STANDARD OPERATING PROCEDURE FOR BOVINE FROZEN SEMEN PRODUCTION

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Revision history

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

Revision history

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STANDARD OPERATING PROCEDURE FOR BOVINE FROZEN SEMEN PRODUCTION

Background

The semen by many is no longer perceived as a dose to impregnate their animals, but perceived as a source of genetics to produce better quality animals. It is realized that while one thinks about expanding the semen production facility in terms of increasing bull housing and semen processing facilities, one has also to think about putting in place quality control and bio-security systems at frozen semen stations (FSS). The minimum standards required for production of quality frozen semen are covered in this SOP. Failure to observe these guidelines could lead to production of poor-quality frozen semen. The Standard Operating Procedures (SOP) provides general guidelines for all the activities that are taking place in a Frozen Semen Production Laboratories which is necessary for operating or implementing a particular system. This guiding document covers the general management of breeding bulls for semen collection as well as standard operating procedure for production of frozen semen in laboratories.

Objectives

To produce quality semen for Artificial Insemination

Requirements

- Breeding Bulls for Semen Collection
- Equipment
  - Dummy bulls
  - Floor mats
  - 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. GENERAL RULES FOR HYGIENE AND SANITATION

A. PREMISES

- Floor mats (preferably washable) are installed at all laboratory entrances and entry to the laboratory 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 area of laboratory are fixed with a common operating switch outside the laboratory. These lights are kept 'on' at least for one hour prior to 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 processing of semen are cleaned with 70% alcohol before commencing the work and after completion of work.
- Wearing of clean laboratory foot-wears, clean aprons, 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 visitor is not allowed during semen collection.

B. 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 0.1% savlon or use disposable gloves or do both.
- Use a new pair of disposable gloves for each collection. If not, wash and disinfect hands after each semen collection.
- Use a sterilized napkin to wipe hands and change 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

C. EQUIPMENT

- The exterior surface of all equipment, 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 microscope is gently cleaned daily with lint-free lens cleaning tissue paper.
- Incubators to maintain artificial vagina are cleaned and disinfected with 70% alcohol.
- Single distilled water or pure rain water is used in Autoclave and thermo-controlled water bath.
- The water bath is cleaned and filled with fresh water on a regular basis.
- The thermometer, kept immersed in water bath is cleaned dally to have precise temperature reading.

D. PERSONNEL

- Keep finger nails trimmed and clean, wash hands, and wear clean lab coat and caps.
- Do not eat, drink or smoke in lab.
- Wash hands with soap and water and rinse with 70% alcohol before commencing work in the laboratory.
- Dry hands with clean towel.
- Never touch semen bottles with wet hands.
- The workers in the FSS should be screened for TB and brucellosis every year.
- Personals should ware appropriate color coated apron - Lab Personal: White, Collector: Light Blue, Handler: Dark Blue
- Technical exposure/training of semen collection should be conducted every year.

E. BULLS

- Trim hairs around the prepuce.
- Wash bulls with brush every day.
- Prepuce is cleaned with 0.9% Normal saline solution fortnightly and dried with clean sterilized napkins.

II. PREPARATION

Preparation of Bulls and collection yard

(A) Preparation of bulls and collection yard for semen collection

1. Grooming at shed for the bulls under semen collection and wiping is done with sterilized napkins.
2. One day before the semen collection the bulls are exercised for at least 30 minutes.
3. Sterilized bull aprons to be tied for all bulls under semen collection.
4. At least two false mounts should be conducted prior to semen collection.
5. Wash the collection yard one day before prior to semen collection.
6. The area of collection yard is disinfected regularly with disinfectant (Laboline).
7. Clean the non-live fixed dummy with 70% ethanol.

(B) Collect semen from only a clean and groomed bull

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

(C) Provide adequate sexual preparation for the bull

a. Adequate sexual preparation eliminates the need for intensive collection.
b. Select appropriately sized stimulus animal and present it in an area affording the bull good footing.
c. The bull's nose is tender. Therefore, the lead rope should not be jerked violently.
d. Novel stimulus elicits sexual response in bulls 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.

e. Bull should be allowed at least two false mounts with two minutes restraint before taking collection.

