubation.
- Samples showing higher percentage of motile spermatozoa for longer periods of incubation at 37^0 C are considered as better samples in terms of viability of spermatozoa.

0 hour motility -	40-50%
1st hour motility-	35%
2nd hour motility –	20%
3rd hour motility-	10%

- Samples not meeting the above specifications should be discarded.
- Bullss having low percentage of viability are advised for sexual rest and correcting the causative factors.

Annex 7
Assessing Live and Dead Spermatozoa

Assessment of percentage of dead spermatozoa and abnormalities in sperms. This test, utilizing eosin-nigrosin stain, is efficient in determining the exact percentage of dead and abnormal spermatozoa.

Preparation of the stain:

- Eosin (water soluble) - 1.67 g
- Nigrosin - 10 g
- Sodium citrate - 2.9 g
- Distilled water - 100 ml

Weigh the components and dissolve completely in a suitable flask at 50 C, by o stirring. Cool and Filter. Store at 4^0C.

- Use clean slide. Keep on warm stage
- On one side keep 2-3 drops of prepared stain
- Add one drop neat semen. Mix for 10 seconds
- Prepare a thin smear in a forward movement with the help of a spreader (another new slide) and allowed to dry in the air.
- Observe under high power
- Examine 100 spermatozoa from different fields on the slide.
- Dead spermatozoa will stain pink. Alive will remain colorless or slightly pinkish. Nigrosin forms a background which makes easy identification.
- It is recommended that the potential sire may contain 20-30% dead spermatozoa and about 15-20% abnormal spermatozoa in the first ejaculate. The numbers decrease with the increasing number of collections. Bulls having more than 20% dead sperms in neat semen are advised for sexual rest and correction causative factors.

Annex 8
Minimum Quality Parameters of Frozen Semen

The following chemicals with lab grade only shall be used for preparing buffer. **Tris**, **Citric Acid** Monohydrate GR, **D-Fructose** /D-Glucose GR, **Glycerol**. Whenever a new brand of chemical is to be introduced in the routine process, examine for post thaw revival rates after conducting few split ejaculate trials (maintaining a control) with the new chemical.

- Whenever a new brand of chemical is to be introduced in the routine process, examine for post thaw revival rates after conducting few split ejaculate trials (maintaining a control) with the new chemical.
- Concentration of sperms in frozen semen doses, calculated by photometer should be checked periodically using Hemocytometer.
- Bulls having more than 20% dead sperms in neat semen are advised for sexual rest and correction causative factors.
- Bulls having more than 15% disintegrity are re-examined for three consecutive tests and are suggested for culling
- Samples showing higher percentage of motile spermatozoa for longer periods of incubation at 37^0 C are considered as better samples in terms of viability of spermatozoa.
- Samples not meeting the prescribed national specifications should be discarded.
- Bulls having less than 40% Hypo-Osmotic Swelling sperms are advised for sexual rest and correcting the causative factors.
- Microbial examination should be conducted frequently. Aerial count test, Rinse Test, Working Solution Test and Batch Test are generally carried out.

A summary of quality tests to be conducted for frozen semen and their cut-off values

S.N.	QC Parameters	Minimum Standard
1	Sperm concentration	20 million spermatozoa per dose (0.25 ml mini straw) for bulls
2	Post-thaw motility (0 hour)	≥ 45%
3	Fresh semen motility	≥ 70%
4	Dead spermatozoa	≤ 20%
5	Bacterial load	5000 CFUs /ml
6	Hypo osmotic swelling test (HOST)	≥40%
7	Acrosome integrity (Fresh Semen)	≥ 70%