Minimum Standards

For

Production of Bovine Frozen Semen

2022

DEPARTMENT OF ANIMAL HUSBANDRY & DAIRYING

MINISTRY OF FISHERIES, ANIMAL HUSBANDRY & DAIRYING

GOVERNMENT OF INDIA

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MINIMUM STANDARDS FOR PRODUCTION OF BOVINE FROZEN SEMEN

Artificial Insemination with frozen semen has been proved to be the best tool worldwide for mass genetic improvement through dissemination of superior germplasm. This objective can be achieved only if the frozen semen used in

A.I programme conforms to certain prescribed quality standards. For production of quality semen, it is most important that the bulls used in A.I programme meet prescribed norms, and are disease- free and that the semen is harvested and processed in accordance with the standard protocols. The minimum essential protocols required for production of quality semen are described in this manual. Failure to observe these guidelines may result in production of poor quality semen making it unsafe as well as unfit for use in breeding through artificial insemination programme.

1. Standards for Genetic Merit of Breeding Bulls

All the bulls of Gir, Murrah, Mehsana, HF crossbreed and Jersey crossbreed shall be procured based on breeding value w.e.f 1st April 2023. All the bulls with negative breeding values have to be disposed off in a phased manner. No semen station shall produce semen from bulls with negative breeding value from 1st April 2023 onwards, for the breeds where the breeding value is tested and available. In case, bulls with GBV are not available or the PT programme for a particular breed is not being run, the bulls for semen production may be procured based on the dam's Standard lactation yield till the GBV is established for those breeds. The reliability of the data available with respect to performance recording must be kept in view. The minimum dam's lactation yield for different breeds is given in Table 2.The Lactation yield may be arrived at by recording the animal at monthly interval continuously for 11 times or until the animal becomes dry but not less than 8 recordings. Standard Lactation Yield of the animal subjected to performance recording should be calculated using the Test Interval Method (A4) described at Section 2.1.5.1 of the International Agreement of Recording Practices published by International Committee for Animal Recording (ICAR). Bulls to be procured from 1st April, 2023 onwards to be tested for Parentage using DNA finger printing particularly bulls devoid of breeding value. Imported bulls are exempted from Parentage verification.

Table: 1 Weightage for different categories of bull for calculating score for genetics in CMU evaluation

Breed Category	**No. of bulls**	**Weightage**	**% of bulls qualifying for category**
For the Breeds which are imported and/or supplied by GOI	100 80	1.0	80
For Breeds where Breeding Value Estimation Committee can provide GBV (Murrah, Mehsana, Gir, HFCB, JCB)	110 50	1.0	46
For Breeds that are not evaluated by BVEC and has to be procured based on dam's yield Bulls that qualifies based on Dam's yield (On prorata basis)	60 30	1.0	50
For Breeds which are supplied by the GOI under PTS	40 20	1.0	50
	310		226
Total Marks allocated for Genetics in score sheet	**50**		73
Marks obtained by Semen Station for Genetics			**37.0**

* The score card prepared for CMU Evaluation shall have at least 50% weightage of total score for the Genetic Worth of the Semen Station.

Table: 2 Standards for Dam's lactation

Breed	Dam's Lactation yield (Kgs)		
	First	Best	Fat %
Holstein Friesian	7000	10000	3.5
Jersey	5000	6000	5.0
Sahiwal	2400	3000	4.0
Red Sindhi	2000	2500	4.5
Gir	2400	3000	4.5
Kankrej	2000	2500	4.5
Tharparkar	2000	2500	4.0
Hariana	1600	2000	4.0
Rathi	1600	2000	4.0
Ongole	1100	1600	4.0
Deoni	800	1000	4.0
Khillar	380	500	4.0
Dangi	400	530	4.0
Amritmahal	400	500	4.0
HF Cross- F2	5000	6000	4.0
Jersey Cross- F2	3500	4500	4.5
Sunandini	2500	3000	3.5
Murrah	2400	3000	7.0
Mehsana	2400	3000	7.0
Nili Ravi	2400	3000	7.0
Jaffrabadi	2800	3500	8.0
Surti	1600	2000	7.0
Banni	2400	3000	7.0
Bhadawari	1300	1600	8.0
Pandharpuri	1300	1600	7.0

The standard for Dam's lactation yield for F1 cross bulls will be the same as that of respective indigenous bull dam i.e. Gir, Sahiwal, Kankrej, Red Sindhi, etc.

For Breeds not mentioned in above table, concerned state government may notify the min. Dam's lactation details and Breed code.

For imported bulls and embryos, the standards for import of germplasm as prescribed in the "Guidelines for export / import of bovine germplasm" issued and amended from time -to -time by DAHD, Ministry of Fisheries, Animal Husbandry & Dairying, Government of India shall be applicable.

2 (a). Bull Identification

a). Unique identification number having 12 digits with bar code shall be practiced for identification of bulls across all Semen Stations. It should be compatible for registration under INAPH, presently being managed by NDDB, Anand.

b). The bar coded ear tag with unique 12 digit laser printed number will be physically applied to the bull and will remain on it for life. If the bull is sold to other SS, the tag no. i.e the bull identification will remain unchanged.

c). In case the tag falls, is lost or destroyed, it will be changed with a new 12 digit Bull ID following standard procedure of "Ear Tag Change" under INAPH.

d). In addition, each bull will be assigned an alphanumeric ID by the Semen Station incorporating three character Semen Station (SS) code (table 04); 2 to 4 character breed code (table 05) and unique I.D. or name given to bull. For Example: BAS-HFCB-320198; ALM-HF-ARJUN etc. This will be a mandatory and will have to be printed on the straw. In case, a bull is sold to other SS, the new SS will assign a fresh alphanumeric ID.

e). Management of data for semen production and artificial insemination will become easier by capturing real time bull detail, using bar coded semen straws and ear tags.

2 (b). Physical Examination

Before procuring new bull calves/bulls for the semen station, a thorough physical and andrological examination shall be conducted by an experienced Veterinarian with respect to breed characteristics, general health and suitability as a breeding bull.

Standards for scrotal circumference and weight gain index for various breeds need to be evolved and in particular for indigenous breeds by recording the scrotal circumference once in three months and body weight once a month, by the semen stations. For every new bull calf procured, the measurement of scrotal circumference and body weight needs to be initiated immediately.

Prior to introduction of any bull for semen collection, breeding soundness examination should be conducted and documented.

3. Karyotying and testing for Genetic disorders/ diseases

It is necessary that all bulls be karyotyped to rule out any chromosomal defects except imported bulls. In addition, breed specific Genetic disorders such as Factor XI deficiency syndrome, Bovine Leukocyte Adhesion Deficiency (BLAD), Citrullinemia, and Deficiency of Uridine Monophosphate Synthase (DUMPS) will be tested in HF and HF crossbred bulls and as a precaution in Jersey and their crosses (to rule out possibility of HF blood/crossing at any stage)

4. Quarantine

A minimum quarantine period of **60 days** is compulsory before bringing new bulls into a semen station. Only after favourable results from the health control point, the bulls shall be admitted to the semen station. Relevant definitions are given in Annexure- 1

a) In the quarantine station, new animals shall be housed for a minimum of **60** days in a place which is effectively separated and away from (preferably at a distance of 5 km) the facilities occupied by resident bulls. Manpower deployed and all equipment used in handling, feeding, watering and cleaning the new bulls shall not be shared with the resident herd(s).

b) Each new animal in quarantine station will be tested against major contagious diseases before its entry to resident herd namely TB, JD, Brucellosis, Campylobacteriosis, Trichomoniasis, Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhoea. All tests shall be done by an accredited agency or disease diagnostic laboratory as indicated in Annexure- 2.

c) During the quarantine period, the bulls shall be vaccinated against FMD, HS, BQ, Theileriosis and Anthrax. However, vaccinations against bacterial diseases shall be done only if there is an outbreak or prevalence of a particular disease in the area.

Once the quarantine period is over, all bulls shall be introduced to the young bull rearing station or to the Semen Station depending upon the age of bulls.

**The procedure and duration for quarantine in different situations is given in Annexure- 3A, 3B, 3C & 3D.*

5. Testing of Bulls

The testing protocols/ procedures for bulls against Tuberculosis, Johne's disease, Brucellosis, Campylobacteriosis, Trichomoniasis, Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhoea are given in Annexure- 4 to 10. The breeding bulls should be free from above mentioned diseases. Though Johne's disease is not a sexually transmitted disease but being a chronic, infectious and incurable disease, it has been included and the breeding bulls found positive for Johne's need to be removed. The bulls in the quarantine/ rearing station and the resident herd should go through periodical testing and vaccinations as per the schedule listed in the manual.

6. Vaccination Schedule

The bulls shall be vaccinated against FMD, HS, BQ, Theileriosis and Anthrax. However, vaccinations against bacterial diseases shall be done only if there is an outbreak or prevalence of a particular disease in the area.

a. Theileriosis – Exotic and crossbred bulls shall be vaccinated as per the vaccine manufacturer's recommendation.
b. Sero monitoring of 10% of the bulls at the semen station for FMD vaccination. (Pre & Post monitoring)
c. Radius for ring vaccination will be 10 kms from Semen station.

To reduce lay off time, the bulls shall be vaccinated on the rest day or on the day after completing semen collection. Sexual rest may not be required unless febrile condition is noticed.

The semen station shall arrange for carrying out ring vaccinations of all cloven footed animals including swine against FMD within a radius of 10 km around the semen station. Vaccinations against HS and BQ shall be carried out in the areas having incidence of these diseases.

