METHODS OF SEMEN COLLECTION

I- Collection of semen from vagina or the uterus:
   • Using sponge method
   • Aspiration method

Disadvantage:
   1- Mixing of the ejaculate with the vaginal mucus result in the following disadvantage
      - There is a great chance of transmission of genital diseases.
      - Physical properties of the semen sample are changed and the microscopical examination is so difficult.
      - The sample is not suitable for storage.
      - The sample cannot be used to predict the fertility of the bull because it is diluted and not representative.

   2- Cannot be used in male suffer from Lack or complete loss of sexual desire or Impotantia quand.
   3- Result in damage large number of the sperm cells during handling of the semen.

Trials to overcome the disadvantage:
   1- If possible use female out of estrus and in equine, don’t use the mare in foaling estrus.
   2- Make vaginal douching before collection.
   3- Genital examination for female to be sure that it is free from genital disease.
   4- Use special female for each male.

II- Condom (breeding bag):
Application of special rubber bag over the completely erected penis before mounting. Used for animals achieve complete erection while it still on the ground (equine).

NB: To use this method in other species the breeding bag is inserted in the vagina before mating to collect the semen in it

III- Artificial vagina (AV):

Advantage:
   1- The collected ejaculate is clean and concentrated.
   2- The collected ejaculate give good indication about spermatogenic activity of the testis.
   3- Collection by AV give good indication about the sexual desire and potency of the male.
   4- It require simple equipment.
Semen collection

Disadvantage:
1- It cannot be used in male suffer from Lack or complete loss of sexual desire or Impotentia quando.
2- It requires some training for the bull.
3- Some bull may not ejaculate properly in to AV.
4- There is possibility for Injuries of the operator.

Parts of AV:
1- **Heavy thick outer cylinder or rigid rubber casing**
   It mostly formed from rigid rubber, iron or any other strong material. The length and diameter is varied
   among different animal species (bull 35- 40 X 7 cm, Stallion 80 X12-17).
   The thick outer cylinder has a compound valve (double valve) which used for introduction of air and
   hot water during preparation of AV.
   In stallion, the thick outer cylinder is provided by a handle or strap to help the collector to support its
   weight during vigorous thrusting of the stallion.
   **NB:** The length of the outer cylinder (casing) and to some degree its diameter, should be to fit the males.
   For better sanitation, it is important that the end of the glans penis extend well into the collecting funnel
   at the time of ejaculation.

2- **Thin inner rubber sleeve or inner rubber liner or latex inner liner**
   Made from soft rubber and it is usually longer than the outer thick cylinder.

3- **Rubber cone or funnel.**

4- **Collecting graduated tube or bottle** according to the expected volume of the ejaculate.

5- **Broad rubber band** that used to fix the thin inner sleeve and/or rubber cone over the thick outer
   cylinder.

6- **Insulating jacket** that used keep the temperature of the rubber cone and the collecting tube at the
   optimum temperature especially at the cold area.

7- **Filter:** In species give fractionated ejaculate (stallion, boar) special filter is applied to separate the
    postspermic viscous portion.
Assembling of the AV:
- The inner sleeve is introduced inside the thick outer cylinder.
- The inner sleeve is turned back over the ends of the outer cylinder and then tightly fixed by a broad rubber bands or rings to avoid escaping of the water and/or air during intromission.
- In case of using disposable liner, it should be inserted to inside the rubber inner liner.
- The rubber cone is fixed by rubber band on one side of the thick outer cylinder.
- Fixation of the graduated tube to the rubber cone. In case of equine collecting tube may be used with disposable collecting bag and filter.
- In cold area, the rubber cone and collecting tube is covered by the insulating jacket. This jacket also protects the sample from the direct sun light.

Simulation of the artificial vagina to the natural vagina:
The AV must simulate the natural vagina in some conditions that is essential to stimulate the sensory nerve ending in the glans penis to complete the ejaculation into the AV.

1- **Warming:**
The temperature must be 42-45 °C at time of collection. Warming is achieved by filling 1/2- 2/3 of the space between inner sleeve and outer cylinder with warm water (60°C in summer and 70°C in winter) through the large valve (lower valve).
Low temperature usually result in failure of ejaculation while higher temperature may result in
   - Pain to the bull during collection which may cause psychological problem and the bull will refuse to collect by this method again.
   - Killing large number of the sperms if the ejaculate gets in contact with the inner sleeve.
**NB:** The temperature of AV measured by chemical thermometer from the aperture (free end) of the AV.

2- **Pressure:**
Pressure is achieved by Inflation of air form the small valve (upper valve) and the water between the thick outer cylinder and the thin inner sleeve.
Air must be inflate to the level that allow passage of one finger under pressure.
Application of low pressure may result in incomplete ejaculation or bull refuse to ejaculate.
Application of higher pressure may result in
- Laceration of the penis during intromission or even broken penis
- Bursting of the inner sleeve during thrust
- Slipping of the rubber band during thrust

3- Lubrication:
The caudal third of the inner sleeve must be lubricated (vaseline, mineral oil, paraffin oil) to facilitate the intromission of the penis into the AV.
Excessive lubricant will contaminate the semen changing its physical properties, causing clumping of the sperm and making its examination very difficult.

Preparation of the male:
In bull and ram: The preputial hair tuft should be clipped; the underline and the external preputial orifice should be cleaned by brushing, washing and drying.
In stallion: The stallion should be sexually teased till achieve full erection and penis must be washed with warm water and non irritant soap to remove the debris form the prepuce invagination.
In the Boar: The content of the preputial diverticulum should be evacuated.

Type of teaser mount and its Preparation:
The mount may be either Female in oestrus, Quiet female, Teaser bull, Dummies either with or without bullet in AV.
- Restraining (to minimize lateral and forward movements):
This mainly achieved by placing the mount in breeding rack or even using head gate. In equine, application of breeding hopplies is necessary. In canine, the female must be muzzled to avoid pitting the collector. In camel, the teaser female camel kneels in the collection yard with its forelegs tied with a rope around its neck and the hind legs tied with a rope around its lumbar region.
- Cleaning the hind quarter:
In all types of mount, the hind quarter and external genitalia must be washed and cleaned by warm water and soap, then dried by clean towel. In some cases, the hind quarter may be covered by rubber apron which swapped by ethyl alcohol. In mare warping the tail with gauze is necessary. All these precautions must be taken in consideration to avoid contamination of the penis during seeking.

