SEMEN DILUTION

The main aim of semen dilution is to extension of the volume and creation of optimal media for living of the sperm.

Preparation of the semen diluent:

1- Egg yolk diluent:

1- **Egg yolk sodium citrate:**

The buffer in this diluent constitutes form 2.9% sodium citrate dihydrate. It is prepared by dissolving 2.9 gm of sodium citrate dihydrate in 100 ml glass bidistilled water in glass beaker. The solution should be filtrated and stored in a water bath at 25-37°C.

The egg yolk is prepared from fresh non-fertilized egg collected from a herd free from salmonella. You have to select eggs with normal shape, normal size, free from any cracks. The egg must be cleaned by water and detergent, then swabbed with 70% alcohol and dried in air. The egg shell is opened at the middle line. The egg white is removed by transferring the egg white and yolk from half shell to another. Traces of egg white must be removed by transporting the egg yolk in a filter paper till complete dryness. The egg yolk membrane is penetrated by sterile needle or pipette and begins to suck the egg yolk and transfer it to clean measuring cylinder without the shell membrane. The egg yolk should be homogenized by homogenizer or roaring by glass rode to obtain a smooth emulsion.

Before preparing the final diluent, the egg yolk and sodium citrate must be kept in the same water path to acquire the same temperature to avoid salting out of the buffer during mixing both ingredients with each other.

The egg yolk is added to sodium citrate with proportion of 10-25 %. The mixture is filtrated within the water bath. The antibiotics are added at the recommended concentration.

If the diluent will be used for preservation of frozen semen, cryoprotectant (glycerol) will be added.

2- **Egg yolk sodium phosphate:**

The buffer in this diluent constitute from 2.09 gm sodium phosphate, 0.2 gm potassium phosphate dissolved in 100 ml glass bidistilled water. The egg yolk is prepared and added to this buffer by the same method mentioned in the previous diluent.

The egg yolk sodium citrate is more preferable than egg yolk sodium phosphate because sodium citrate has a strong chelating activity so it chelate the heavy metal as Ca, Mg preventing the sperm agglutination. The sodium citrate disperses fat and protein globules in egg yolk so egg yolk sodium citrate is clear. This make microscopical examination of the semen samples diluted with egg yolk sodium citrate easier than those diluted with egg yolk sodium phosphate.
II- Milk based diluent:

1- Whole homogenized, skimmed homogenized milk diluent:

The milk must be heated up to 90-92°C for 10 minutes by direct heat method or double heater. Heating of milk aims to destroy the lactenin which has spermicidal action and to liberate sulfahydreal group which have antioxidant activity. Boiling of the milk should be avoided because it will destroy nutrients present in the milk. The milk should be cooled then homogenized by using homogenizer.

The milk can be centrifuged at 800 rpm for 30 minutes before use to remove fat globules which hinder the microscopical examination.

The egg yolk, glycerol and antibiotic should be added at the optimum concentration.

2- Dried skimmed milk powder:

The milk diluent can be reconstituted from the dried milk powder by dissolving 10 gm of milk powder in 100 ml glass bidistilled water. Heat the milk at 90°C for 10 minutes.

NB: Addition of 12.5% of egg yolk to milk diluent found to improve the fertility of the diluted semen. Addition of the egg yolk to milk-based extenders seemed to inactivate the toxic factor in milk.

III- Tris based diluent (Tris-egg yolk diluent):

Tris (hydroxymethyl amniomethan) has been extensively used as buffer medium in semen diluent of different species. The buffer has been prepared in various moraility and pH. A 0.2 mM concentration and pH of 6.5 plus 1% fructose give best result.

To prepare the buffer add 3.028 gm tris and 1 gm fructose in 200 ml beaker and added 75 ml of glass bidistilled water. Adjust the pH to 6.5 with 10% citric acid; complete the volume to 100 ml.