7. Culling of Bulls and Semen Doses due to Specific Diseases

Diseases	Bulls	Semen doses
FMD	Retain	Last one month's doses to be discarded, refer Annexure-11
Brucellosis	Castrate & remove as per prescribed scientific method	FS doses in stock to be discarded since the last negative test
TB	Castrate & remove as per prescribed scientific method	FS doses in stock to be discarded since the last negative test
JD	Remove as per prescribed scientific method	FS doses in stock to be discarded since the last negative test
Infectious Bovine Rhinotracheitis (IBR)	i) Castrate and Remove for IBR – free S.S. and retest all remaining bulls until all tested negative ii) Isolate the bull and process and store semen separately for IBR positive semen stations. All IBR positive SS should aim at becoming IBR negative as soon as possible. Only IBR sero- negative bulls will be introduced at SS.	i) Test each batch/ ejaculate since last negative test by RT-PCR. Semen found positive shall be destroyed by incineration /autoclaving. Use only Semen that has tested negative by RT-PCR. ii) Test each batch / ejaculate by RT-PCR. Semen found positive shall be destroyed by incineration/ autoclaving. Use only Semen that has tested negative by RT-PCR.
Bovine Viral Diarrhoea (BVD)	Isolate and remove Persistently Infected bulls. Only PI negative Animals will enter the SS	Destroy by incineration Frozen Semen doses of the PI positive bulls.
Campylobac teriosis	Isolate and remove	FS doses in stock to be discarded since the last negative test
Trichomoniasis	Isolate and remove	FS doses in stock to be discarded since the last negative test

The semen station must remove bulls (within 48 hours) which are positive for Brucellosis, TB, JD, IBR (IBR negative SS) and PI BVD.

Guidelines issued by the Department of Animal Husbandry & Dairying, Ministry of Fisheries, Animal Husbandry & Dairying, Government of India for progressive IBR/ BVD control and as amended from time-to-time shall be followed in letter and spirit by all semen stations.

Besides, the semen station are advised to cull those bulls which have completed eight years of productive period or 3 lakh semen doses, whichever is earlier unless the bull is of exceptional genetic merit. In addition, the bulls with poor libido, poor semen quality, incurable lameness, etc. may also be culled on regular basis.

8. Housing

Bull sheds shall have spacious individual pens with adequate loafing area, manger and water trough with access to drinking water all time. Adequate shade around the bull shed shall be provided. The roof shall be made of asbestos or suitable materials. During summer, cooling system with sprinklers and fans is required particularly for the buffaloes and exotic bulls. Disinfectants like **formalin or phenyl** based compounds **shall not be used** in the bull sheds. Alternatively, compounds containing Gluteraldehyde may be used. Weekly spraying of Sodium Carbonate (4% solution) shall also be practiced. The floor should be sterilized at least once a year by a blow lamp or by burning straws. At one corner of the farm, there shall be an isolation shed for separating ailing / sick bull(s) for treatment. Bull(s) once diagnosed suffering from any of the infectious diseases shall be removed immediately from semen station to contain its spread to other bulls.

There should be separate staff and separate bio-security arrangements for semen station and female herd, if any.

9. Management of Bulls

Proper management of breeding bulls at all times is essential to keep them in good health and to ensure a satisfactory state of cleanliness. The following guidelines should be considered:

a) The bulls shall be kept under hygienic conditions at all times.
b) The coat of the bulls shall be kept clean and generally short. The hooves shall be regularly trimmed.

c) The length of the tuft of hairs at the preputial orifice, which is invariably soiled, shall be cut to about 2 cm. The hair would not be removed altogether, because of its protective role. If cut too short, it may cause irritation of the preputial mucosa.

d) Bulls shall be brushed and groomed regularly, and where necessary, special attention shall be given to the under-belly, a day prior to semen collection.

e) Cleaning of the prepuce with sterile normal saline solution prior to the day of collection can be practiced if the microbial load in frozen semen is beyond the prescribed limit otherwise occasional cleaning is advised as per need. The person carrying out preputial wash must use disposable gloves and separate sterilized nozzle for each bull to avoid transmission of infection from one bull to another.

f) In the event of obvious soiling, careful cleaning of the preputial orifice and the adjoining areas with soap or a detergent is recommended followed by thorough washing and drying.

g) Scientific feeding schedule shall be followed for the bulls. A general guideline is attached as Annexure-12. Semen station is advised to carryout routine quality analysis of feed and fodder to ensure balanced ration.

10. Semen Collection

a) Ideally, the floor of the collection yard shall be made of concrete layer at a depth of one foot from the ground level. Mixture of sand and limestone shall be used to fill up to ground level and pressed firmly. If it is not possible to renovate the entire collection arena, at least the mounting area shall have sand and limestone mixture for proper footing of bulls. Alternatively, good quality rubber mat (with interlocking arrangement) or coir mat shall be put into concrete groove of the mounting area for adequate cushioning effect. After collection, the area must be thoroughly cleaned and odorless disinfectant solution (Colloidal iodine) be sprayed. A dusty floor shall be avoided to prevent dust falling on the A.V / semen samples.

b) On the day of collection, before collecting semen, the bulls shall be properly washed and cleaned. After that, the prepuce shall be cleaned externally with normal saline and a sterilized paper napkin or sterilized cloth napkin soaked in normal saline to remove any sand or dust particles. For each bull a separate napkin shall be used.

c) The person carrying out perpetual wash must use disposable gloves and separate sterilized nozzle for each bull to avoid transmission of infection from one bull to another. Preputial washing should be carried out if the bull is soiled otherwise it is better to avoid.

d) Semen collection should be individualized based on the bull behavior.

e) Sexual preparation (number of false mounts and restraint) of the bulls may not be generalized but decided based on the behavior of the individual bulls. For this purpose, the sexual behavior of the individual bulls shall be studied and documented

f) As a general rule, bulls shall be sexually prepared by giving two / three false mounts followed by restraint. The gap between two ejaculates shall be preferably approximately fifteen minutes to half an hour depending on the bull. Second ejaculate shall be taken following proper stimulation and preparation of bulls.

g) Sterilized bull aprons shall be used to avoid penis touching hindquarter of the dummy.

h) Before every collection, the semen collector shall wash his hands with suitable antiseptic solution or use disposable gloves or do both. The semen collector shall not touch the penis.

i) Semen should be collected either by the Veterinarian himself or by a suitably trained technician / staff under his close supervision. While taking collection, it shall be ensured that AV is not thrust on penis of bull, instead the penis is gently guided into the AV.

j) Immediately after collection, the AVs shall be thoroughly cleaned by non-spermicidal neutral detergent. Separate AVs shall be used for each ejaculation. The AV shall be changed even if the bull has inserted its penis without successful ejaculation. The same AV shall not be used twice. The AVs shall always be kept inverted and the collection tube shall be covered with felt / water jacket (plastic bottle filled with warm water at 34o C) to avoid cold shock. The open end of sterilized AVs shall be covered with aluminum foil, which would be removed at the time when bull is ready for giving semen.

k) Appropriate size AVs, ranging from 8-14", shall be used for cattle and buffaloes ensuring that the semen is ejaculated in the cone. The cone shall be of good quality Neoprene rubber/Silicone rubber.

l) Use of lubricant shall be avoided. If it is extremely essential to use lubricant, separate sterilized glass rods shall be used for smearing K-Y Jelly on individual AVs.

m) The AV shall not to be shaken after ejaculation and carried to the pass box with open end slightly inclined downward to avoid flowing down and mixing of lubricant and debris with the semen samples.

n) As soon as the first ejaculate is taken, the bull apron should be removed and dipped in the plastic tub filled with warm detergent solution. For second ejaculate, a fresh, sterilized bull apron should be used.

o) The entry of visitors and staff / labourers (other than those involved in semen collection) shall be strictly prohibited in the collection arena at the time of semen collection.

p) Protective clothing (barn coat) and gumboots shall be used by the veterinarians and staff during semen collection. Gumboots and barn coat should be washed immediately after completion of semen collection work.

q) Semen stations must follow the norm of a minimum of two ejaculates per collection and two collections per bull per week, resulting into at least 90 collections and 180 ejaculates annually from each adult bull. However, a maximum number of collections per bull would depend on the individual capacity of the bull.

11. Handling, processing & freezing of semen

11 (A) Premises

a) Sufficient trees shall be planted and lawns prepared around the semen station to minimize dust in the premises.

b) The ceiling and walls of the laboratory shall be made up of non- porous materials. All cracks and crevices shall be sealed to control pests and insects.

c) Entry of persons to the laboratory, other than laboratory personnel, shall be strictly restricted. Airlock system or anti-room shall be provided to avoid direct entry to the semen-processing laboratory.

d) Laboratory windows shall preferably be made of fixed double glass sheet with aluminum frame. The glass panes shall be plastered with sun control films to avoid direct sunlight. The doors shall be kept closed, especially during dilutor preparation and semen processing.

e) Preferably cassette type or, split type air conditioners fitted with air purifying system with remote temperature control mechanism should be installed to maintain the room temperature at 20°C–22°C. The number of ACs to be fixed to sustain this temperature shall depend on the size of the processing room. Maintaining this temperature is an essential requirement to achieve the optimum results when single step dilution method is followed for freezing semen. The flow of air from AC must not be towards the front side of the Laminar Air Flow Unit. Adequate number of thermometers shall be kept at suitable locations in the laboratory to monitor room temperature.