Location of the semen collection:
- Must have enough space for free movement of the collector and male animal.
- The location of semen collection must have a method for restraining the live mount.
- The collection area must provide good footing to prevent slipping and injury of the male animal.
- The location must be free from dust, dirts and mud to prevent semen contamination.
- It should be close to the lab.
• **Collection from bulls:**

**Sexual stimulation**

Before beginning of the actual mounting sexual stimulation of the male is necessary to:

- Insure that male will mount and ejaculate in a reasonable period of time.
- Improve the quality of semen sample including the volume, the sperm cell concentration and the motility percent.
- Clean the pathway of the semen.

Sexual stimulation for male with normal libido can be achieved either by

- Exposing the bull to teaser animal for several minutes. A period of 10 – 15 minutes of teasing stimulates most bulls.
- Allowing the bull to mount the teaser animal without ejaculation (False mounts).
- The combination of a false mount followed by a few minutes of teasing, plus one or two additional false mount. (False mount and/or teasing for 5 – 10 minutes are effective).

**The actual collection**

- The right hand operator should stand in the right side of the mount and catch the AV in the right hand with palm upward and the free end of the AV in lower level than the end connected to the cone.
- The bull is introduced to the mount and leaves him till covering the mount. At the moment of seeking, the operator begins to redirect the penis to the AV by catching it by the left hand from the part covered by prepuce. During this step the operator should avoid catching of penis from the exposed part out of the sheath as it may result in rapid ejaculation before intromission of the penis in the AV or rapid retraction of the penis without ejaculation or Pain to the bull, so it will refuse to collect by this method again.
- During redirection of the penis to the AV, the operator should bring the glans penis in contact with the inner sleeve then allow the bull to thrusting and complete the intromission.
- During thrusting, the AV should be allowed to move with the thrust and must be kept in the same line of the penis to avoid sharp bending of the penis.
- The AV must keep on the penis till the bull begins to dismount.
- After dismounting, the position of AV must be rapidly changed to vertical position to allow passage of the sample to the collecting tube. Removing some of air may be necessary to allow the passage of semen sample to the collecting tube if the ejaculation has occurred inside the inner sleeve.
- The semen sample must be rapidly transferred to the laboratory and kept at 25 – 37°C till the process of evaluation and processing.

• **Collection from stallion:**

- Brace the artificial vagina against the thigh or buttock of the mare or the mount and hold it parallel to the direction of his thrust.
- The anterior end of the AV should be lowered sufficiently to allow semen to flow into the collection bottle supplied with filter to separate the coagulum (postspermic fraction) from the rest of semen.
- Ejaculation is complete in about 12 to 25 seconds.

**IV- Collection of semen by rectal massage to ampullae and seminal glands:**

**Idea:** The manual massage of the accessory genital glands specially seminal vesicles and ampullae results in dribbling of the semen but mostly without erection.

**Preparation of the bull:**

In this method the semen mostly dribbles through the prepuce and drips from the preputial hairs. So, in addition to the cleaning of the external preputial orifice, the sheath should be carefully douched with physiological saline solution to clean it from remnant of epithelial cells, smegma, dirt and remnant of urine. Moreover, douching of the prepuce will induce possible urination before collection since urine is toxic to sperm.

**Technique:**

- Collection in presence of female may result in satisfactory results.
- All precautions of rectal examination must be followed.
- The operator takes the pelvic urethra as a guide to reach the neck of urinary bladder where seminal vesicle and ampulla are found and begin to milk or strip the ampullae toward the urethra.
- Massage of the seminal gland or even striking it against the pelvic brim may be indicated, in some conditions massage of pelvic urethra is indicated.
- The semen sample is collected by an assistant with a funnel and collecting tubes fixed at the external preputial orifice. This tube may be fixed with long holder

**Advantages:**

This method can be used for semen collection from bull suffer from Lack or loss of sexual desire or Impotantia quandi. Used for semen collection from bull not respond properly to the AV.

**Disadvantages:**

- Collected semen sample is watery with low sperm cell concentration.
- High possibility of contamination.
- It requires special experience or trained persons.
- Not all bulls give satisfactory response to this method.
- Urination can takes place during manipulation and urine mixed with semen.

**V- Collection of semen by electro ejaculation:**

**Idea:** Rhythmic electrical stimulation of the nerve supply of the genital organ results in ejaculation with erection in 90% percent of cases.

The electro-ejaculator is formed from a source of alternating electric current attached to probe or
electrode (30-35X6-9 cm in bull and camel bull, 20X 2 cm in ram).

- **Electro-ejaculation in bull**

  **Preparation of the bull**
  - As in massage technique in addition to provide some support under the ribcage to help support the bull’s weight.

  **Technique**
  - Insert the lubricated electrode, begin to stimulate the bull with low voltage, which is gradually increased, a few volts at a time alternated with 4-second rest periods in which the voltage is returned to 0. This low voltage stimulation is ended with erection of the penis and dribbling of the clear bulbo-urethral secretion. Increasing the voltage too rapidly can result in ejaculation without erection and the prepuce will contaminate the semen. If the bull fails to erect its penis try to change the site of probe or press the sigmoid flexure behind scrotum.
  - Begin to increase the voltage but not more than 15 voltages in the same rhythmic manner. Most bulls are stimulated to ejaculate in a period ranging from 2 to 5 minutes. The clear secretion is changed to milky or opaque secretion.
  - The semen sample is collected by an assistant with a funnel and collecting tubes fixed at the external preputial orifice. This tube may be fixed with long holder.

  **Advantages:** As the massage method.

  **Disadvantages:**
  1- Ejaculates obtained by the electro-ejaculation method are usually larger in volume but lower in concentration than those obtained with AV.
  2- Some may ejaculate without erection which make high possibility for contamination.
3- In some cases lay down during collection.
4- Not all the bulls respond efficiently to this method.