An alternative tris buffer can be made by mixing 2.42 gm of tris, 1.38 gm of citric acid monohydrate and 1 gm fructose with sufficient distilled water to make 100 ml. The egg yolk should be added to a proportion up to 20%

IV- Commercial available patent diluent:

Laiciphos 271: It is a skim milk-based powder medium (50 gm) supplied with antibiotic fractions, requiring addition of water.
SEMEN PRESERVATION

The main aim of semen preservation is to prolong the life span of spermatozoa without reducing its fertilizing capacity.

Methods of semen preservation:

I- Preservation at room temperature:
This method depends on the reduction of metabolic activity by lowering the pH of the semen by incorporating CO$_2$ into a suitable diluent and stored the diluted semen in sealed ampules.

Method:
The most common diluent used for preservation of room temperature is the Illini Variable Temperature diluent (IVT). The diluent is saturated with CO$_2$ by bubbling the gas through it for ten minutes (or until the pH was reduced to about 6.35). The antibiotic and egg yolk is added at optimum concentration. Each 1 ml of the diluted semen should be sealed in glass ampules and stored in the dark at room temperatures (18°C to 24°C). Before using the metabolic activity of the sperm cells is stimulated by elevating the pH or increasing the temperature.

This method can successfully preserve the motility and fertility of bovine semen for several days but semen not used after 72 hrs. due to aging of the sperm (detachment of acrosome).

II- Preservation at below body temperature (liquid or chilled semen):
In this method, decreasing the metabolic activity of the sperm cells is achieved lowering the temperature of the semen sample to about 4-5°C.

Methods:

1. Preparation of the diluent:
These diluent must contain a component that can protect the sperm cells from the cooled shock during lowering the temperature of the sample. These components are the phospholipids originated mainly from egg yolk or the milk.

2. Semen collection:

3. Semen examination and evaluation:

4. Semen dilution:
Calculate the dilution rate and the amount of diluent:
The dilution rate should be calculated according to the following factors

- The number of motile sperm present in each one ml of the ejaculate (This will varied according to the ejaculate)

- The number of the sperm cells required in each inseminating does (This number must be at lest equal to the minimal number of the sperm cells required to achieve maximum conception rate).
Semen preservation

In bovine about 10-12 x 10^6 motile sperm.

- The volume of the inseminating dose in liquid semen this dose usually 1 ml.

\[ Dilution\ rate = \frac{\text{Number of sperm cell concentration/ml} \times \text{Motility \%} \times \text{Volume of inseminating dose}}{\text{Number of sperm cells in inseminating dose}} \]

After calculating the dilution rate the total volume of the diluent is calculated as the following:

The total amount of the diluents = dilution rate X ejaculate volume.

Technique of dilution:
The diluent and the semen should be held together in warm bath for sufficient time to balance the temperature of semen and diluent before mixing to avoid thermal shock. The diluent should be added to the semen gradually not vice versa to avoid dilution shock. This can be achieved adding by transporting the semen sample to a wide mouth container and the diluent is added in a drop wise manner or in four steps (unequal volume). The diluent should be added gradually on the wall of the container then genitally mixed with the semen sample (avoid agitation). For equilibration, about 20-25 minutes should be allowed between each step.

5. Cooling:
The semen must be cooled gradually form the initial temperature (25-35°C) to the storage temperature (4°C) to avoid cold shock. This can be achieved either by using automatic controlled refrigerator or by adding small ice cups to the water path. Cooling may also be achieved by putting the semen container in another container contain water at 30°C. The container is then placed in cold handling cabinet controlled at 4°C. The cooling process may take 0.5-2hrs. As generally slower cooling result in better survival rate.

6. Packaging:
The liquid semen usually stored in sterilized 1 ml glass vials. This vial must be completely filled with semen to avoid semen agitation during transportation. This vial should be closed by plastic cap with different color and labeled with a label containing information about name of the bull, date of collection, AI centre.

7. Storage and re-evaluation:
The liquid semen is stored in a refrigerator at 4°C. After 6 hours, random sample must be selected, warmed and examined. Liquid semen should not be used before elapsing 6 hrs to allow the action of the antibiotic. The liquid semen can used within 72 hrs not more even the sperm cells still maintain its motility for longer period.