Alternatively, central cooling with 10 to 15 air changes should be fixed, especially for the semen processing laboratory. This helps to control the bacterial load in the semen-processing laboratory and in removing obnoxious odour. The processing laboratory should ideally maintain around 55% relative humidity.

f) Sink drains shall be decontaminated routinely with a disinfectant. Sink shall not be placed in the semen processing room.

g) The floors shall be preferably made up of vitrified tiles. Floors and horizontal surfaces shall be cleaned and mopped with a disinfectant solution, as dirt and dust, which settle on these surfaces, are the main sources of contamination.

h) Unwanted furniture, equipment and materials shall not be kept in the laboratory as these only provide additional area for dust and spores to accumulate.

i) Appropriate number of germicidal UV lights (2470A) may be provided in the laboratory (Optional), laminar airflow unit, apron and laboratory footwear cabinet etc with a common operating switch outside the laboratory. These lights shall be switched 'on' at least 8 hours prior to commencement of work in the laboratory and shall be switched 'off' before beginning the work. The date of installation of the UV lights shall be recorded to facilitate timely replacement as the life of UV tube is of 2000 hours. A logbook should be maintained for UV lights.

j) The laboratory shall be fumigated twice a week with Cold Fumigant, using humidifier.

k) The efficacy of Fumigation should be regularly monitored by undertaking bacterial load test (pre and post fumigation) of the laboratory environment. The bacterial load shall be measured every week to monitor contamination of the laboratory atmosphere.

l) The working platforms, the exposed parts of equipments and other items to be handled during processing of semen, shall be cleaned with 70% alcohol (Iso Propyl) or Glutaril. It is advisable to repeat the cleaning exercise after completing processing of semen.

m) Clean laboratory footwear, apron/ coats, hand gloves, mask and caps shall be compulsorily put on while working in the laboratory.

n) Eating, drinking, smoking, etc. shall be strictly prohibited in the laboratory. Unnecessary conversation should also be discouraged in the laboratory. Besides, entry of unauthorized persons shall be strictly restricted.

o) Long exposure of semen to ultraviolet rays, visible light in direct sunlight and white florescent light causes chromosomal damage and hence, direct exposure to such sources of light shall be avoided. Hence, there shall be a provision for indirect or diffused lighting inside the semen processing room. Care shall also be taken not to switch on tube lights in cold handling cabinet and laminar air flow unit (LAFU). However, at the time of filling and sealing of straws in LAFU, diffused light could be used.

11 (B) Equipment

a) The exteriors of all equipment and furniture shall be cleaned weekly. The equipment shall be kept covered by plastic covers when not in use.

b) The pre-filter of Laminar Airflow unit shall be cleaned Quarterly. Routine servicing and DOP testing twice a year will ensure efficiency of HEPA filters. Alternatively, culture plate test to monitor bacterial load of the air passed through the filters shall be carried out at weekly intervals to assess its efficacy/ functioning.

c) Digital photometer / Computer aided Spectrophotometer shall be validated with Haemocytometer readings for sperm concentration twice a year separately for cattle and buffalo (20 samples each).

d) The automatic semen straw filling and sealing machine shall be thoroughly cleaned, immediately after use.

e) The microscope lens shall be gently cleaned daily with a piece of cotton soaked in a mixture of ethyl and methyl alcohol (1:1) or a mixture of 80% ethyl alcohol and 20% ether.

f) The bio-freezer shall be defrosted and thoroughly cleaned and dried, immediately after use.

g) Incubators to maintain artificial vagina shall be cleaned and disinfected regularly with 70% alcohol (Iso Propyl).

h) Single distilled water shall be used in autoclave and thermo-controlled water bath. The water bath shall be cleaned and filled with single distilled water on a regular basis.

i) The thermometer kept immersed in water bath shall be cleaned daily to ensure recording of correct temperature. Alternately, water bath fitted with digital display temperature indicator should be used.

j) The Liquid Nitrogen containers returned / received from outside shall be disinfected thoroughly with 4% Sodium Carbonate solution and finally with 1 to 4% formaldehyde.

k) The refrigerator meant for storing eggs, antibiotics and buffer shall only for storing these materials. While vaccines, medicines and other materials shall be stored at a place away from the semen laboratory. The refrigerator used for storing eggs, etc. shall be cleaned and sterilized every week using alcohol swab.

l) The following equipment should be validated/ calibrated by Manufacturer/ supplier or by NABL certified laboratories once in a year:

i. Thermometers
ii. Water Bath
iii. Weighing Balance
iv. Incubator
v. Autoclave
vi. Hot Air Oven
vii. Slide Warmer
viii. Micropipettes
ix. pH Meter
x. Cold Handling Cabinet
xi. Laminar Air Flow Units
xii. biological Freezer
xiii. Microjet Ink Printer
xiv. Filling & Sealing Machine
xv. Photometer
xvi. Triple distillation unit

m) All equipment used in semen processing should be covered under Annual Maintenance Contracts.

11 (C) Personnel Hygiene

Clothing, skin and hair of laboratory personnel are the recognized sources of contamination. Hence, everyone should wear laboratory aprons, footwear and take other necessary precautions all the time while working in the laboratory. Hands shall be washed with soap and water and rinsed with 70% alcohol before commencing work in the laboratory.

The bull attendants and officials coming in regular contact with bulls must undergo test for Tuberculosis (TB) and Brucellosis every year.

The other staff working in farm should be tested for TB and Brucellosis once in two years.

11 (D) Diluents

a) Buffer and diluents should be prepared in a separate classified room/ zone.

b) All disposable and reusable supplies coming in contact with the semen and dilutor must be sterile and devoid of toxins and pyrogens.

c) Prolonged storage of purified water is not recommended because water purity deteriorates progressively over a period of time as heavy metals leach from some glass and plastic storage vials / containers.

d) Glassware, collection tubes, etc. shall not be held from their rim / mouth.

e) Manual pipetting shall be replaced with automatic adjustable micropipettes with sterile disposable tips.

f) After adding all the components of buffer viz. TRIS, Citric Acid, Glycerol and Fructose in ultra–pure water (Autoclaved) or triple glass/ double distilled water it should be sterilized by microfiltration (Optional). If the buffer is prepared on the previous day, it should be stored in the refrigerator and antibiotics are to be added next day in the morning after warming it to 34^{o}C.

g) Antibiotics in diluents: A combination of Penicillin and Streptomycin may be used in diluents. However, it is better to use a combination of Gentamycin, Tylosin, Lincomycin & Spectinomycin (GTLS), having the additional benefit of controlling Mycoplasma. The antibiotics should preferably be of Tissue culture grade or laboratory grade and not those used for therapeutic purpose as injections.

h) Only fresh eggs shall be used for making dilutor. The eggs shall be stored in refrigerator after wiping with dry cotton. Just before preparation of dilutor, eggs shall be wiped with 70% alcohol (Iso Propyl). To ensure the quality, eggs shall be purchased from known sources.

i) The required quantity of yolk shall be separated from albumin on sterile (autoclaved) standard filter papers (No.1) and yolk membrane shall be punctured using sterile glass rod, Pasteur pipette or sterile straws/ tip under the Laminar Air Flow Unit. Only fresh semen extender/dilutor shall be used since some constituents may get deteriorated during storage. Even a day old extender should not be used.

11 (E) Evaluations & Processing

a) The tube containing the freshly collected semen should be capped with aluminum foil as soon as it is placed in the dynamic pass box before transferring to the laboratory. The collection tube should preferably remain capped until processed.

b) As soon as the neat semen is received, it shall be kept in a thermo- controlled water bath at 34^{o}C under Laminar Air Flow Unit, after recording the volume of semen.

c) After examination of sperm concentration and initial motility as soon as possible, the semen samples shall be primarily diluted (1 :1) with dilutor maintained at 34°C.

After dilution of semen in the ratio of 1:1, it shall be extended further (final) in appropriately sized flasks after with dilutor / flasks maintained at the same temperature (34°C), under the Laminar air flow unit. The neat semen samples should not be allowed to get accumulated for long time in water bath to minimize adverse effects on viability.

After the final dilution the flask should be kept at room temperature for 10-15 minutes so that temperature reaches 20-22^{o}C.

d) Sperm concentration shall be checked by a digital photometer with auto dilutor, manufactured by a reputed company. Semen samples having concentration of less than 500 million / ml sperm concentration shall be discarded. The dilution unit of photometer should be placed under laminar air flow unit.

The volume of straws should be checked as it may vary between manufacturer/ type of straw. While determining the dilution rate as per the photometric reading, the correct volume of the French mini straw should be fed to the photometer. The volume of randomly drawn straws from a day's production should be checked as part of quality assurance and be documented. Each straw (dose) should possess a minimum of 20 million spermatozoa.

e) Semen samples selected for processing should have a minimum of 70% initial progressive motility.

f) Filling and sealing of semen shall be done under Laminar Air Flow Unit using sterile straws, filling nozzles and disposable rubber tubing's. Rubber tubing's shall not be reused in any case.

g) Unused straws shall be repacked (air-tight) under Laminar Air Flow Unit before storage. Immediately after use, all the glass ware and other re-usable articles shall be immersed in lukewarm neutral detergent solution (in a plastic tub near the Laminar Air Flow Unit).

h) The freezing should be carried out as per the recommended protocols for freezing cattle and buffalo semen. After freezing gets over, the straws should be collected from the racks using scoop tongs. The operator should wear appropriate protective gloves to avoid frost injury

11 (F) Colour Specifications of straws:

All semen stations shall follow the following colour codes for filling of semen in straws:

Table: 3 **Colour Specifications**

Breed	**Colour**
Holstein	Pink/Rose
HF Crossbred/Frieswal/ Karanswiss	Pistachio Green (light green)
Jersey	Yellow
Jersey Crossbred	Salmon
Indigenous cattle	Orange
Sunandini	Blue
Buffalo	Grey
Gir	Purple
Sahiwal	Orange
Red sindhi	Putty
Rathi	White
Tharparkar	Light yellow

- The new color code will come into existence from 1st April 2023
- If any of above mentioned colour is not available, then transparent straws shall be used.

11 (G) Printing of Straws

Each Semen Station shall print certain key information on the straw in the following Sequence, starting from the factory end.

1. SS Bull ID – Alphanumeric
2. Batch No with Ejaculation No in Parentheses
3. Dam's Lactation Yield and source (e.g. 3380- PT)
4. Brand image
5. Brand Code

Items serially numbered from 1 to 3 shall be mandatory for all stations. Items 4 and 5 may be optional.