**Electro-ejaculation of the boar:**
The level of voltage required for ejaculation is greater in the boar than either the bull or the ram due to the insulating effect of body fat.

**VI-Collection by digital manipulation of the penis in dog:**
Depend on application of pressure and message for the penis. Teaser bitch may help considerably.

**VII- Gloved hand method in boar:**
It the most common method used to collect semen in boar. It did not require special equipment. It is very important to filter the ejaculate of the boar during or after collection using a filter disc or cheesecloth. This prevents the gelatin plug from inhibiting further processing, evaluation and extension of semen.

<table>
<thead>
<tr>
<th>Method</th>
<th>Bull</th>
<th>Ram</th>
<th>Stallion</th>
<th>Camel-bull</th>
<th>Dog</th>
<th>Boar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection from vagina</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Breeding bag</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>AV</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Rectal manipulation</td>
<td>✓</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Electro-ejaculator</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Digital manipulation</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>✗</td>
</tr>
<tr>
<td>Gloved hand</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>✓</td>
</tr>
</tbody>
</table>
I- Gross or macroscopical examination:

1- Volume:

<table>
<thead>
<tr>
<th>Species</th>
<th>Volume</th>
<th>Species</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull</td>
<td>2-8 ml</td>
<td>Stallion</td>
<td>50-150 ml</td>
</tr>
<tr>
<td>Buffalo-bull</td>
<td>3-6 ml</td>
<td>Boar</td>
<td>150-250 ml</td>
</tr>
<tr>
<td>Ram</td>
<td>0.5-1 ml</td>
<td>Dog</td>
<td>10 ml</td>
</tr>
<tr>
<td>Camel – bull</td>
<td>2-12 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factors affecting the volume of ejaculate in bull:

- **Age:** The volume of the ejaculate increase from puberty till reach the maximum volume about 2 years post maturity then begin to decline after 8 years of age.

- **Breed:** Dairy bulls tend to produce larger ejaculate than do beef bulls.

- **Body size:** The greater the body size the greater the volume.

- **Nutrition:** Well nourished bull give larger volume than the bad nourished one.

- **Season:** The volume in the summer is larger than in the winter.

- **Frequency of collection:** The volume decreased by increasing the number of the ejaculates at short interval. However when two ejaculates are collected at a short interval the second is usually larger, This is due to increased sexual excitement.

- **Sexual excitation and proper preparation of bull (teasing):** Sexual stimulation of the bull before the actual mounting result in higher volume and higher sperm cell concentration.

- **Method of semen collection:** Samples collected by electroejaculator usually have larger volume than those collected by manual manipulation larger than those collected by AV.
Abnormal low volume of ejaculate may be observed in case of excessive use of the bull, improper preparation of the AV, Uni or bi-lateral aplasia of seminal vesicles and/or Uni or bi-lateral seminal vesiculitis.

**2- Color and opacity:**

The opacity of the semen sample varied according to the sperm cell concentration. Ejaculate with high sperm cell concentration is opaque while that with lower sperm cell concentration is translucent.

<table>
<thead>
<tr>
<th>Species</th>
<th>Color and opacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow-bull</td>
<td>Opaque white to yellowish. Yellowish coloration is due to the presence of lipochromes (riboflavin or flavin) pigments.</td>
</tr>
<tr>
<td>Buffalo-bull</td>
<td>Opaque white</td>
</tr>
<tr>
<td>Ram</td>
<td>Dark opaque white</td>
</tr>
<tr>
<td>Stallion</td>
<td>Translucent, greyish white</td>
</tr>
<tr>
<td>Boar</td>
<td>Translucent, greyish white</td>
</tr>
<tr>
<td>Dog</td>
<td>Light grey to white</td>
</tr>
<tr>
<td>Camel-bull</td>
<td>Differ from translucent grayish to opaque white</td>
</tr>
</tbody>
</table>

**NB:** Abnormal color gives indication about the hygienic quality of the semen sample.

<table>
<thead>
<tr>
<th>Color</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink or reddish</td>
<td>Indicates admixture of fresh blood e.g. from wound on the surface of the penis</td>
</tr>
<tr>
<td>Dark red or brown</td>
<td>Indicates admixture of degenerated blood and tissue</td>
</tr>
<tr>
<td>Yellow</td>
<td>Indicate presence of purulent material (pus)</td>
</tr>
<tr>
<td>Greenish</td>
<td>Indicates admixture of fecal matter or pus</td>
</tr>
<tr>
<td>Grayish</td>
<td>Indicates presence of prespermic cells, giant cells, and protoplasmic droplets with decreasing number of sperm cells</td>
</tr>
<tr>
<td>Dark gray</td>
<td>Indicates presence of dust</td>
</tr>
</tbody>
</table>

**3- Density (Viscosity, Consistency):**

The density of the ejaculate gives good indication about the sperm cell concentration. The thicker ejaculate usually has higher sperm cell concentration. The normal ejaculate of the bull, buffalo-bull and ram are creamy while those of stallion, boar and dog are watery to viscous.

The density of semen sample may be descriptively evaluated by naked eye or numerically evaluated by the viscometer. The normal bull ejaculate is about 3.7 centipoise (the water is 1 centipoise).