- During the false mount the bull should be encouraged to mount directly from the rear.
- The semen collector should ensure that whenever a bull mounts every effort must be made to assure that the mount animal does not move.

f. Use of bull aprons during false mount will avoid the penis touching hindquarter of the stimulus animal.
g. Tie the bull aprons just before starting the false mount. Use separate bull aprons for each ejaculation and each bull.

(D) Management of Bulls during collection

a. The semen collector should understand the individual bull's sexual behavior and physiology.
b. The semen collector and bull attendants should not be changed frequently.
c. Never mistreat or abuse a bull at the time of semen collection.
d. Too high temperature of AV, forcing of the AV on penis, grasping of the penis instead of sheath are painful conditions leading to sexual suppression.

Preparation equipment and other accessories

(A) Preparations of equipment

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

(B) Preparation of artificial vagina

1. The water jacket of AV is filled up to 0.5 to 0.75 levels with 50^0C and 55^0C water in summer and winter respectively.
2. Air is blown into the jacket through the air valve in order to get additional pressure.
3. Temperature of the AV cones and semen collection tubes is maintained around 34-37^0C
4. To maintain the temperature and to protect the ejaculated semen from ambient temperature, an insulation bag is attached to the AV hose.

(C) Preparation of buffer

Is to be done in a separate buffer / dilutor preparation room which has been sterilized regularly by hydrogen peroxide-based fumigation day before collection. The buffer should be prepared by a competent person of the laboratory (lab technician / lab assistant) in worktable / laminar airflow unit (LAFU) after sterilized by use of 70% alcohol or UV light.

a) Take 500 ml. of sterilized double distilled water or Ultra-pure water in the sterilized volumetric flask.

b) To this, add following (Weighed by an analytical electronic balance for high accuracy in order to arrive at the desired pH and osmolarity)

24.220 grams of Tris (hydroxy methyl amino methane)
13.600 grams of Citric acid (Citric acid monohydrate)
10 grams of D-Fructose or D-Glucose
64-70 ml. of Glycerol

c) Allow to dissolve by shaking.

d) Make up the total volume to 800 ml. by adding double distilled water.

e) Autoclave this solution at 5 psi pressure for 20 minutes.

f) The autoclaved buffer is cooled. If not used immediately it should be stored in a refrigerator at 4 to 8°C.

(E) Preparation of semen extender:

a. The eggs should be stored in a refrigerator after wiping with dry cotton not for 1 days and just prior to dilutor preparation eggs shall be wiped with 70% alcohol.

b. Egg yolk shall be separated from albumin using an egg yolk separator onto autoclaved filter paper (Whatman filter paper No. 1) and rolled over to remove the entire albumin before adding to the buffer.

c. 200 ml of egg yolk is taken in a 500ml graduated measuring cylinder and added to Buffer present in volumetric flask (prepared the previous day) to make the final volume 1000 ml.

d. The final semen extender is prepared by adding egg yolk and antibiotics to the prepared buffer.

e. The extender can be prepared fresh on the day of collection early in the morning or on the previous day in the evening.

f. If prepared on the previous day, egg yolk should be added only after cooling buffer at 20^0C and antibiotics should be added in the morning (on collection day) after warming prepared extender at 34°C, prior to use.

g. A combination of antibiotics can be added with a bactericidal activity to the dilutor: Benzyl penicillin 1000 IU/ml and Streptomycin Sulphate 1000 micro gram/ml or a combination of Gentamicin 250 micro gram/ml, Tylosin 50 micro gram/ml and Lincomycin spectinomycin

150/300 micro gram/ml.

h. Add a sterilized magnet (surgical sprit can be used to sterilize magnet) to extender containing volumetric flask and placed on magnetic stirrer for 30 min for homogenization.

i. PH of final extender should be between 6.7 and 6.9.

j. This extender is maintained at 37^0C in water bath before commencing of semen processing.