NOTE - canister holder-label **may preferably** have information of Bull No., Breed, Source-PT/PS/ET/IM/O, Dam lactation yield/breeding Value and Name of Semen Station

Table: 4 Three Character Semen Station Codes

Sr. No.	Semen Station	State	Type	SS Code
1	ABC, Salon	Uttar Pradesh	Trust	ABC
2	AMUL	Gujarat	Coop.	AMU
3	BAIF	Maharashtra	Trust	BAF
4	Bassi	Rajasthan	Coop.	BAS
5	Bhopal	Madhya Pradesh	Govt.	BHO
6	Banavasi	Andhra Pradesh	Govt.	BNV
7	CFSP&TI, Hessarghatta	Karnataka	GOI	CSF
8	Dhoni	Kerala	Govt.	DHO
9	Banas new	Gujarat	Coop.	DSU
10	Jagudan	Gujarat	Coop.	DUR
11	Haringhata	West Bengal	Govt.	HGT
12	Hisar	Haryana	Govt.	HIS
13	Nandini	Karnataka	Coop.	KMF
14	Karimnagar	Andhra Pradesh	Govt.	KNG
15	Mattupatty	Kerala	Govt.	MUT
16	Nabha	Punjab	Govt.	NBH
17	NJF, Ooty	Tamil Nadu	Coop.	NJF
18	FSPS, Ooty	Tamil Nadu	Govt.	OTY
19	Patan	Gujarat	Govt.	PAT

Sr. No.	Semen Station	State	Type	SS Code
20	SAG Bidaj	Gujarat	Trust	BDJ
21	Salboni	West Bengal	Govt.	SAL
22	Rishikesh	Uttaranchal	Govt.	ULD
23	Anjora	Chhattisgadh	Govt.	ANJ
24	Aurangabad	Maharashtra	Govt.	AUR
25	Babugarh	Uttar Pradesh	Govt.	BAB
26	Beldanga	West Bengal	Govt.	BEL
27	Bhatian	Punjab	Coop.	BHA
28	Chitale	Maharashtra	Pvt.	CHI
29	Jind, BAIF	Haryana	NGO	JND
30	Cuttack	Odisha	Govt.	CTK
31	Deep Frozen Semen Station Rehmankhera, Lucknow	Uttar Pradesh	Govt.	RKH
32	Dharwad	Karnataka	Govt.	DHA
33	Echenkottai	Tamil Nadu	Govt.	EKT
34	Gurgaon	Haryana	Govt.	GUR
35	Hakkal, Jammu	Jammu & Kashmir	Govt.	HKL
36	Hosur	Tamil Nadu	Govt.	HOS
37	Jagadhari	Haryana	Govt.	JAG
38	Barapeta (Khanapara)	Assam	Govt.	BPT
39	Kulathupuzha	Kerala	Govt.	KUL
40	Nagpur	Maharashtra	Govt.	NAG
41	Nandyal	Andhra Pradesh	Govt.	NDL
42	Palampur	Himachal Pradesh	Govt.	PLM
43	Pune	Maharashtra	Govt.	PNE
44	Rohtak	Haryana	Coop.	ROH
45	Ropar	Punjab	Govt.	ROP
46	SLBTC, Hessarghatta	Karnataka	Govt.	SLB
47	Srinagar	Jammu & Kashmir	Govt.	SRI
48	SSCC, Hessarghatta	Karnataka	Govt.	SSC
49	Vizag	Andhra Pradesh	Govt.	VZG
50	Germplasm Station, Narwa, Jodhpur	Rajasthan	Coop.	NWA
51	Central Semen Bank, Upper Shillong.	Meghalaya	Govt.	SLG
52	Alamadhi Semen, Station, Chennai.	Tamil Nadu	Trust	ALM
53	Rahuri Semen Station, Ahmednagar.	Maharashtra	Trust	RHR
54	Hisar Bovine Research	Haryana	Private	HBR

Breed Code formation

Sr. No.	SS Bull ID Breed Code formation
1.	Every Breed has its own two character code as Breed Code. For Exp.- Jersey=JY or Red Sindhi= RS etc. For Non-Descript breed use **ND** as breed code. Refer trailing table for breed code list.
2.	If there is no cross with the breed then that breed has 100% blood level of its own. For e.g. Breed code for 100% pure Jersey animal will be 'JY' . Therefore, SS Bull ID of a 100% pure jersey bull at ABC Semen Station will be 'ABC-JY-34'.
3.	For crossbred, having two or more than two type of breed involve, Breed code and SS Bull ID will be formed in the following order.
	(a) In case exotic breed is available then breed code of the exotic breed having maximum percentage will be preferred and concatenated with 'CB'. Eg. (1) a crossbred bull having HF(75%) and Sahiwal (25%) breed will have breed code 'HFCB' and SS Bull ID as 'ABC-HFCB-97'. Eg. (2) a crossbred bull having HF(25%) and Sahiwal(75%) breed will have breed code 'HFCB' and SS Bull ID as 'ABC-HFCB-90'. Eg (3) a crossbred bull having HF(50%) and Sahiwal(50%) breed will have breed code 'HFCB' and SS Bull ID as 'ABC-HFCB-971'. So the breed code in all the three cases will remain same as 'HFCB'
	(b) Incase, exotic breed is not available then breed code of the indigenous breed having maximum percentage will be considered and concatenated with 'CB'. For e.g. a crossbred bull having Sahiwal (50%), Red Sindhi(25%) and Gir(25%) breed will have breed code 'SHCB' and SS Bull ID as 'ABC-SHCB-97'.

Table: 5 **Breed codes**

Sr. No.	SpeciesName	BreedName	CODE
1	Buffalo	Bhadawari	BW
2	Buffalo	Jaffarabadi	JF
3	Buffalo	Marathwadi	MD
4	Buffalo	Mehsana	MH
5	Buffalo	Murrah	MR
6	Buffalo	Nagpuri	NP
7	Buffalo	Nili-Ravi	NR
8	Buffalo	Pandharpuri	PD
9	Buffalo	Surti	SU
10	Buffalo	Toda	TD
11	Buffalo	Banni	BN
12	Buffalo	Chilika	CK
13	Buffalo	Kalahandi	KD
14	Buffalo	Bargur	BG
15	Buffalo	Luit(Swamp)	LU
16	Cattle	HF	HF
17	Cattle	Jersey	JY
18	Cattle	Amritmahal	AM
19	Cattle	Bachaur	BC
20	Cattle	Bargur	BR
21	Cattle	Dangi	DN
22	Cattle	Deoni	DO
23	Cattle	Gaolao	GL
24	Cattle	Gir	GR

Sr. No.	SpeciesName	BreedName	CODE
25	Cattle	Hallikar	HK
26	Cattle	Hariana	HR
27	Cattle	Kangayam	KY
28	Cattle	Kankrej	KN
29	Cattle	Kenkantha	KK
30	Cattle	Kherigarh	KG
31	Cattle	Khillar	KH
32	Cattle	Krishna Valley	KV
33	Cattle	Malvi	MV
34	Cattle	Mewati	MW
35	Cattle	Nagori	NG
36	Cattle	Nimari	NM
37	Cattle	Ongole	OG
38	Cattle	Ponwar	PW
39	Cattle	Punganur	PG
40	Cattle	Rathi	RT
41	Cattle	Red Kandhari	RK
42	Cattle	Red Sindhi	RS
43	Cattle	Sahiwal	SH
44	Cattle	Siri	SR
45	Cattle	Tharparkar	TH
46	Cattle	Umblachery	UB
47	Cattle	Vechur	VC
48	Cattle	Motu	MO
49	Cattle	Ghumusari	GH

Sr. No.	SpeciesName	BreedName	CODE
50	Cattle	Binjharpuri	BH
51	Cattle	Khariar	KR
52	Cattle	Pulikulam	PU
53	Cattle	Kosali	KS
54	Cattle	Malnad Gidda	MG
55	Cattle	Belahi	BL
56	Cattle	Gangatiri	GT
57	Cattle	Badri	BD
58	Cattle	Ladakhi	LD
59	Cattle	Konkan Kapila	KP
60	Cattle	Lakhimi	LK

11 (H) Post thaw motility

After freezing, the semen straws shall be stored in a separate container. Post-thaw motility of semen should be examined at 24 hours (after freezing). Differences in observations shall be updated and recorded for the purpose of accepting a particular batch of semen doses. Whenever there is any doubt, post-thaw motility shall be examined by two experienced persons. Preferably, the person involved in evaluation of neat semen, shall not check the post thaw motility. For a minimum concentration of 20 million per dose, minimum acceptable post thaw motility shall be 50%. Semen doses below 50% motility shall be discarded. Semen samples with circular and Jerky movements and with any other abnormal motility shall be discarded.

11 (I) Quality Checks for frozen semen

The quality control measures are sub divided into mandatory and optional tests and include continuous monitoring of semen production at various steps/sub processes essential for quality semen production. It shall be mandatory to document quarterly summary of each test including the number of samples tested and the number of samples not meeting standard with the follow up action taken. The quarterly summary will be authenticated by the ***Veterinarian*** of the Semen Station.