**✓ Correlation between the density and the sperm cell concentration in the ejaculate.**

- The first or the Russian method:

<table>
<thead>
<tr>
<th>Density</th>
<th>Description</th>
<th>Approximate concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Thin sample, almost watery</td>
<td>Up to 400,000 /mm³</td>
</tr>
<tr>
<td>Dd</td>
<td>More creamy sample with large number of sperm cells</td>
<td>400,000-1,000,000 / mm³</td>
</tr>
<tr>
<td>ddd</td>
<td>Thick creamy sample with very large number of sperm</td>
<td>More than 1,000,000 / mm³</td>
</tr>
</tbody>
</table>
- The second method uses figures:

<table>
<thead>
<tr>
<th>Color</th>
<th>Density</th>
<th>Approximate concentration/mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creamy</td>
<td>3</td>
<td>More than 1,000,000</td>
</tr>
<tr>
<td>Thick milky</td>
<td>2</td>
<td>500,000 - 1,000,000</td>
</tr>
<tr>
<td>Milky</td>
<td>1</td>
<td>200,000 - 500,000</td>
</tr>
<tr>
<td>Watery</td>
<td>0</td>
<td>Less than 200,000</td>
</tr>
</tbody>
</table>

4- Presence of the foreign bodies or foreign materials:

<table>
<thead>
<tr>
<th>Source</th>
<th>Particle</th>
<th>Method of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male genital tract</td>
<td>Inflammatory cells (orchitis, epididymitis, seminal vesiculities)</td>
<td>- Changing the color to yellow color</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Snowflakes particles adjacent to the wall of the tube</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Stained film (Miscellaneous abnormalities)</td>
</tr>
<tr>
<td></td>
<td>Red blood cells (injury in the genital tract)</td>
<td>- Changing the color to red color</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Stained film (Miscellaneous abnormalities)</td>
</tr>
<tr>
<td></td>
<td>Prespermic cells (testicular degeneration)</td>
<td>- Changing the color to Dirty gray</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Stained film (Miscellaneous abnormalities)</td>
</tr>
<tr>
<td>Prepuce</td>
<td>Fecal matter and hair</td>
<td>- Changing the color to Dirty gray</td>
</tr>
<tr>
<td>Artificial vagina</td>
<td>Lubricant and m.o.</td>
<td>- Clot of lubricant material</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Fresh film</td>
</tr>
<tr>
<td>Collection place</td>
<td>Dust and dirties</td>
<td>- Changing the color to dark gray</td>
</tr>
</tbody>
</table>

5- Gross motility:

If light is allowed to fall on the semen sample containing high sperm cell concentration and higher proportion of motile sperm, the layer adjacent to the tube wall will take a granular appearance or swirl movement.

NB: Gross motility can be observed only in bull or ram ejaculate but cannot be observed in stallion, boar ejaculate or bull semen collected with electroejaculator or massage technique.

6- Estimation of the pH:

It is one of the most rapidly changed parameters, so it must be measured as quickly as possible. pH can be measured by pH paper, pH meter, bromothymol blue test (Catalase test).

Factors affecting the initial pH of semen sample:

- Biochemical character of seminal plasma.
- Proportion of the epididymal secretion into the whole seminal plasma.
- Contribution of different accessory gland in secretion of seminal plasma. (epididymal and seminal gland secretion is acidic, while prostate and cowper’s is alkaline).
- Number of sperm cell concentration.

When two ejaculates are collected within a short interval the second tends to be more acidic, due to higher sperm cell concentration in such sample.
<table>
<thead>
<tr>
<th>Species</th>
<th>Initial pH</th>
<th>Species</th>
<th>Initial pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull</td>
<td>6.4-6.9</td>
<td>Stallion</td>
<td>7-7.8</td>
</tr>
<tr>
<td>Buffalo-bull</td>
<td>6.4-6.9</td>
<td>Boar</td>
<td>6.9-7.9</td>
</tr>
<tr>
<td>Ram</td>
<td>5.9-6.3</td>
<td>Camel-bull</td>
<td>7.6-7.8</td>
</tr>
<tr>
<td>Dog</td>
<td>6-6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Changing the initial pH of the bull semen toward alkalinity mostly occur in case of:
  - Inflammatory conditions as seminal vesiculitis, epididymitis, orchitis.
  - Excessive use of the bull.
  - Incomplete ejaculation (mostly seminal plasma).
- Changing the initial pH of bull semen toward acidity mostly occurs in case of:
  Increase sperm cell concentration, Delayed examination with bad storage.

II- Microscopical examination:

1- Examination of unstained fresh semen (Sperm- motility):

Motility must be done as quickly as possible, the equipment (slides, cover slides) or diluents should be warmed up to 37°C and the temperature of the sample should be maintained at 37°C during examination. This can be achieved by storage of the diluents, slides and cover slides in an incubator set at 37°C. Using a microscope supplied with a hot stage (thermostage microscope).

A- Mass motility:

The strength of the mass motility (wavy motion) is varied according to the sperm cell concentration, the number of motile sperms and the strength of their motility.

Technique:

- Large drop of undiluted semen is transferred to clean dry warm slide by clean dry pipette
- Examine under low power (x4) with a closed diaphragm.

<table>
<thead>
<tr>
<th>Descriptive scale</th>
<th>Numerical</th>
<th>Appearance of pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>5</td>
<td>Extremely rapid and vigorous wavy motion (over than 90% motile sperm)</td>
</tr>
<tr>
<td>Very good</td>
<td>4</td>
<td>Apparently rapid dark distinct wave (~90% motile sperm)</td>
</tr>
<tr>
<td>Good</td>
<td>3</td>
<td>Slowly wavy motion (50-80% motile sperm)</td>
</tr>
<tr>
<td>Fair</td>
<td>2</td>
<td>Only strong rotatory movement (less than 50% motile sperm)</td>
</tr>
<tr>
<td>Poor</td>
<td>1</td>
<td>Week rotatory movement</td>
</tr>
<tr>
<td>Very poor</td>
<td>0</td>
<td>Waves not present, sperm cells are immotile</td>
</tr>
</tbody>
</table>

Bull ejaculate should show be good at the descriptive scale to be acceptable
Semen evaluation

**NB:** No mass motility in stallion and boar semen or in bull semen collected with electroejaculator or massage technique.

**B- Individual motility:**

Aimed to descriptively estimate the proportion of anterior forward progressive motile sperm. Presence of large proportion of abnormal motile sperm may indicate high proportion of sperm abnormalities those including the tail.

**Technique:**

- The semen sample is diluted by either warm sodium citrate 2.9% or warm saline 0.9%.
- A drop of diluted sample is transferred to a clean and dry prewarmed slide by clean dry pipette.
- Covering the drop by clean and dry cover slide to achieve a very thin uniform film, delays drying of the smear and prevent contamination of the objective lens of the microscope by the sample.
- Examine under high power (X40) with partially opened diaphragm.

