

# Steps for Freezing Bovine Semen: From Semen Collection to the Liquid Nitrogen Tank

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## Introduction

Artificial insemination (AI) has contributed enormously to the genetic improvement of beef and dairy cattle in the last 50 years. Frozen semen in 0.5 ml or 0.25 ml straws has become the universally accepted unit of storage and transfer of bovine genetics to cattle producers. This document reviews the steps performed for freezing bull semen in straws, beginning with semen collection through storage in liquid nitrogen tanks.

## Semen Collection

Bulls are ready for semen collection and freezing procedures after they have passed a complete physical examination, the required federal health tests and a breeding soundness evaluation. There are different methods that can be used to collect semen from bulls.

## Semen Collection by Artificial Vagina (AV)

This method is used almost exclusively in AI centers for practical reasons and because it produces physiological semen samples.

The usual components of a bovine AV include a rigid casing, inner rubber liner, rubber bands, valve, director cone, semen vial and warming bag (Fig. 1). After all the AV components are put together, the space located between the rigid casing and the inner rubber liner is filled with warm water. Bulls for semen collection require an AV temperature of 42 - 50°C. AV pressure is also an important factor for obtaining ejaculates of optimum quality. Pressure is adjusted by adding air through a valve in the rigid casing until the AV liner protrudes slightly from the ends. A small amount of non-spermicidal lubricant is applied to the inner liner before the collection is performed.



Figure 1. Bovine artificial vagina ready for semen collection. (Courtesy of Minitube International).

Two people are required for bull semen collection by AV, the bull handler and the semen collector who carries the AV. Bulls to be collected by AV must be halter broken and should be nose ring trained. Since semen collection by AV imitates natural breeding, the bull must mount either a cow in heat, a steer (Fig. 2), a dummy cow (Fig. 3 and Fig. 4), or a bull while the collection is being performed. If cows are available, but not in heat, a tranquilizer may be administered to facilitate bull mounting and semen collection.

When the bull has mounted, the collector must grasp the penis through the sheath and direct it to the opening of the lubricated AV (Fig. 2). The bull will make seeking motions and thrust into the AV. The thrust should be vigorous to ensure an ejaculate has been collected.



Figure 2. Top: The semen collector grasps the penis through the sheath and directs it to the opening of the AV. Bottom: The bull's penis enters the AV before thrusting (Photos courtesy of Gencor - The Genetics Corporation).



Figure 3. Mobile dummy designed for semen collection by AV. (Courtesy of Minitube International).



Figure 4. Semen collection with a mobile dummy. The collector with the AV is seated inside the dummy. (Courtesy of Minitube International).

## Semen Collection by Transrectal Massage

This technique requires two people, one to do the massage and one to collect the semen. The bull is held in a chute. After removal of feces from the rectum, a longitudinal back and forth massage is applied mainly over the ampullae, drawing semen toward the pelvic urethra. When the urethral muscle begins to pulsate the massaging action should be in synchrony with the pulsations. The semen collector must collect the cloudy fluid into a warm receptacle as it dribbles from the penis or prepuce. The extended penis may be held by the semen collector during rectal massage to facilitate collection of a clean semen sample.

### Advantages:

1. No expensive equipment is required.
2. The technique avoids the potentially painful aspects of electroejaculation.

### Disadvantages:

1. A skilled palpator is required.
2. Libido, mating ability, penile erectile function and the ability to ejaculate are not evaluated.
3. Semen samples may be contaminated with epithelial cells, bacteria, and dirt especially when it dribbles through the prepuce and off preputial hairs.
4. Semen volume and concentration are very variable.

## Semen Collection by Electroejaculation

### Equipment

## Equipment

Electroejaculators are designed to stimulate the pelvic sympathetic and parasympathetic nerves with pulses of low voltage and amperage to induce penile erection and ejaculation.

An electroejaculator set has the following components: carrying case, rectal probe, control unit, battery charger, power cord, probe cord, semen collection handle, collection cone and a collection vial (Fig. 5).



Figure 5. Complete electroejaculator set. (Courtesy of Minitube International).

There are different kinds of electroejaculators available in the market. They may be battery operated or plugged into an electrical outlet. They may be manually operated, operate from a built in program and may be programmable; some have all three options. Most bulls ejaculate with electrical stimuli of < 9 volts. There are also different kinds of rectal probes that vary in diameter, weight, orientation of electrodes and in the number of electrodes. All modern probes have three ventrally oriented electrodes. Most probes have a U-shaped extension that fits around the bull's tail when the probe is inside the rectum. The bull's tail keeps the probe oriented correctly. To obtain the best results with each individual bull it is important to choose the right probe size since its weight and diameter influence transmission of the stimulus to the nerves. Probes with larger diameter produce a stronger response to stimuli of a given electrical output than smaller diameter probes.