Part-A **Mandatory Testing** **Part-B** **Optional Testing**

1.0 Quality Control of Sub Processes

2.0 Quality Control of Sub Products

3. 0 Quality Control of End Product

Part-A **Mandatory Testing**

1.0 <u>Quality Control of Sub Processes</u>

1.1 <u>Microbial load Testing</u>

Sr. No.	Sub Process	Name of test to be carried out	Frequency	Acceptance criteria/ Action` to be taken
1.1(a)	**Sterilization**	Microbial Load Testing of Glassware, Collection Tube, Dilution Bottles	Once a week	No growth is to be observed. Failed sample–rewash and sterilize the whole lot of Glassware again on the date of result assuming that the same error in sterilization process is continuing. Daily use of Sterilization indicator tape is recommended.
1.1(b)	**Dilution & Filling/ sealing LAFU**	Microbial load Testing	Once a week	Failed Test – Appearance of even a single CFU following incubation for 48 hrs. Action taken – calibration & maintenance check of LAFU.
1.1(c)	**AV**	Microbial Load Testing of AV Wash	Once a week	No growth is to be observed. Failed sample-rewash and sterilize the whole lot of AVs again on the date of result assuming that the same error in sterilization process is continuing. Daily use of Sterilization indicator tape is recommended.
1.1 (d)	**Microbial load**	Laboratory environment	Once a week	< 30 CFU

1.2 Calibration of Equipment:

Sr. No.	Activity	Frequency	Method
1.2(a)	**Calibration of Equipment**	Annual	Outsourced from NABL accredited lab/ OEM
1.2(b)	**Calibration of Photometer**	Six monthly	By the manufacturer

1.3 Validation Process :

Sr. No.	Activity	Frequency	Method	Acceptance criteria/ Action` to be taken
1.3 (a)	**Random checking of sperm concentration in straws**	Once at an interval of six months (20 samples - preferably all breeds).	As per SOP	Acceptance criteria-10 % variation (18-22 million) Dilution activity to be monitored & Calibration of photometer to be taken up with AMC/ OEM Provider.
1.3 (b)	**Validation of Photometer**	Six monthly	As per SOP	Acceptance criteria Variation of < 20% is accepted

2.0 Quality Control of Sub Products

Sr. No.	Sub Product	Test to be carried Out	Frequency	Acceptance criteria/Action` to be taken
2.1(a)	QC of chemicals	Whenever a new batch of chemical is introduced.	As per SOP	As per the advice of QCO
2.1(b)	**Diluter**	pH – testing	Daily Each lot of Buffer & diluter prepared.	Failed sample to be Discarded, recorded and details to be given in quarterly report. Acceptance criteria Buffer:6.7 to 7.1 Dilutor:6.7-6.9

3. Quality Control of End Product

3.1 Frozen Semen Straws:

Sr. No.	Name of test to be carried out	Frequency	Acceptance criteria/ Action` to be taken
3.1(a)	**Incubation Test**	Once in a quarter for all bulls- equally distributed in all the months in a quarter	Acceptance criteria as per the table below

Interpretation of Incubation test

Incubation– Time	Observation and Action taken
0 min (PTM)	Pass sample = >/= 50%
120 min	Pass sample = >/= 20% Failed sample to be discarded, recorded and details to be given in quarterly report

Sr. No.	Name of test to be carried out	Frequency	Method	Acceptance criteria/ Action` to be taken
3.1(b)	**Bacterial load Test**	Daily 3 bulls – (all bulls in a quarter-equally distributed in all the months in a quarter.)	As per SOP	Acceptance criteria. If all three samples have higher no. of CFU (>5000 CFU/ml) The entire batch to be retested. Failed samples to be discarded, recorded and details to be given in quarterly report.
3.1(c)	PIA	Once in a quarter for all bulls- equally distributed in all the months in a quarter	As per SOP	Acceptance criteria- Acrosome integrity (Neat semen) > 70 % Percent intact acrosome (FSD) > 65 %.
3.1(d)	HOS Test	Once at an interval of six months for all bulls	As per SOP	Acceptance criteria- HOST test cut off value > 40 %

3.2 Use of Equipments for Advance quality Check: (Wherever laboratory is equipped)

Sr. No.	Activity	Frequency	Method	Acceptance criteria/ Action` to be taken
3.3(a)	**CASA Evaluation of neat/ frozen semen**	Number of samples to be tested shall be decided by officer in charge/QCO	As per manufacturer's instructions / QCO may decide on the procedures	NIL
3.3(b)	**Evaluation by Flow cytometer**			

Part- B Optional testing:

Sr. No.	Activity	Frequency	Method	Acceptance criteria/ Action` to be taken
1. (a)	HOS-G TEST	Once at an interval of 6 months for all bulls	As per ANNEXURE-13	Criteria for High fertile bull semen Neat semen- 40% HPAP (HOST positive and Acrosome positive). Frozen semen-20% HPAP (HOST positive and Acrosome positive.)
1. (b)	Sperm Morphology	Once at an interval of six months for all bulls Young bulls-first six ejaculates	As per SOP	Acceptance criteria- Total abnormalities < 20% Head & Midpiece alone < 7%
1. (c)	Live and dead count	Once at an interval of six months for all bulls Young bulls-first six ejaculates	As per SOP	**>=70%**

11 (J) Information System

In order to facilitate the information system, all the bulls maintained by the semen station must be identified using unique 12 digit, bar coded system compatible with INAPH and each bull be given an alphanumeric identification by the respective SS as described in earlier section to enable on-line reporting and real time data capture of semen production and artificial insemination.

The semen stations shall use suitable software to record data pertaining to various activities and also should have online facility for the same. The software should be able to identify and trace the bulls and their ejaculates, production, storage and dispatch of semen etc.

a) Record for volume of semen, motility, sperm concentration, dilution rate, total extended volume, post-thaw motility (24 hrs after freezing), and total number of doses produced, etc. shall be maintained.

b) Miscellaneous information regarding actual reason(s) for not donating semen, undesired percentage of gross morphological defects, semen pH, presence of dirt, dust, blood, pus, etc. in semen samples shall be noted and recorded.

c) Details of semen supplied to various agencies, including post-thaw motility at the time of dispatch, shall be recorded.

d) Fertility data of bulls, conception rate, records of the progeny associated with any genetic defect, percent male / female born, etc. shall be noted and recorded.

e) Data/ record of microbiological examination of semen samples shall be maintained.

f) Record of all quality tests for neat and frozen semen samples shall be maintained.

11. (K) Semen Storage

To avoid accidental spread of diseases, the semen station shall follow the procedure of preserving semen doses for at least 30 days (quarantine) after production. Frozen semen doses produced at least 30 days prior to the date of dispatch should only be supplied for AI.

After checking post-thaw motility and found acceptable, frozen semen straws shall be stored in temporary storage container for 30 days. After temporary storage, the semen goblets shall be transferred to the bulk storage containers with proper recording of position in the canisters. After each dispatch, records redefining the position of remaining doses shall be updated.

Two reference samples of the doses dispatched should be drawn and retained for six months or a screen shot of randomly selected sample should be stored and a soft copy of which should be given to the customer.

The goblets containing the semen should be well identified and precaution should be taken to see that each goblet has sufficient space for liquid nitrogen. Mini straws need special care and should not be exposed above liquid nitrogen even for a short time (10 seconds) as they get warm faster and any exposure causes irreversible damage to sperm viability.

Liquid Nitrogen shall be replenished at regular intervals depending on the liquid nitrogen evaporation rate of the container.

12 Bio–security

The risk of disease spread has grown manifold with increasing number of bulls maintained at the semen production center. With the expected higher risk, implementation of strict bio–security measures at the semen stations assumes greater significance. Every semen station should have a well defined Bio–security protocol put in place across all its activities. There shall be a designated Bio–security Officer–Veterinarian at each Sperm Station. The premises should be demarcated into high, medium and low risk zones.

The detailed protocol for Bio-security circulated to all SS may be referred for strict compliance.

13 Cleaning and Sterilization

All the items to be washed shall be initially cleaned with running tap water and soaked in warm neutral detergent for at least 30 minutes. These items will then be thoroughly cleaned under running tap water using a brush. Filling nozzles shall be cleaned with pressure using 20 ml syringe. These materials shall be rinsed thoroughly with de-ionized/ distilled water (3 changes) to completely remove detergent residues and other impurities. Appropriate procedure for sterilization of different materials, recommended for use in the semen station follows.

13.1 Laboratory and other areas

Cold fumigation solution is ideal for fumigation of laboratory and other areas. It should be done as per SOP/ manufactures guidelines.

13.2 Artificial Vagina (AV)

a) Cone from the AV and water from AV jacket shall be removed before washing.

b) Cones and AVs shall be cleaned thoroughly with a soft sponge brush under running tap water and then soaked in warm neutral detergent for about 30 minutes, followed by proper rinsing in warm and clean water and then three times rinsing with double distilled water.

c) For sterilization, fully assembled AVs shall be autoclaved at 5 p.s.i. pressure for 20 minutes. During sterilization, the valve of AV shall be kept open. Alternatively, use AV sterilizer (using double distilled water in the sterilizer) for proper sterilization of AVs.

d) Finally, AVs filled with water and covered with aluminium foil, at both ends shall be stored overnight in an incubator at 45^{o} C.

e) To achieve best cleaning effect, AVs shall be cleaned immediately after use, preferably by non-spermicidal neutral detergent.

13.3 Glassware

a) The glassware shall be washed thoroughly with running tap water and soaked in warm, non-spermicidal neutral detergent solution for about 30 minutes.

b) Using appropriate nylon brush, the glassware shall be cleaned and rinsed with running tap water. The collection tubes shall be brushed at least 3 times and thoroughly cleaned and rinsed with distilled water.

c) Finally the glassware shall be rinsed three times with double distilled water and allowed to dry by keeping them inverted on a blotting paper or a drying stand made of SS/ plastic.

d) The open end/s of the dried glassware shall be covered with aluminium foil and sterilized in hot air oven at 160°C (holding) for one hour or at 180°C for 30 minutes. One item should be wrapped with newspaper and its mild charring will indicate proper sterilization.

13.4 Rubber wares

The washing and cleaning procedure of rubber wares is similar to that of glass ware. Care shall be taken to clean the rubber wares with sponge brush instead of nylon brush. Plastic tips shall be cleaned by water jet with force using a syringe. Sterilization technique, however, differs owing to the thermo-sensitivity of the rubber items. Thermo-resistant rubber wares shall be sterilized by autoclaving at 3 - 4 p.s.i. for 10 minutes. (**The rubber tubing for semen filling shall not be reused).**

13.5 Distilled Water

Fresh triple glass distilled water or Ultra pure water shall be autoclaved at 15 p.s.i. for 15 minutes and used for preparation of the dilutor.