<table>
<thead>
<tr>
<th>Motile cells %</th>
<th>Descriptive value</th>
<th>Numerical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 – 100</td>
<td>Very good</td>
<td>5</td>
</tr>
<tr>
<td>60 – 80</td>
<td>Good</td>
<td>4</td>
</tr>
<tr>
<td>40 – 60</td>
<td>Fair</td>
<td>3</td>
</tr>
<tr>
<td>20 – 40</td>
<td>Poor</td>
<td>2</td>
</tr>
<tr>
<td>0 – 20</td>
<td>Very poor</td>
<td>1</td>
</tr>
</tbody>
</table>

In fresh bull ejaculate at least 70% of the motile cells should exhibit the progressive motility. But in frozen thawed sample 50% or even lower is acceptable.

The motility test is considered to provide the most significant information about the quality of semen.

**2- Examination of stained film:**

**A- Live/dead percent or Eosin/nigrosin test or vital stain test:**

**Idea:** The sign of death in living cells is the destruction of the cell wall. Differentiation between live and dead sperm depends on staining the sperm cell with special stain (vital stains) that able to penetrate the cell wall of dead spermatozoa but unable to penetrate the cell membrane of the live spermatozoa. By this method the dead sperm will take the stain and the live one will remain unstained. For easy distinguish the stained and the unstained sperm addition of counter stain is helpful.

There are many vital stains for example (bluish eosin) and many stains can be used as a counter stain for example Nigrosin.
Semen evaluation

Technique:

One step protocol

√

- One drop of the semen is added to several drop of the prewarmed stain mixture (5% nigrosin, 0.6% eosin, 2.9% sodium citrate) in a clean, dry warm slide.
- Make genital mixing and leave for few seconds up to one minute.
- From the last mixture begins to smear out several films.
- Begin to dry the film rapidly on flam, hot plate, or hot electric fan.
- Examine under the oil immersion lens. Dead sperm will take up the eosin and show up a distinct red color, whereas, the live sperms remain uncolored or transparent against a brownish purple background. At least 70% of sperm cells must be live.

Two step protocol

- Place a drop of semen on a clean dry slide.
- A drop of eosin (5% aqua’s solution) about double the size of the semen drop is placed on the slide.
- A drop of nigrosin (10% aqua’s solution) about twice the size of the eosin is placed on the slide.
- Mix the semen with eosin and wait for one minute then mix the mixture with nigrosin stain.
- Complete as described in the one step technique.

B- Examination of sperm abnormalities (sperm morphology):

Aimed to examine the shape of sperm cell and detect the proportion of the sperm cells which are atypical in the morphological appearance. Although the strong relationship between the morphological appearance of the sperm cells and its fertilizing capacity, examination of the sperm cell morphology is not routinely applied in AI center. Examination of sperm cell morphology is indicated in the following cases:
Semen evaluation

1- If the sample is examined to predict the fertility of the male (during male examination).
2- When the bull shows obvious reduction in the fertility.
3- When high proportion of abnormal motile sperm is observed during examination of the motility.
4- Monthly examination of the bull ejaculate

A large variety of stains had been described for the staining of semen samples to examine all sperm abnormalities, the following are the most satisfactory and widely used in the semen laboratory are alkaline methyle violet, indian ink, opale blue, nigrosin stain, eosin-nigrosin stain.

**Alkaline methyl violet:**
- The semen sample is diluted with 1 % sodium chloride by mixing 1 part semen with 4 - 5 parts diluents.
- Dry in air at room temperature and fix by passing through a flame or by immersion in alcohol.
- The slide is moistened by distilled water.
- The slide is immersed in freshly prepared mixture of (9 parts of 1 % aqueous methyl violet and one part of 1 % aqueous sodium carbonate solution) for 4 - 5 minutes then rinsed with distal water to remove excess of stain.
- Dry by filter paper or passing the slide two or three times through the flame.
- Examine under oil immersion lens. The sperm cell will appear violet.

**Indian ink, nigrosin or opal blue**
The sperm cells are seen light against a dark back ground in Indian ink or nigrosin or unstained against a blue back ground in case of opal blue.

<table>
<thead>
<tr>
<th>Primary sperm cell abnormalities</th>
<th>Secondary sperm cell abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origin</strong></td>
<td><strong>During the process of spermatogenesis</strong></td>
</tr>
<tr>
<td></td>
<td><strong>(dysfunction of the testes).</strong></td>
</tr>
<tr>
<td></td>
<td><strong>During passage of sperm in the duct</strong></td>
</tr>
<tr>
<td></td>
<td><strong>system or during bad manipulation of the</strong></td>
</tr>
<tr>
<td></td>
<td><strong>semen sample in the lab (after the sperm</strong></td>
</tr>
<tr>
<td></td>
<td><strong>has left the seminephrous tubules)</strong></td>
</tr>
<tr>
<td><strong>Accepted %</strong></td>
<td><strong>10-15 %</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Whole sperm</strong></td>
<td><strong>Giant sperm.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Dwarf sperm.</strong></td>
</tr>
<tr>
<td><strong>Sperm head</strong></td>
<td><strong>Small head (microcephalic).</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Large head (macrocephalic).</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Double head.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Pyriform or pear head.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Elongated or narrow or lancet head.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Round head.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Tapered or spindle head.</strong></td>
</tr>
<tr>
<td><strong>Middle piece</strong></td>
<td><strong>Abaxial attachment.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Abnormal long.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Abnormal short.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Bent middle piece.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Cork screw or zigzag middle piece.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Bent middle piece with or without</strong></td>
</tr>
<tr>
<td></td>
<td><strong>protoplasmic droplets.</strong></td>
</tr>
</tbody>
</table>
### Main piece