8. Using of the liquid semen:
   a. Transportation:
Liquid semen can be transported for short distance (within the same governorate). During transportation,
the temperature of semen sample must be kept at 4°C till be used. This can be achieved by wrapping the vial in paper and kept it in insulated thermos with amount of ice wrapped in a paper. This thermos is transferred in a foam or carton container. When receiving the liquid semen in the farm you have to confirm that the ice is not melted. If the inseminator receive the shipment with the ice is completely melted he must examine random samples before using.

**b. Reactivation:**

By increase the temperature of the sample using a water bath at 37°C for 30 seconds or friction between the hands.

**c. Insemination:**

Using disposable inseminating pipette connected to syringes.

**Disadvantages of the liquid semen:**

1. Short life span of the sperm cells, about 72 hours.
2. Can be transported only for short distance.
3. Many sperm cells may be exposed to cold shock.
4. Some micro-organisms can be established at 5°C.

**Advantages of the liquid semen:**

The only advantage of liquid semen over the frozen semen occurs when there is a high demand for the semen of certain bull within small region. The same ejaculate can be used to produce higher number of inseminating doses in liquid semen than in frozen semen.

**III- Preservation of the semen in a frozen state (Frozen Semen):**

This method depends on preservation of semen in frozen condition in liquid nitrogen (-196°C). This very low temperature results in complete cessation of the sperm metabolic activity which allows storage of the sperm cells for very long time.

**1- Straw technique:**

<table>
<thead>
<tr>
<th></th>
<th>Medium straw</th>
<th>Mini straw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>133 mm</td>
<td>133 mm</td>
</tr>
<tr>
<td>Diameter</td>
<td>2.8 mm</td>
<td>2 mm</td>
</tr>
<tr>
<td>Volume</td>
<td>0.5 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Inseminating dose</td>
<td>18-25×10⁶</td>
<td>30×10⁶</td>
</tr>
<tr>
<td>Freezing temperature</td>
<td>-150°C</td>
<td>-120°C</td>
</tr>
<tr>
<td>Freezing time</td>
<td>7 min</td>
<td>9 min</td>
</tr>
</tbody>
</table>

**NB:** There are 20 different colors of straw and 7 different colors of polyvinyl alcohol powder.

**Technique:**

1- **Preparation of the diluent:**
Any diluent can be used after addition glycerol (cryoprotectants) by 7% concentration. Glycerol minimize the adverse effect of freezing process through:

1- Lowering the freezing point of the diluent (from -0.53°C to -3°C)
2- Replaces the intracellular water. Therefore, it decreasing the intracellular ice crystal formation.
3- They have bactericidal effect.
4- Source of nutrient, which increase the motility and fertilizing capacity of sperm cells.

**NB:** Glycerol is highly toxic to the living cells if it is added at a high concentration at room temperature. Therefore, addition of glycerol occurs after cooling of the sperm cells to 4°C.

**Preparation of Laiciphos 271**
- Empty a packet of 50 gm of Laiciphos 271 into flask. Add 400 ml of glass bidistilled water at 40°C and shake vigorously to dissolve completely.
- Dissolve 50 ml of fresh egg yolk in 50 ml glass bidistilled water at 50°C.
- Added them to the Laiciphos 271.
- Divide the preparation in equal fractions and add glycerin as follows:

<table>
<thead>
<tr>
<th>Portion A</th>
<th>Portion B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laiciphos 271</td>
<td>Laiciphos 271</td>
</tr>
<tr>
<td>Glycerin 3 %</td>
<td>Glycerin 11 %</td>
</tr>
<tr>
<td>Egg yolk 10 %</td>
<td>Egg yolk 10 %</td>
</tr>
</tbody>
</table>

- Portion A is kept at 30°C in the water bath and portion B is kept at 4°C in a refrigerator.