13.6 Buffer

Buffer shall be sterilized by microfiltration on 0,2 µm membrane filter.(optional)

13.7 Bacteriological Media

It is to be autoclaved at 15 p.s.i. pressure for 15 minutes.

13.8 Filter Papers

A bunch of clean filter papers of standard brand - No. 1 (thrashed to remove dirt, if any) shall be wrapped in thick cotton cloth for sterilization in an autoclave at 5 p.s.i. pressure for 20 minutes. Alternately, these can be sterilized dry in suitably sized petri dishes in hot air oven at 180 degree Centigrade for 30 minutes.

14 Summary of Sterilization

Table: 6 Autoclave

Sr. No.	Item	Pressure (p.s.i.)	Time (Min.)
1.	Artificial Vagina	5	20
2.	Plastic Tips	5	20
3.	Filter Papers	5	20
4.	Bull Apron	5	20
5.	Thermo-resistant Rubber Wares	3-4	10
6.	Bacteriological Media	15	15
7.	Distilled Water	15	15
8.	Surgical Equipment	10	10
(The rubber wares can withstand above pressure and duration, provided the quality is good)			

Table: 7 Hot Air Oven

Sr. No.	Item	Temperature	Time (min.)
1.	Glass wares	160° C / 180° C	60/30
2.	Filling Nozzles	160° C / 180° C	60/30

c) AV Steriliser

Wherever Autoclave is not used, AVs and rubber cones shall be sterilised using AV sterilizer. After sterilizer starts boiling, 30 minutes vapour sterilisation shall be do

d) Validation of Sterilization

To ensure that potentially infectious agents are destroyed; the efficacy of sterilization regimes may be to validation through -Physical, Chemical or Biological tests, if required.

15 Quality Control of Consumables

Chemicals

The chemicals of only highest purity of either, Analytical Reagent **(AR)** or Guaranteed Reagent **(GR),** from reputed manufacturing companies shall be used. Whenever a new chemical is to be introduced in the routine process, it is recommended to examine the post-thaw revival rates after conducting a few spilt ejaculate trials (maintaining a control) with the new chemical. Assay of chemicals shall be >99%, having less impurities.

Straws

1. Straws manufactured by highly reputed companies are safer to use for production of quality semen. While buying straws, package volume and microbial load in straws shall be checked randomly from the consignment. In addition, some empty straws should be placed in filling and sealing machine and the machine should be run to see the sealing quality of the straws. In case of any foul smell, it should be presumed that the straws are manufactured from poor plastic which could be toxic to the spermatozoa and can even result in reduced motility on long storage.

2. The factory plug should not be loose. The factory seal should be impenetrable and the seal formed should be homogeneous and compact.

3. The straws should be intact (without cracks / dents, etc.) and should not get damaged during filling /sealing and after freezing / thawing.

4. The movement of straws along the printing machine should be free and print should be clear and sharp. Print should not fade as a result of freezing and subsequent thawing.

5. The use of dark coloured straws should be avoided, as they are not transparent enough. Due to this, it is difficult to distinguish between filled / semi-filled straws.

6. Movement of the factory plug should be free.

7. Straws should be routinely checked for microbial load.

Note**:** The semen stations should avoid purchase of consumables/ straws simply **on lowest quotation basis**. The quality should never be compromised in view of the stakes involved. Only time-tested products may be used keeping in view the storage for a very long period. For example: To produce top quality semen, it is better to use AR / GR reagents manufactured by reputed companies (for eg- Sigma, Merck, Himedia etc) whose products are reliable and time- tested. This is true with other consumables used for semen production as well.

16 Manpower Requirement for semen production

Table: 8

Designation	Up to 10 Lakh Doses	>10-25 Lakh Doses	>25-50 Lakh Doses	>50 Lakh Doses	Mega Semen Station >10m Doses
Officer In-charge	1	1	1	1	
QCO/QAO	1	1	1	1	1
Vet. Officer	1	2	3	3-4	5-6
Biosecurity Officer-Vet	1	1	1	1	1
Agriculture Officer	1	1	1	1	1
Data Mgmt. Officer	--	1	1	1	1
Accts. & Adm. Officer	--	1	1	1-2	1-2
Office Assistant	1	2	3	5	6-7
Livestock Assistant	1	2	3	4	5
Agri. Assistant					
Lab Technician	1	2	3-4	5-6	8-10
Lab Technician(QC)	1	1	1	1	1
Vehicle/Tractor Driver	1	2	3	4	5
Lab Attendant	2	3	3-5	7-8	10-12
Bull Attendant	1 person per 7- 8 bulls				
Agri. Labourers	15-20/100 acres depending on mechanization level				

The manpower structure suggested above is meant only for semen/fodder production. For other activities, manpower may be positioned as per the need. For dispatch of semen, facility should be created preferably away from semen station and operated by person/s not involved in semen production. The GOI / Department of AH / Livestock Boards / NGO / Private agencies / Cooperatives owning the semen station shall review the requirement of manpower position for each semen station and finalize the staff structure for recruiting additional manpower. After recruitment, all new persons shall be trained at any of the recognized institutes like Central frozen semen production and training institute, Hessarghatta, Bengaluru; Sabarmati ashram Gaushala, Bidaj and Kerala livestock development board, Matupatty. Once trained, they shall continue to work in the semen station at least for five years.

It is **recommended** that Semen Station In-charge should have at least 05 years experience in bovine semen production

Refresher training / visit to other semen lab: technical exposure of semen station personnel working in the semen lab must be arranged compulsorily once in two to three years at reputed institutions like CFSP&TI - Hessarghatta, KLDB - Mattupatty, and Sabarmati ashram Gaushala, Bidaj. As semen production activity is a highly professional/technical work, job rotation of personnel could be detrimental in maintaining the quality of semen. Therefore, personnel working in a semen station should not be transferred at least for five years. If it is inevitable, a proper replacement should be identified at least six months in advance and shall be trained in semen production technology.

Annexure- 1

DEFINITIONS FOR USE IN THE HEALTH PROTOCOL

Bull	Adult male cattle or buffalo used for collection of semen. Teasers and other animals resident in the semen stations are also subjected to similar disease testing, vaccination and medications for maintaining their health status.
Bull Calf	A male cattle or buffalo which has not yet reached sexual maturity.
Known health Status	Animals originating from a semen station or rearing station that is strictly complying with the guidelines mentioned in the MSP.
MSP diseases	MSP diseases are the set of diseases – the causative organism of which should not be present in the semen – or preferably in the bull. These diseases include Bovine Brucellosis, Tuberculosis (TB), Paratuberculosis (JD), Bovine Genital Campylobacteriosis, Trichomoniasis and Foot and Mouth Disease (FMD) and Infectious Bovine Rhinotracheitis (IBR).
Quarantine station	A farm where bulls or bull calves are isolated and examined to assess the health status before shifting to the semen station or rearing station. A series of clinical and laboratory examinations, vaccinations and medications etc. are undertaken during quarantine.
Rearing station	A farm where bull-calves or young bulls, coming from quarantine station are reared till they attain sexual maturity and subsequently get shifted to semen station. A series of clinical and laboratory examinations, vaccinations and medications etc. are undertaken during the stay of bull calves in the rearing station to maintain their health status.
Semen station	A farm along with semen processing facilities where adult bulls are housed for semen collection and processing. A series of clinical and laboratory examinations, vaccinations and medications etc. are undertaken during the stay of bulls in the semen station to maintain their health status.
Unknown health status	Animals originating from village or farm where all the animals of the farm or the village have not been tested against the MSP diseases

Annexure- 2

Details of the tests to be conducted

Disease	**Test**	**Sample**	**Tested by officers of**
Brucellosis	ELISA	Serum	CDDL/RDDL/ NDDB/ State Veterinary Universities
TB*	DTH- Tuberculin PPD	Intra-dermal on the bull	Semen Station/ CDDL/RDDL/ NDDB/ State Veterinary Universities
JD*	DTH- Johnin PPD	Intra-dermal on the bull	Semen Station/ CDDL/RDDL/ NDDB / State Veterinary Universities
Trichomoniasis	Agent identification	Preputial washings / semen	CDDL/RDDL/ NDDB/ State Veterinary Universities
Bovine Genital Campylobacteriosis	Agent identification	Preputial washings	CDDL/RDDL/ NDDB/ State Veterinary Universities
FMD	ELISA	Serum	PD-FMD, Mukteshwar and its laboratories/ NDDB /State Veterinary Universities
IBR	ELISA Real time- PCR	Serum for ELISA (9 months age) Semen for RT-PCR	CDDL/RDDL/ NDDB/ State Veterinary Universities
BVD	ELISA 2 times at 30 days interval (RT-PCR up to 6 months age)	Serum	CDDL/RDDL/ NDDB/ State Veterinary Universities

*** TB and JD testing at Quarantine Station as well as Rearing Station shall be performed by the officers of the Semen Station. However, the testing at the Semen Station shall be done by the Officers of the CDDL/RDDL/NDDB/** NABL accredited **State Veterinary Universities and approved by CMU**

Annexure- 3A

Quarantine Guidelines

A. Quarantine of adult bulls of unknown health status		
Quarantine period	Minimum 60 days or long enough to allow at least two tests for MSP diseases to be performed during quarantine with a minimum interval of 30 days between the two tests. In case of TB and JD the interval between the two tests should not be less than 62 days.	
Shifting of bulls from the quarantine	Within 30 days from the date when the last test was performed and all bulls were found negative.	
Action on finding a positive result	Brucellosis, TB, JD, Bovine Genital Campylobacteriosis, Trichomoniasis, IBR, BVD (PI)	Cull / remove the positive bull and put all the remaining bulls under extended quarantine.
Extended quarantine	For a period of minimum 60 days or long enough to allow at least two tests for the diseases mentioned above to be performed, from the day last positive bull was culled/ removed. Perform one test within the last 30 days of the extended quarantine.	
Action on finding a positive during extended quarantine	During Quarantine, if the bulls are housed and managed Individually - Remove only the positive bull. In groups (not more than 3 animals in each group) – Remove all bulls in the group in which positive was detected. Free and not in groups- Remove all the bulls.	