<table>
<thead>
<tr>
<th>Description</th>
<th>Image 1</th>
<th>Image 2</th>
<th>Image 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double middle piece.</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Filamentous middle piece.</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Diffuse or local swelling in middle piece.</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Double tail.</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Long tail.</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>Short tail or absence.</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>Forked tail.</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
</tr>
<tr>
<td>Coiled tail.</td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
<tr>
<td>Detached tail.</td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
</tr>
<tr>
<td>Broken tail.</td>
<td><img src="image28" alt="Image" /></td>
<td><img src="image29" alt="Image" /></td>
<td><img src="image30" alt="Image" /></td>
</tr>
<tr>
<td>Bent tail, with or without protoplasmic droplet.</td>
<td><img src="image31" alt="Image" /></td>
<td><img src="image32" alt="Image" /></td>
<td><img src="image33" alt="Image" /></td>
</tr>
<tr>
<td>Looped tail.</td>
<td><img src="image34" alt="Image" /></td>
<td><img src="image35" alt="Image" /></td>
<td><img src="image36" alt="Image" /></td>
</tr>
</tbody>
</table>

*To differentiate between the secondary abnormalities developed in male genitalia and that developed due to bad handling, you must examine a second ejaculate which handled properly.*

### Miscellaneous abnormalities (cells other than spermatozoa):

- **Leucocytes:** Indicates inflammation in the genital tract.
- **Erythrocytes:** Indicate presence of injury in the mail genital system.
- **Prespermic cells:** as spermatides and spermatocytes, presence of these cells indicate serious problem including the testis.
- **Medusa cells:** It present due to sloughing of the ciliated epithelial cells of the duct system mainly epididymis. In normal condition, these cells may present in semen as 1:10000. Presence of higher proportion of such cells indicates serious damage in the duct system.
- **Protoplasmic or cytoplasmic droplets:** (no significance).
- **Multi-nucleated giant cells:** It is large cell has 6-8 nuclei formed mainly due to abnormal division during spermatocytogenesis. It may be observed in case of orchitis, testicular degeneration.
- **Clusters of degenerated sperms:** In degeneration and hypoplasia of testis.
- **Squamous epithelial cells:** both nucleated and non-nucleated, that usually originated from epithelium of prepuce and urogenital tract.
- **Micro-organisms:** Semen must be free from any m.o but m.o. may be present in case of genital infection or as a result of ejaculate contamination.
C- Examination of sperm ripeness (protoplasmic droplet):
The protoplasmic droplet is a remnant of cytoplasm formed between the neck and the middle piece during the process of spermiogenesis. During passage of the sperm cells through the duct system the droplet moves along the middle piece till completely loosed. The proportion of the unripe sperm cells those carries proximal protoplasmic droplets should not exceed 2-3%.

Presence of large number of sperm with a distal droplet may be due to:
- large number of services in a very short time (sexual exhaltion).
- Disease that affects the epididymal functions (epididymitis).

Technique:
Detection of protoplasmic droplet requires special stain as nigrosin, opale blue, which stain the background while the sperm cell appears bright in color.
- A drop of semen is diluted with 5 times as much with stain solution.
- Several films are spread over a clean dry slide.
- The slide is dried in air.
- Examine under oil immersion lens.

You should differentiate the protoplasmic droplets from the swelling in the middle piece. The protoplasmic droplet characterized by quite spherical, stainless intensely.
3- **Estimation of the sperm cell concentration (sp. cc):**

**Definition:**
The number of the sperm cells per unit volume and it is usually expressed by n/mm$^3$.

**Importance:**
SP.cc is very important routine examination because it gives good indication about the spermatogenic activity of the testis and it is very important for calculating the dilution rate during semen processing.

**Methods:**

**A- Density:**
As generally, the density gives an indication but not accurate estimation for the for the sp.cc. (see macroscopic examination).

**B- Visual microscopic estimation (Blom’s comparing chamber):**
Undiluted semen is distributed evenly in central and ring chambers by a pastier pipette. When the cover slip is added the peripheral chamber is formed and filled with capillary action.

Individual motility can be assessed by examination of the peripheral chamber, mass motility can be assessed by examination of ring chamber. The sperm cell concentration is determined by comparing the density of the sperm cells in central chamber with pictures of known concentration samples.

<table>
<thead>
<tr>
<th>Density</th>
<th>Description</th>
<th>Approximate concentration/mm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Sperma densum</td>
<td>1,000,000 or more</td>
</tr>
<tr>
<td>SD</td>
<td>Sperma semidensum</td>
<td>500,000 - 1,000,000</td>
</tr>
<tr>
<td>R</td>
<td>Sperma rarum</td>
<td>200,000 - 500,000</td>
</tr>
<tr>
<td>OS</td>
<td>Oligospermia</td>
<td>Less than 200,000</td>
</tr>
<tr>
<td>A</td>
<td>Aspermia</td>
<td>Complete absence of sperm</td>
</tr>
</tbody>
</table>

**C- Opacity tubes:**
This method depends on comparing the opacity of the examined sample with the opacity of known concentration (matching).

**D- Colorometric, photometric, Absorptiometer, spectrophotometer method:**
The sperm cells absorb the light and subsequently the amount of the light passed through the semen sample to the photo cell is inversely related to the sperm cell concentration. Compare optic density with standard curve.

**E- Direct cell count (Haemocytometer):**
The haemocytometer has two counting chambers; each chamber formed from four peripheral chambers used four counting WBCs and one central chamber used for counting RBCs counting.
The area of RBCs counting chamber (primary square) is 1 mm$^2$. This chamber is divided into 400 tertiary squares. The area of each of them is 1/400 mm$^2$. When this chamber is covered by a cover slip the depth of the chamber is 0.1 mm. Therefore, each tertiary square is filled with 1/4000 mm$^3$ of semen. Each 16 square 4x4 is separated either by thick line or triple line to form what is called secondary square.