**2- Semen collection:**

**3- Semen evaluation:**

**4- Semen dilution and cooling:**

\[
Dilution \ rate = \frac{\text{Number of sperm cell concentration/ml} \times \text{Motility} \% \times \text{Volume of inseminating dose}}{\text{Number of sperm cells in inseminating dose}}
\]

After calculating the dilution rate the total volume of the diluent is calculated as the following

The total amount of the diluents = dilution rate X ejaculate volume

Gradual adding of fraction A to semen at 37°C then semen is gradually cooled to 4°C. Fraction B is added gradually to the semen extended with Fraction A. The final volume of extended semen will contain 7% glycerol concentration. This process called glycerolyzation.

**5- Equilibration and packaging:**

Equilibration is the total time during which spermatozoa remain in contact with glycerol before freezing to give chance for glycerol to penetrates into the sperm cell to establish a balanced intracellular and extracellular concentration. It ranged from 4-8 hrs. During this time, the straws could be filled with extended semen, sealed and placed on racks for freezing and counting.
Semen preservation

The equilibration and packaging is done in a cold handling cabinet (4°C). Straws can be labeled either manually or with computerized equipment before filling. The label should contain name of the country, name of the AI center, name of the bull, breed, date of semen collection.

Preprinted straws may be filled manually or automatically.

Every 15 medium or 20 mini straws is clamped with each others with metal clamp and handled as one unite.

After filling of the straws, begin to create air space in the proximal 4 – 5 mm. This is done by introducing a plastic comb at the opened end of the straw. This air space is very important to prevent the damage of the straws during freezing and to give space for closure of the free end.

After filling, the straws may be sealed automatically or manually using polyvinyl alcoholic powder which solidified just after contact with liquid media. The powder for sealing is spread and compressed firmly over a glass plate to a thickness of 5 mm. The straws are held in bundle in hand and immersed in bottom of water bath to remove the excessive powder and solidification of the powder.

6- Freezing:

Sealed straws may be frozen using a traditional vapor freezing method (using wide mouth liquid nitrogen container supplied with fenestrated net) or by computerized programmable freezers.

The straws are placed on racks and held horizontally above the surface of liquid nitrogen (4-5.5 cm). The temperature of liquid nitrogen vapor is set at -120°C for mini and –150°C for medium straws. The temperature is checked with a thermo-couple. The mini straws are kept for 9 minutes and the medium straws are kept for 7 minutes.

7- Storage and post freezing evaluation:

The straws are plugged into the goblet that is filled with the liquid nitrogen (each 5 straws from the same bull are placed in a goblet); each two goblets are clipped onto a metal rack. The rack is identified with the bull’s code number printed on top of the rack. The racks are kept in a canister. The canister is secured inside the liquid nitrogen tank with the index ring at the top and index spider in the bottom. After 48 hours a sample is examined microscopically.

8- Thawing and insemination:

In warm water thaw (35-37°C) for 30-45 sec. Insemination using inseminating gun.

Advantages of straw technique:

1. Easy labeled.
2. No chance for bacterial contamination.
3. Need less storage space.
4. Easy thawed.
5. Easy insemination.
6. The straw permits more uniform control of the freezing and thawing process, which has led to improved sperm cell recovery.

2- Pellet technique:

1- Preparation of the diluent: pellet need special diluent.
   - Lactose 11% 75.3 ml
   - Egg yolk 20 ml
   - Glycerol 4.7 ml
   - Antibiotics 0.5 mg streptomycin and 500 IU penicillin/ml

2- Semen collection:

3- Semen evaluation:

4- Semen dilution, cooling, equilibration:
The dilution rate is calculated to achieve 12 – 30 million sperm/ 0.2 ml. whole volume of the diluent is added gradually to semen at 30°C. The diluted semen is cooled to 4°C gradually. The diluted semen is kept for equilibration for 2-6 hrs.