B. Quarantine of adult bulls of known health status	
Quarantine period	Minimum 30 days or long enough to allow at least one test for all MSP diseases
Shifting of bulls from the quarantine	Within 30 days of the last negative test
Action on finding a positive result	Same as in Annex- 3A
Extended quarantine	For a period of minimum 30 days from the day last positive bull was culled/ removed. Perform one test within the last 30 days of the extended quarantine.
Action on finding a positive during extended quarantine	Same as in Annex- 3A

C. Quarantine of adult bulls to be shifted between the farms managed by the same administration ■ For shifting between semen stations for semen production ■ From a rearing station that implements Quarantine (Annexure-3D) before allowing entry of calves for rearing	
Quarantine period	Minimum 30 days or sufficient to allow at least one test for MSP diseases
Shifting of bulls from the quarantine	Within 30 days of the last negative test
Action on finding a positive result	Same as in Annexure- 3A
Extended quarantine	For a period of 30 days from the day last positive bull was culled/ removed. Perform one test within the last 30 days of the extended quarantine.
Action on finding a positive during extended quarantine	Same as in Annexure- 3A

<table>
<tr><td colspan="3">D. Quarantine of calves above 2 months of age</td></tr>
<tr><td>Quarantine period</td><td colspan="2">Minimum 60 days or sufficient to allow at least two tests for each of the MSP diseases to be performed with a minimum interval of 30 days between the tests. In case of TB and JD the interval between the two tests should not be less than 62 days.</td></tr>
<tr><td>Shifting of calves from quarantine</td><td colspan="2">Within 30 days of negative results.</td></tr>
<tr><td rowspan="3">Action taken on finding positive calf</td><td>TB, JD</td><td>Remove the positive calf and put all the remaining calves under extended quarantine.</td></tr>
<tr><td>Bovine Genital Campylobacteriosis and Trichomoniasis</td><td>Tests conducted only on calves older than 6 months.
Remove the positive calf and put all the remaining calves under extended quarantine.</td></tr>
<tr><td>Brucellosis, IBR,
BVD (PI)</td><td>Remove the positive calf irrespective of age and put all the remaining calves under extended quarantine.
OR
For Brucellosis and IBR: If the positive calf is less than 9 months old, isolate the calf till it is 9 month old and retest.
Calf positive at retesting should be removed.</td></tr>
<tr><td>Extended quarantine</td><td colspan="2">For a period of minimum 60 days from the day last positive calf was removed. Perform one test within the last 30 days of the extended quarantine</td></tr>
<tr><td>Action on finding a positive during extended quarantine</td><td colspan="2">Same as in Annexure- 3A</td></tr>
</table>

Annexure- 4

Disease testing and management of Bovine Tuberculosis in Semen Station

Name of test	Delayed Hypersensitivity – Single Intra Dermal (SID) Test
Reagent used	Bovine tuberculin PPD
Manufacturer	IVRI, Izatnagar
Testing done	On site, where animals are housed
Result criteria	***Positive:*** Increase in skin thickness of 4 mm or more, or presence of clinical signs viz. exudation, necrosis, pain, and inflammation of the lymphatic duct of that region or the lymph node, 72 hours post-inoculation. ***Negative***: Increase in skin thickness less than 2 mm & without clinical signs viz. exudation, necrosis, pain, inflammation of the lymphatic duct of that region or the lymph node, 72 hours post- inoculation. ***Inconclusive***: Increase in skin thickness more than 2mm & less than 4mm, absence of above clinical signs, 72 hours post-inoculation. Bull with inconclusive result should be immediately isolated. Only if the animal is negative during the testing in isolation, it should be brought back to the semen station.
Eligible animals	Animals above 2 months of age.
Action to be taken on Positive animal	Immediate isolation and removal from herd (within 2 days).
Frozen semen doses of the positive animal	Destroy frozen semen doses of the positive animal since the last negative test.
Positive herd testing	Testing not before 42 days after culling of last positive animal.
Negative herd testing	Six monthly (± 1 week) testing after last whole herd negative testing.
TB free herd	Herd found negative on two consecutive tuberculin tests carried out at an interval of 6 months, the first being performed 6 months after the culling of last affected animal. If frequency of testing is more than two in a year, the testing should establish that all animals in the herd have been negative for the last 6 months beginning from 6 months after culling the last affected animal.

Annexure- 5

Disease testing and management of Para tuberculosis (JD) in Semen Station

Name of test	Delayed Hypersensitivity – Single Intra Dermal (SID) Test
Reagent used	Johnin PPD
Manufacturer	IVRI, Izatnagar
Testing done	On site, where animals are housed
Result criteria	***Positive:*** Increase in skin thickness of 4 mm or more, or presence of clinical signs viz. exudation, necrosis, pain, and inflammation of the lymphatic duct of that region or the lymph node, 72 hours post-inoculation. ***Negative***: Increase in skin thickness less than 2 mm & without clinical signs viz. exudation, necrosis, pain, inflammation of the lymphatic duct of that region or the lymph node, 72 hours post- inoculation. ***Inconclusive***: Increase in skin thickness more than 2mm & less than 4mm, absence of above clinical signs, 72 hours post-inoculation. Bull with inconclusive result should be immediately isolated. Only if the animal is negative during the testing in isolation, it should be brought back to the semen station.
Eligible animals	Animals above 2 months of age
Action to be taken on Positive animal	Immediate isolation and removal from herd (within 2 days)
Frozen semen doses of the positive animal	Destroy frozen semen doses of the positive animal since the last negative test.
Positive herd testing	Testing not before 42 days after culling of last positive animal.
Negative herd testing	Six monthly (± 1 week) testing after last whole herd negative testing.
JD negative herd	Herd found negative on two consecutive Johnin tests carried out at an interval of 6 months, the first being performed 6 months after culling of the last affected animal. If frequency of testing is more than 2 in a year, the testing should establish that all animals in the herd have been negative for the last 6 months beginning from 6 months after culling the last affected animal.

Annexure- 6

Disease testing and management of Bovine Brucellosis in Semen Station

Name of test	Enzyme Linked Immunosorbent Assay (ELISA)
Sample required	Serum
Eligible animals	All animals. However, animals up to 9 months of age may have maternal antibodies.
Action to be taken on the positive animal	Immediate isolation and removal from herd after castration (within 2 days)
Frozen semen doses of the positive animal	Destroy frozen semen doses of the positive animal since the last negative test.
Positive herd testing	Testing 30 to 60 days after culling of last positive animal.
Negative herd testing	Six monthly (± 1 week) testing after last whole herd negative testing.
Brucellosis free herd	Herd found negative on two consecutive annual tests. If the frequency of testing is more than one in a year, the testing should demonstrate that the herd has been negative for the last one year

Annexure- 7

Disease testing and management of Bovine Genital Campylobacteriosis (BGC) in Semen Station

Name of test	Agent –Identification
Sample required	Preputial washing/ semen
Eligible animals	Animals above 24 months of age (when there is free penile movement)
Positive animal	Immediate isolation and removal from herd (within 2 days)
Frozen semen doses of the positive animal	Destroy frozen semen doses of the positive animal since the last negative test.
Positive herd testing	Minimum of 30 days after treatment/culling of last positive animal.
Negative herd testing	Annual (± 1 week) testing after last whole herd negative testing.
Bovine Genital Campylobacterio sis free herd	All animals are negative on two consecutive annual testing.

Annexure- 8

Disease testing and management of Bovine Trichomonosis in Semen Station

Name of test	Agent –Identification
Sample required	Preputial washing
Eligible animals	Animals above 24 months of age (when there is free penile movement)
Action to be taken on Positive animal	Immediate isolation and removal from herd (within 2 days)
Frozen semen doses of the positive animal	Destroy frozen semen doses of the positive animal since last negative test.
Positive herd testing	Minimum of 30 days after treatment/culling of last positive animal.
Negative herd testing	Annual (± 1 week) testing after last whole herd negative testing.
Bovine Trichomonias is free herd	All animals are negative on two consecutive annual testing.

Annexure- 9

Testing and management of Infectious Bovine Rhinotracheitis (IBR) at semen stations

Name of the test	Enzyme Linked Immuno absorbent Assay (ELISA), Real-time PCR
Sample (s) required	Serum for ELISA, semen for real-time PCR
Induction of new animals into herd/ semen stations	Only negative animals will be inducted. All the animals to be inducted irrespective of their age should be put on hold and inducted only if found test negative after the age of 9 months.
Sero positive bulls at IBR positive semen station	Action in order of priority:- Immediately cull sero-positive animals and castrate them. If culling is not possible, immediately isolate the animal and process and store their semen separately. Test each ejaculate by real-time PCR (rt-PCR). Semen positive by real-time PCR shall be destroyed by incineration. Use only semen that has tested negative by RT-PCR.) Test all the animals at three months interval.
Action to be taken on bulls at the IBR free Semen Stations**	All positive bulls culled immediately. Retest remaining bulls at 30 - 60 days after culling last positive animals. Repeat (i) & (ii) until the remaining herd is tested negative. Thereafter test at 6 monthly interval.) The negative herd should be tested at 6 monthly interval.
Documentation	Records of all the ejaculates collected from sero- positive bulls, the results of real-time PCR, details of real time PCR positive ejaculates destroyed and details of agencies where semen has been distributed shall be maintained.