**Technique:**
- A clean and dry haemocytometer is fixed on the microscope stage. The counting chamber is adjusted using the low power.
- A clean and dry cover slide is placed over the counting chamber, but must leave a distance between the edge of cover slide and the edge of haemocytometer.
- Live sperm cells must be killed in fresh sample to prevent its motion and to be easily stained.
- The semen sample under examination must be diluted (1:200 in bull semen, while stallion and boar semen can be diluted to 1:10 - 1:20 after removal of the gelatinous portion). The dilution rate 1:200 can be achieved by using RBCs Pipette and the dilution rate 1:20 can be achieved by using the WBCs pipette.
- The diluents may be saline to which 0.01% mercuric chloride, 2% sodium hydroxide to suppress the motility of the sperm, One drop of eosin or methylene blue was found to be useful for staining the sperm and making them more distinct and to be easily seen in the chamber.
- A small drop of diluted semen is added at the edge of the cover-slip over the center of the haemocytometer and the diluted semen allowed to be drawn under the cover slip by capillary action.
- The haemocytometer is left for 5 minutes, so that all the cells settle.
- The sperm cells are counted under a 40X objective in 80 tertiary squares (5 secondary squares). It is preferable to count in the 4 peripheral and one center square or in 5 squares in the diagonal line.

**Calculation:**
The total number of sperm cells in 5 secondary or 80 tertiary = \( R \)

\[
R = \text{the number of sperm cell in } 1/50 \text{ mm}^3 (80 \times 1/4000 \text{ or } 5 \times 1/250)
\]

Number of sperm cells in mm$^3 = R \times 50 \times \text{dilution rate}
<table>
<thead>
<tr>
<th>Species</th>
<th>Sperm cell concentration</th>
<th>Species</th>
<th>Sperm cell concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull</td>
<td>800,000 – 1,000,000 / mm³</td>
<td>Stallion</td>
<td>50,000 – 200,000 / mm³</td>
</tr>
<tr>
<td>Buffalo-bull</td>
<td>600,000 – 1,000,000 / mm³</td>
<td>Boar</td>
<td>100,000 – 200,000 / mm³</td>
</tr>
<tr>
<td>Ram</td>
<td>2,000,000 – 4,000,000 / mm³</td>
<td>Dog</td>
<td>50,000 – 100,000 / mm³</td>
</tr>
<tr>
<td>Camel-bull</td>
<td>500,000 – 600,000 / mm³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**F- Electrical practical counter:**

Depends on the passage of a volume of semen through a capillary tube that only allows passage of one sperm cell at a time. This tube present between two electrodes. Passage of sperm head causes abrupt increase in the resistance between the 2 electrodes which is registered in a counter.

**G- Haematocrit tube (PCV tube):**

Put the semen sample in PCV tube → centrifugation → compare with slandered curve.

**H- Computer assisted semen analyzer (CASA):**

The CASA is an automated reproducible high-performance sperm analysis instrument that can provide precise and accurate information about 16 clinical parameters within 75-second. These parameters include count, motility (A+B+C), morphology, velocity, and functional sperm.

**III- Estimation of hygienic quality:**

**Definition:** The hygienic quality means the degree of purity of the semen sample from any contaminant.

**Methods:**

1. **Macroscopical examination (color)**

2. **Microscopical examination (miscellaneous abnormalities)**

3. **Bromothymol blue (Catalase test):** Used to assess the pH and hygienic quality of the ejaculate.

The idea of this test depends on the fact that normal, clean and hygienically collected semen contains only a small amount of catalase enzyme (hydrogen peroxidase splitting enzyme). In the opposite, most of the suspected contaminants (RBCs, WBCs, M.O. fecal matter) contain higher amount of this enzyme.

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase enzyme}} 2\text{H}_2\text{O} + \text{nascent O}_2
\]

Measuring the amount of the catalase enzyme in the semen sample could be taken as an indicator for the hygienic quality of the semen sample. This can be done through measuring the amount of the nascent oxygen liberated within a specific time after addition of \( \text{H}_2\text{O}_2 \) to the semen sample.

**Technique:**

This test is performed in a special tube called catalase tube. This tube is graduated from 200 at the bottom of the tube till 2000 at the opened end. Near the opened end there is a mark of double rings, little downwards there is another mark of single ring.
Semen evaluation

- One ml of the undiluted semen sample is transferred to clean and dry catalase tube.
- Five drops of bromothymol blue indicator are added (2% in 96% alcohol). This will result in special colors indicating pH of the semen sample:

<table>
<thead>
<tr>
<th>Color</th>
<th>Yellow</th>
<th>Green</th>
<th>Medium green</th>
<th>Blue green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.0</td>
<td>About 6.4</td>
<td>About 6.8</td>
<td>About 7.2</td>
<td>7.6</td>
</tr>
</tbody>
</table>

- Add 2-3 % hydrogen peroxide solution until the single ring on the tube.
- Close the tube with a special stopper provided with a capillary tube. The stopper usually reach the double ring. The catalase tube is then shacked vigorously then incubated in inverted position for 20 minutes and shacked every 5 minutes.
- The catalase enzyme will split the H₂O₂ into H₂O + nascent O. The nascent O₂ will move upward and pass on the H₂O to escape from the capillary tube.
- After 20 minutes tack the reading (catalase number).

Judgment:
If the reading 300 or less------------- good semen sample.
If the reading 300 – 400 --------------- suspicious.
If the reading more than 400 --------- the sample must be rejected.

NB: This test not used in stallion and boar semen.

IV- Estimation of metabolic activity:

1- **Fructolytic activity:**

It is the main metabolic process performed by the bull sperm cells under anaerobic conditions. In which the sperm cells utilize the fructose (main component of seminal plasma in bull and ram) to produce metabolic energy.

Fructose → Lactic acid + energy (2ATP) → CO₂ + H₂O + energy (38 ATP)

Assessment of anaerobic fructolysis

1- The rate of lactic acid accumulation (pH) give qualitative indication about the fructolysis
2- The rate of fructose disappearance
Semen evaluation

**Sawaf and Barakat method**

**Idea:**
If ammonium molybdate reagent is added to semen sample containing fructose and the sample is heated, this reagent will react with the fructose and result in a blue color. The intensity of this color will varied according to the fructose concentration in the semen sample.

There are two problems facing this test. The first is that the citric or ascorbic acid can react with the reagent and result in a blue color, therefore the citric or ascorbic acid must be removed from the semen sample before beginning the test. This can be achieved by addition of manganese dioxide.