III. SEMEN COLLECTION

(A) Collection of semen using artificial vagina (AV)

a) Donor bull with normal libido is brought in collection yard near the suitable dummy.

b) Bull is stimulated by giving two false mounts. Collection should be attempted only if the bull is properly sexually prepared.

c) Check temperature of the artificial vagina before collection. Ideal temperature is 45ºC for adult bulls and between 42-45ºC for young bulls. (Pressure- 45 to 55 mm of Hg. Younger bulls require higher pressure.) Normally, 2/3-part warm water and 1/3-part air should be kept inside artificial vagina.

e) The collector should not touch the penis of the bull, the touching of the penis by the collector causes shying of the bull and the bull will not readily mount. The glans penis is guided into firmly held sterilized and well-prepared AV.

f) The AV shall not be shaken after ejaculation to avoid the lubricant and debris may mix with the ejaculated sample. Protect the semen collection tube from sunlight. Never use chipped or defective collection tube.

g) Separate AV should be used for each bull and for each ejaculate and even if the bull inserts the penis in the AV without ejaculation the AV should be changed. To reduce possible loss of sperm do not remove the artificial vagina from the penis too quickly after the bull has ejaculated. (Especially in buffalo)

h) The collected semen tube with ejaculate details and identification read by sensing gun via microchip is delivered to the laboratory through Pas Box as soon as possible, for further processing.

i) For every ejaculation thrust, the AV should be changed. Immediately after collection dip the AV's in a container having neutral detergent solution for cleaning.

j) Use 12" or 14" AV (For Bovine) according to the breed and size of the penis. Generally, for Buffalo bulls 12", for Jersey and its crosses bulls 14" and for Holstein bulls 16" AV are preferred.

k) The second ejaculate is collected about minimum of 30-40 minutes interval.

l) Teamwork among bull handler, semen collector and other staffs is essential to assure correct bull 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.

m) Avoid distractions during semen collection. The entry of visitors shall be prohibited.

IV. EVALUATION AND EXTENSION OF NEAT SEMEN

- Labelling: Collection tubes containing semen should be labeled with Barcode using traceability software for collection detailed (bull numbers, collection no, collection time for identification)
- Storing raw Semen: As soon as the raw semen is received, it shall be kept under thermo controlled water bath at the temperature of 34-37^0C under Laminar Flow unit.
- Macroscopic Evaluation
  - ✓ Color: Color of the semen is noted. Milky white, Creamy white and slightly yellow color is considered as normal for further processing.
  - ✓ Volume: Volume of the neat semen is recorded precisely. For Bovine species less than 0.5 ml and above 15 ml is discarded.
  - ✓ Presence of foreign matter: Foreign materials, if any, the semen is discarded.

- ✓ Density: Visual density of the semen is recorded as 1, 2 or 3 (1 for thin and 3 for thick by normal visual detection).
- ✓ Sperm concentration with photometer: Samples having sperm concentration below 500 million per ml are discarded. For a minimum concentration of 20 million per dose should be allocated.

- **Microscopic evaluation**
  - *Mass activity***:** A drop of raw semen is put on the warm slide without applying cover-slip and mass activity is observed under low power microscope (10X). Good semen samples show a series of rapidly changing swirls waves like clouds and current of sperm motion which is graded on a scale of + to + + + + depending on the vigor in wave (+ for weak vigor and ++++ for strong vigor).
  - *Initial Motility***:** A drop of semen is diluted in 2 ml of extender maintained at 37^0C, One small drop of this diluted semen is put on a warm slide placed on the slide warmer of microscope maintained at 37^0C and covered with a cover slip.
  - Approximate percentage of progressively motile spermatozoa is observed in different fields of slide at 10 & 20X under a phase contrast microscope.
  - Any sample showing below 70% progressive initial motility is discarded from further processing.
  - *Percentage of live and dead sperm***:** Percentage of dead sperms is estimated during motility evaluation and semen with less than 80% live sperm is discarded.
  - *Percentage of abnormal sperms***:** Similarly, 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 on the basis of inputs we supplied) in sterilized conical flask so as to pack 20 million sperms/Straw.
- Single-step extension should be followed and the extended semen is immediately transferred to filling, sealing and printing machine for further processing of semen at room temperature.
- Pre-filling motility test**:** Pre-filling motility of the extended semen should be examined and semen with less than 60% motility should be discarded from further processing

V. FILLING, SEALING & PRINTING OF STRAWS

- **Filling and sealing of straws:** Using automatic filling, sealing and printing machine, the extended semen (having concentration of 20 million per dose) is filled in straws using sterilized filling nozzles and sealed by using heat and pressure and then printed straw are automatically collected in collection bucket (Annex).