**Please refer to the Guidelines for progressive IBR/ BVD control issued by DAHD, Government of India and as amended from time-to-time.

Annexure-10

Testing and management of Bovine Viral Diarrhoea (BVD) at semen stations

Name of the test	Enzyme Linked Immunosorbent Assay (ELISA) for detection of antigen (Ag-ELISA)/real time PCR (RT–PCR).
Sample	Serum
Induction of new animals into herd/semen stations	Test the animal for Persistent Infection (PI) by testing two times at an interval of at least 30 days by Ag- ELISA. Test by rt-PCR instead of Ag-ELISA for animals up to 6 months of age. If the animal is positive on both the tests, the animal is considered positive for PI. Only PI negative animals shall be inducted.
Action to be taken for PI positive animals	Immediately isolate and cull
Semen doses of PI positive animals	Destroy by incineration of frozen semen doses of the PI positive bulls.
Bulls at the semen stations	(i) Test all the bulls for PI (if not already tested for PI status) by testing two times at an interval of at least 30 days. If the bull is found positive on both the tests, then the bull is considered positive for PI. All PI positive bulls shall be culled immediately. (ii)Test all new bulls entering the semen station for PI. Only PI negative bulls should enter the semen station.

** Please refer to the Guidelines for progressive IBR/ BVD control issued by DAHD, Government of India and as amended from time-to-time.

Annexure-11

Management of Foot & Mouth Disease (FMD) in Semen Station

FMD outbreak in Semen Station	
Immediate action to be taken	Immediate disinfection of premises and fomites. Destruction of contaminated feed & fodder by burning.
Frozen semen doses of FMD infected animal	Destroy frozen semen collected from infected animal up to one month prior to onset of outbreak.
Action to be taken on FMD infected animal	Isolate the affected bull immediately Affected bull is treated and rested for 90 days after recovery from clinical symptoms. No semen collection from any infected animal during the infection and up to 3 months after last case has recovered in the farm.
Animals in the farm not affected by FMD	No semen collection from healthy bulls during the outbreak and no semen collection up to one month after the last case has recovered.
Semen Sale	If frozen semen sale is from the same campus of the SS where FMD is recorded, suspend semen sale till 30 days after the last case has recovered.
FMD outbreak in areas surrounding the SS	
Ring vaccination	Arrange immediate ring vaccination within a radius of 10 Km around the focus of infection starting from the perimeter towards the focus.
Disinfection	Disinfection of the roadsides adjacent to the farm on a daily basis.
Movement of fodder	Stop all fodder movement through areas of infection.
Animal movement	Stop animal movement of semen station through areas of infection.

Annexure - 12

Feeding Growing and Mature Bulls

Daily nutrient requirements of growing and mature bulls *

Body wt	gain/day	DM/day	C.P.	TDN	Ca (g)	P (g)	Vit. A
(kg)	(g)	(kg)	(g)	(kg)	(g)	(g)	(1000 IU)
Growing bulls							
100	750	2.8	390	1.9	11	8	4
150	750	4.3	460	2.7	15	11	6
200	750	5.7	530	3.4	18	14	8
250	750	7	610	4	21	16	10
300	750	8.2	680	4.6	23	17	13
350	750	9.3	760	5.2	24	18	15
400	700	10.2	820	5.7	25	19	17
450	600	10.4	875	5.8	26	20	19
500	400	10	885	5.6	26	20	21
550	250	10	845	5.6	25	19	23
600	100	9.8	800	5.5	24	18	26
Maintenance of mature breeding bulls							
500	-	8.3	640	4.6	20	15	21
600	-	9.6	735	5.4	22	17	26
700	-	10.9	830	6.1	25	19	30

Daily ration for Bulls

Body wt. (kg)		Calf starter (kg)	C.F. (kg)	B.P.F. (kg)	Hay (kg)	Green Fodder (kg)
Growing bulls						
100		2	-	-	0.5	6-8
150		-	-	2	0	8-10
200		-	-	2	0.5	15
300		-		2	1	ad lib.
400	a)	-		2	3	ad lib.
	b)	-	2.5	-	3	ad lib.
500	a)	-	-	2.5	2-4	ad lib.
	b)	-	3	-	2-4	ad lib.
600	a)	-	-	2.5	2-4	ad lib.
	b)	-	3	-	2-4	ad lib.
Mature breeding bulls						
500	a)	-	2.5	-	2-4	ad lib.
	b)	-	-	2	2-4	ad lib.
600		----------do----------				
700		----------do----------				

Note: **1) Mineral mixture should be supplemented as follows:**

- 50 g mineral mixture for bulls up to 200 kg body weight
- 70 g mineral mixture for bulls between 200 to 350 kg body weight.
- 100 g mineral mixture for bulls above 350 kg body weight

2) Fresh water should be made available 24 hrs.

Green fodder requirement of 10 mature bulls would be approx. 125 MT per year, which can be grown in 1 hectare of land by intensive farming.

*** Source:** Ranjhan, S.K (1980). Animal nutrition & feeding practices in India, 2nd Ed., p196-212

Nutrients available in feed & fodder

	Calf starter	**C.F.**	**B.P.F.**	**Green fodder**	**Hay**
DM %	90	90	90	20-25	90
CP %	22-23	18-19	22-23	5-6	5-6
TDN %	70	62-64	65-68	55-60	55

Annexure: 13

Hypoosmotic swelling-Giemsa test (HOS-G Test)

Importance:

Hypoosmotic swelling-Giemsa (HOS-G) test evaluates the sperm subpopulations positive for both functional membrane integrity and acrosome integrity in an individual spermatozoon. This test is based on the principle that when spermatozoon is subjected to hypo-osmotic environment, the sperm with functionally intact plasma membrane swell causing the flexible motor apparatus of the tail to form a hairpin bend. Additionally, staining of these semen samples with Giemsa stain reveals acrosome intactness in the sperm subjected to HOS test. To achieve fertilization, the sperm have to swim through the female reproductive tract and reach the ovum at oviduct for which plasma membrane intactness is necessary. Subsequently, for penetration through zona pellucida, acrosome intactness is of paramount importance. Thus, this test assesses the most vital sperm attributes such as functional membrane integrity and acrosomal integrity, that are reflective of sperm fertilizing ability.

Reagents required

a. 300 mOsm media

Trisodium citrate	-1.47g
Fructose	-2.7g
Distilled water (make upto)	-100ml

b. 150 mOsm media

Trisodium citrate	- 0.73g
Fructose	- 1.35g
Distilled water (make upto)	-100ml

c. 100 mOsm media

Trisodium citrate	- 0.49g
Fructose	- 0.99g
Distilled water (make upto)	-100ml

d. Buffered formal saline (BFS)

Disodium hydrogen phosphate	- 6.194g
Potassium dihydrogen phosphate	- 2.543g
Sodium chloride	- 5.406g
Formalin (35%)	- 125ml
Distilled water (make upto)	-1000ml

e. Giemsa stain

Preparation of Stock: 1g of Giemsa in 60ml of glycerol + 66 ml of absolute methanol as described in Chapter 7.

Working standard: 3ml of stock Giemsa stain + 2ml of BFS + 45 ml distilled water.

Methodology:

1. Maintain all reagents at 37°C before use.
2. Add 450µL of 300 mOsm and 150 mOsm for cattle or 100 mOsm for buffalo media each in two different 1.5 ml microcentrifuge tubes and incubate at 37°C.
3. To each tube, add 50µL of the semen sample and incubate at 37°C for 30 min.
4. After 30 min of incubation, make a smear from both the media on two different clean glass slides and dry at room temperature for 15 min.
5. Incubate the slides in BFS for 30 min.
6. Wash the slides in running water for at least 2 min to remove the excess fixative on the slides to stain the acrosome clearly.
7. Dry the slides at room temperature.
8. Incubate the slides in Giemsa working standard solution overnight.
9. Wash the slides gently and observe the sperm tail structure and acrosome intactness under phase contrast microscope.

Observation and calculation:

A minimum of 200 sperm should be counted in a phase contrast microscope either at high-power (40x) or oil immersion (100x). Sperm may display different types of swelling (coiling of tail), but only sperm with hairpin bend in the principal piece is considered as HOS positive. The sperm with evenly distributed Giemsa stain (pink colour) in the acrosomal region is considered as acrosome positive.

During the observation, the cells are classified into four subpopulations

- HOS positive and acrosome positive (HPAP)
- HOS positive and acrosome negative (HPAN)
- HOS negative and acrosome positive (HNAP)
- HOS negative and acrosome negative (HNAN)

Functional membrane integrity (HOS positive):

The HPAP and HPAN subpopulations were added together to calculate HOS positive sperm.

The percentage of sperm with hairpin bend tail in the control (300 mOsm) was subtracted from the percentage of sperm reactive to the HOS (150 mOsm/100mOsm) to obtain the actual proportion of sperm positive for HOS test.

HP = HPAP+HPAN (150 mOsm/100 mOsm) - HPAP+HPAN (300 mOsm)

Acrosomal Integrity:

The HPAP and HNAP subpopulations were added together to calculate acrosome positive sperm.

AP = HPAP (300 mOsm) + HNAP (300 mOsm)

Sperm subpopulation positive for functional membrane integrity and acrosomal integrity:

The percentage of HPAP sperm in the control (300 mOsm) was subtracted from the percentage of HPAP sperm (150 mOsm/100 mOsm) to obtain the actual proportion of HPAP sperm.

HPAP = HPAP (150 mOsm/100 mOsm) - HPAP (300 mOsm)

Interpretation:

The high fertile neat semen should have at least, 40% HPAP positive, 60% HOS positive and 85% intact acrosome.The high fertile frozen thawed bull semen should have at least, 20% HPAP positive, 40% HOS positive and 65% intact acrosome.