5- Freezing and storage:
The freezing process took place on a block of solid Co₂. The upper surface of block is leveled smooth with a hot iron plate. A number of pits 6 mm in diameter and 8 - 10 mm in depth were pressed into the surface of the Co₂ block. 0.2 of diluted semen is dropped into each pit. The pellets of semen are kept in the pits for 7 - 10 minutes. During this period the block is covered with a piece of filter paper to prevent the contamination and vapor condensing from the air. The frozen pellets are then shed from the block into a goblet containing liquid nitrogen by means of a funnel; the goblet with pellets is placed in a storage container into liquid nitrogen. A tag with identification date is placed inside the goblet with pellets.

6- Thawing and insemination:
In tube containing 0.8 ml of sodium citrate 2.9% at 37°C. Insemination using disposable inseminating pipette connected to syringes.

Disadvantages of Pellet:
1. It needs special diluent.
2. It needs different media for freezing (block of solid Co₂), storage (liquid nitrogen).
3. Unsanitary technique.
4. Difficult to identify.

3- Ampule technique:

1- Preparation of diluent: Any diluent can be used after addition glycerol.
2- Semen collection:

3- Semen evaluation:

4- Semen dilution and cooling:
The volume of the inseminating dose usually 1 ml contain about 30x 10^6 sperm cells.

Divide the diluent in equal fractions and add glycerin as follows:

<table>
<thead>
<tr>
<th>Portion A</th>
<th>Portion B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg yolk sod. Citrate</td>
<td>Egg yolk sod. Citrate</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Glycerin 14 %</td>
</tr>
</tbody>
</table>

Portion A is kept at 30°C in the water bath and portion B is kept at 4°C in a refrigerator. Gradual adding of fraction A to semen at 37°C then semen is gradually cooled to 4°C. Fraction B is added gradually to the semen extended with Fraction A. The final volume of extended semen will contain 7% glycerol concentration.

5- Equilibration and packaging:
The semen is packaged in glass ampules made from proper type of glass. After filling the ampoules are sealed. Wait for equilibration time.

6- Freezing and Storage:
Placing the sealed ampules in ethyl alcohol path at 5°C, then temperature lowered at rate 1°C/minute by addition a portions of solid CO₂ till reach –15°C. After that freezing must be done as quickly as possible at rate 5°C/minute till reach –79°C. After freezing completed the ampules is directly fixed in the metal rack which is placed inside the canister which is placed in liquid nitrogen.

7- Thawing and insemination:
Ampules should be thawed in ice water (5°C) for 10 minutes. Insemination using disposable inseminating pipette connected to syringes.

Disadvantages of Ampule:

1- Improper sealing may result in expulsion during freezing.

2- Need more storage space.

3- Need more time for thawing.

4- Large number of sperm cells were lost during thawing.
STRUCTURE AND HANDLING OF LIQUID NITROGEN CONTAINERS

Liquid nitrogen tanks are actually large metal vacuum bottles with extremely efficient insulating system. It consist of two tanks, the insulation of the inside take is achieved by high quality solid insulation material present in between the outer and the inner tank and vacuum in the outer chamber.

Types of narrow mouth liquid nitrogen container:

1- Storage unit:
Its capacity is usually about 35-47 litter. This container usually contains whole semen investment necessary for about 6 month to one year.

2- Portable unit:
The capacity of this unit is usually about 3-10 litter. It usually contains few number of inseminating doses enough for 1-2 days. These units is kept near the inseminating place, may be transported through the yard or even carry form village to another. This unit should be refilled with liquid nitrogen every day or even more than one time every day.

3- Filling unit:
These units have no place for canisters. It used only to transport liquid nitrogen from the factory to the farm to be used to refill the storage and/or portable units.

Liquid nitrogen containers management:
Routinely monitor nitrogen levels and keep a record of nitrogen loss. Measuring the level of the liquid nitrogen is done by removing the neck stopper and immerse centimeter marked dipstick to the bottom of the tank for 10 second. Then pull it out and leave in the air for few second. Reading the lowest part of the U-shape frost line will indicate the level of the liquid nitrogen. Each container has its own safe level. As generally, the safety level in long container that can hold metal rack containing two rows of straws is about 28 cm while in the short container that hold only one raw of the straw the safety level will be about 14 cm.