- **Straw Color Code:** All semen stations shall follow the specific color codes for filling of semen in straws: Holstein- Transparent Pink/Red, Jersey- Transparent light Green, Murrah- Transparent/clear white, Hariyana- Transparent Yellow.

- **Racking of filled straws and equilibration of semen:** Once filling and sealing of straws is done, sealed semen straws are immediately counted in counting chamber and racked on racks by use of the ramp and racks and then placed in a cold handling cabinet (CHC) placed in the freezing room. The straws are maintained at 4^0C and should be kept there for 4 hours before freezing in order to maintain semen equilibration.

VI. FREEZING

After equilibration, straws can be frozen in a biological freezer (Forced Vapor Freezing Method-Annex 1). Alternatively, it can be done custom frozen 4 or 10 cm above liquid nitrogen vapor for 10 minutes and then immersed in liquid nitrogen.

VII. FROZEN SEMEN STORAGE

- Frozen semen doses, if found acceptable on checking post-thaw motility after freezing, would be stored in the goblets of various capacity. These goblets would be stored in mother containers each allotted for separate bulls.
- Liquid Nitrogen shall be replenished at a regular interval depending on the liquid nitrogen evaporation rate of the container (current practice is to refill mother containers every 15^{th} day in summer and every 20^{th} day in other seasons).
- All the frozen semen doses are stored / dipped in liquid nitrogen at least $2/3^{rd}$ of the semen straw.

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.

CLEANING AND STERILIZATION

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. (Annex 9)

ANNEXES

Annex 1
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 in the computerized freezer. The rate of fall of 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 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, once the temperature is stabilized at 4^0C it is started again.
- The rate of fall of temperature is @ 5^0C from 4^0C to minus 10^0C.
- From minus 10^0C the rate of fall has been programmed at @ 40^0C per minute up to minus 100^0C.
- Finally, from minus 100^0C to minus 140^0C at @ 20^0C 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 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
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%

Annex 9

CLEANING AND STERILIZATION AFTER SEMEN COLLECTION

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.

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 liter 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 in hot air oven.
- 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 clean cloth and immerse in a mixture of ethanol and chloroform (8:2). Wipe with clean cloth and keep for next use.

Sterilization in Hot Air Oven

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

- Cover or wrap each washed and dried items 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 or 180^0C for 30 minutes. Keep for an hour and switch off the oven.
- Let the items be inside and allow cooling.
- Remove from the oven and keep them in the storage cupboard or incubator.

Sterilization in Autoclave

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

Autoclaving

- 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 rubber band close 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 buffer, it should be autoclaved at 5 psi pressure for 20 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 15 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 STERILIZED mark.

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 UV chamber.

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

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 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 low pressure AV sterilizer.
- The sterilized AV is stored in the incubator at 45^0C after filling with clean water for next day use.

Summary of Sterilization

(A) Autoclave

S. No.	Item	Pressure (psi)	Time (min)
1	Artificial Vagina	5	15
2	Buffer	5	20
3	Plastic tips and suction tubes	5	20
4	Bull apron, Napkins	5	20
5	Thermo-resistant rubber ware	5	15
6	Bacteriological media	15	15
7	Distilled water	15	20
8	Surgical equipment	10	10
9	Poly propylene collection tubes	5	20

(The rubber wares can withstand above pressure and duration provided the quality is good)

(B) Hot Air Oven

S.N.	Item	Temperature	Time (Min)
1.	Glassware	160/180 Deg. C.	60/30
2	Filling Nozzles (Metal only)	160/180 Deg. C.	60/30
3	Collection glass tubes	160/180 Deg. C.	60/30
4	Filling Needles (Metal only)	160/180 Deg. C.	60/30

(C.) AV sterilizer: Wherever Autoclave is not used, AVs and rubber cones shall be sterilized using AV sterilizer. After sterilizer starts boiling, 30 minutes vapor sterilization shall be done.