The second is that the protein present in the seminal plasma will interfere with measuring the density of the blue color; therefore the protein must be removed from the semen sample before beginning the test. This can be achieved by addition of trichloroacetic acid.

- Evaluate the sperm cell concentration and motility in the semen sample.
- Dilute the semen sample 1:1 with 2.9% sodium citrate (0.4 ml to 0.4 ml).
- Take 0.1 ml from the mixture and incubate the rest at water path at 37°C under anaerobic condition.
- Add 3.9 ml trichloroacetic acid to the 0.1 ml of semen to precipitate the protein then add 0.2 gm manganese dioxide to get rid of citric acid.
- Filtrate the mixture and take 2 ml of the filtrate.
- Add 8 ml of the freshly prepared ammonium molybdate reagent (70 ml of 8% ammonium molybdate+ 60 ml of 10% H2SO4+ 70 ml of 5% HCL).
- Keep the tube in a water path at 80-85°C for 10 minutes then allow to cool
- Estimate the optical density of the developed color by the calorimeter
- To change the optical density of the colored developed to fructose concentration you have to use a standard curve. This curve is prepared by numerous tube contain 2 ml of fructose solution each of them contain 0.2, 0.1, 0.05, 0.025 mg of fructose, respectively. To each of these tubes 8 ml of ammonium molybdate reagent and the experiment is completed as mentioned.
- The fructose concentration mg/dl of semen is calculated by multiplying the amount of fructose determined in 2 ml of filtrate by 4000. This initial fructose concentration in semen sample gives good indication about the activity of the seminal glands and/or the androgenic activity of the testis. the initial fructose concentration in bull ejaculate is about 500-800 mg/dl.
- To calculate the rate of fructose disappearance another 0.1 ml from the semen sample that stored at 37°C under anaerobic condition is taken after 1 hr. The experiment is repeated on this sample to determine the fructose concentration after 1 hr.
- The rate of fructose disappearance (FD) is calculated from the following equation
  - \[ FD = \text{initial fructose concentration (mg/dl)} - \text{fructose concentration (mg/dl)} \text{ after 1 hr} \]
- The amount of the fructose disappeared is consumed by the motile sperm present in dl of the semen (motile sperm/ dl)

- The amount of fructose that consumed by 10^9 motile sperm during 1 hr (fructolysis index ; FI) is calculated as follow

$$\text{FD motile sperm/dl} \rightarrow \text{FI} = \frac{\text{FD x 10}^9}{\text{Motile sperm/dl}}$$

**Fructolysis index for bull or ram ejaculate (1.2 : 2 mg / 10^9 motile sperm kept at 37^oC for 1 hr).**

**NB:** This test not used in stallion because metabolism is aerobically depend mainly on sorbitol.

### 2- Enzymatic activity (dehydrogenase enzyme activity):

This enzyme acts mainly under anaerobic condition to oxidize some component by removing hydrogen ions from it. The amount of the free hydrogen ions liberated in the semen sample can be taken as a good indicator for the activity of the dehydrogenase enzyme.

**Idea:**
The free hydrogen ions liberated from the action of the dehydrogenase activity can reduce the methylene blue to leucomethylene.

**Methods:**
- Semen sample is diluted 1:4 with yolk citrate (0.2 ml of semen with 0.8 ml diluent).
- To 0.9 ml form diluted semen add 0.1 ml from 0.05% methylene blue dissolved in sodium citrate (50 mg in 100 sodium citrate solution).
- The mixture is then covered with 1 cm layer of mineral oil or liquid paraffin.
- The tube is then placed in a temperature controlled water bath held at 46.5^oC and the time required for decolorization of the methylene blue in noted.

<table>
<thead>
<tr>
<th>Species</th>
<th>Bull</th>
<th>Ram</th>
<th>Descriptive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time/minutes</td>
<td>3 – 10</td>
<td>3 - 7</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>11 – 20</td>
<td>8 - 11</td>
<td>Fair</td>
</tr>
<tr>
<td></td>
<td>Over 20</td>
<td>Over 11</td>
<td>Bad</td>
</tr>
</tbody>
</table>

**NB:** This test not used in stallion semen because sperm of stallion is highly sensitive, low SCC, semen sample in stallion highly contaminated, metabolism is mainly aerobic.

### 3- Respiratory activity:

The respiratory index is the amount of oxygen consumed by 10^9 motile sperm cell during one hour when incubated at 37^oC. Measured by manometer, it was found to be 100 to 200 μl for bull and ram semen.
V- **Assessment of the sperm resistance to chemical or environmental changes:**

1- **Viability during low temperature storage:**

- The semen is diluted by egg yolk buffer solution and the initial motility is estimated.
- The sample is stored at 5°C and the sperm motility is examined daily till complete loosening of the motility.
- Estimate the time needed till complete loosening of the motility.

**Judgment:**

In normal semen, the sperm cells may still motile for several weeks although it is not fertile due to detachment of acrosome (aging of the sperm after 72 hrs.)

2- **Stability against cold shock:**

The initial motility of semen sample is estimated and small volume of the sample is rapidly cooled to 4°C or less for 10 minutes then re-estimate the motility.

**Judgment:**

\[
\text{Normality} = \frac{\text{Motility \% after 10 minutes at 4°C}}{\text{Initial motility}}
\]

3- **Stability against heat shock:**

- The semen sample is diluted and stored at different temperatures 46.5°C, 47°C and 47.56°C.
- The motility is examined every 10 – 15 minutes till the sperm cells completely loose their motility.
- Ejaculate with good resistance should keep the motility for 1 hr at 46.5 °C, 3/4 hrs at 47 °C and for 30 minutes at 47.5 °C.

4- **Stability against sodium chloride dilution:**

A known volume of semen is diluted with 1 % sodium chloride solution until the motility of spermatozoa is stopped. The resistance "R" is calculated as the relation between the volume of sodium chloride solution and the volume of semen.

Resistance to sodium chloride = Volume of NaCl / Volume of semen

Good semen sample may resist up to 5000 (3000-20000)