Annex 10

Morphological Studies of Spermatozoa

- Type of Abnormalities: Head, Mid-piece, Tail, Proximal droplets.
- Take a sterilized Borosil glass tube, to it 5 ml. of buffered normal saline is poured.
- Add 1 to 2 drops of neat semen to above 5 ml. of buffered normal saline, gently shake, incubate at 37^0 C for 10 to 20 minutes.
- Put a drop of the sample on clean glass slide, cover with cover slip and examine under oil immersion objective.
- 200 sperms are counted from different fields and types of sperm abnormalities are tabulated.
- Sperm abnormalities and the maximum allowed frequency in normal buck semen are-

a. Head abnormalities- Young Bulls	10%
Old Bulls	20%
b. Mid-piece abnormalities	5%
c. Tail abnormalities	5%
d. Proximal and distal droplets	5%

- Semen should not be used if the sample contains more than 20% abnormalities put together
- Bulls having more than 20% primary abnormalities in neat semen are re-examined for three consecutive tests are suggested for culling.
- Bulls having more than 20% secondary abnormality are advised for sexual rest and correcting the causative factors.
- Microbial examination should be conducted frequently. Five types of examinations are carried out.

1. Aerial count test

1. Sterile (after keeping it in hot air oven at180^0 C for 30 minutes) petri dish is taken.
2. Under laminar air flow 15 ml. autoclaved (15 psi for 20 minutes) Nutrient Agar media for non-pathogens and Brain heart infusion agar for pathogens is poured in the petri dish. The media has to spread so as to it covers the Petri Dish completely.
3. The petri dish is exposed for 10 minutes in the area to be tested
4. The petri dish is closed and incubated for 48 hours at 37^0 C.
5. The colonies are counted after 48 hours.
6. The areas tested are laboratory, extender preparation room, and sterilization room, cold handling Units, Laminar Air Flow Stations, UV Chambers, Autoclave, Incubator, and Hot Air Oven etc.

Random sampling for bacterial load- The standards for acceptable Colony Forming Units (CFU) in processed semen is 5000 per ml as per OIE norm.

2. Rinse Test

1. The instruments and glassware to be tested are rinsed with autoclaved distilled water and collected in a sterile beaker. The instruments and glassware tested are sterilized AV, UV exposed empty straws, conical flask, measuring jar, collection tube, and filling tube.
2. One ml. of rinsed water is taken using a sterile pipette and inoculated in a sterile Petri Dish under the Laminar Air flow.
3. 15 ml. of media is poured in the Petri Dish.
4. Petri Dish is incubated for 48 hours at 37^0 C.
5. After 48 hours the colonies are counted.

The maximum permissible level is 10 colonies.

3. Working Solution Test

1. Under Laminar Air Flow, 1 ml. of autoclaved working solution (Working solutions tested are distilled water, buffer and extender) is inoculated in a sterilized petri dish.
2. 14 ml. of media is poured in the petri dish.
3. The petri dish is incubated at 37^0C for 48 hours.
4. After 48 hours the colonies are counted.

There should not be any colonies in the working solution.

4. Batch Test

1. Single straw from each bulls of same batch taken and pooled in a sterile test tube A.
2. From it 0.1 ml. semen is taken in another test tube B.
3. To this 9.9 ml. of autoclaved distilled water is added.
4. Under laminar air flow 1 ml. is taken from test tube B and inoculated in a petri dish.
5. 15 ml. of autoclaved media is poured.
6. Petri dish is incubated at 37^0C for 48 hours.
7. After 48 hours the colonies are counted.
8. As the dilution rate is 1 in 100 one colony counted = 100 colonies.

There should be no colonies.

5. Bull wise Test

1. A single straw from the bulls to be tested is thawed and taken in a test tube.
2. To it 9.75 ml. of autoclaved distilled water added under laminar air flow
3. From it 1 ml. is inoculated in a petri dish

4. 15 ml. of autoclaved media is poured.
5. The Petri dish is incubated at 37^0C at 48 hours.
6. After 48 hours the colonies are counted.
7. As the dilution rate is 1 in 40 one colony counted = 40 colonies