The recommended probe diameter for bulls weighing 1200 - 2000 lbs (550 - 900 kg) is 6.5 to 7.5 cm. For larger bulls, a 9 cm diameter probe may be necessary to achieve ejaculation.

## Bull Restraint

Ideally, bulls are restrained in a chute with good footing without the head caught in a head gate. If they are caught by the head, they are more likely to lie down during the procedure. In rare instances, it may be necessary to prevent a bull from lying down during electroejaculation by applying a restraining belt under the bull's chest. A 30 inch (76 cm) wide chute can accommodate most bulls. It is extremely important to locate at least one strong pole behind the bull. With only one restraining pole, a height of 28 - 30 inches (71 - 76 cm) may be preferred if the scrotum and testicles are to be examined.

### **Technique (Fig. 6):**

1. Move the bull to the chute and position the pole behind. Set up the electroejaculator beside the bull leaving it turned on and ready to be used.
2. Using a palpation sleeve, the rectum is emptied of feces and a longitudinal massage is applied over the ampullae and urethralis muscle for 1 - 2 minutes.
3. A lubricated probe is introduced into the rectum with the electrodes facing ventrally. Make sure the electroejaculator is turned on before performing this step. If it is turned on after inserting the rectal probe, the bull may receive a strong electrical pulse that will increase the level of stress in the animal.
4. Electrical stimulation begins slowly until the bull shows a minimal response. Consecutive stimuli are then given, each increasing in intensity a small amount. Stimuli should last 1 - 2 seconds and then be discontinued for 0.5 - 1 second before the next one starts.
5. After several stimulations, clear pre-seminal fluid begins to flow from the protruded penis. This clear pre-seminal fraction should not be collected.
6. As soon as the cloudy sperm rich fraction begins to flow from the penis, a collection cone with the test tube is placed over the penis and the sample is collected.
7. After collecting a suitable sample, the stimulation is stopped and the rectal probe is removed. The semen sample is then taken to the lab for evaluation and processing.



Figure 6. Semen collection by electroejaculation. (Courtesy of Dr. Albert Barth).

While performing the procedure is important to obtain penile protrusion for examination of the penis and prepuce. The majority of the bulls emit semen without excessive stimulation. However, if a bull has not ejaculated after reaching the highest level of stimulation, 3 - 4 stimulations in the maximum level can be done, followed by a rest period of 1 - 2 minutes with the probe still inside the rectum. Often on a second attempt to electroejaculate bulls, the penis will not protrude; therefore, while the bull rests, the penis should be held manually with a gauze sponge to prevent retraction into the preputial cavity. All four fingers with the gauze should wrap the glans penis. Do not attempt to catch the penis over the preputial region, as it will roll off into the prepuce. Semen emission commonly occurs during the rest period thus the collector should be attentive to catch the emission. After resting the bull, stimuli beginning at two voltage increments below the maximum are begun in a second attempt to obtain an ejaculate. This is often successful. Bulls should be sexually rested for 1 - 2 days before electroejaculation to allow sperm to accumulate in the ampullae. With good equipment and proper technique, only about 2% of normal fertile bulls fail to emit semen by electroejaculation. Electroejaculation without anesthesia is considered to be painful to bulls and is controversial in some countries. Vocalization during electroejaculation, an elevation of circulating adrenal progesterone and cortisol after electroejaculation are evidence of pain. Therefore, the procedure must only be done by personnel with proper training and always in the gentlest way possible.

# Evaluation of Semen Quality

Semen traits to be evaluated include the following:

## Volume

The capacity to produce spermatozoa per gram of testicular tissue (Daily Sperm Production; DSP) is well correlated to scrotal circumference measurement in young bulls. In bulls on a regular semen collection schedule, volume and sperm concentration indicate a bulls capacity to produce sperm and also allow an AI center to monitor testis function.

## Density

Density may be classified as follows:

- Very Good (VG): creamy, grainy semen with 750 to 1 billion or more spermatozoa per ml
- Good (G): milk-like semen with 400 to 750 million spermatozoa per ml
- Fair (F): skim milk-like semen with 250 to 400 million spermatozoa per ml
- Poor (P): translucent semen with less than 250 million spermatozoa per ml

Semen collected by AV may be more concentrated, and cleaner, than samples collected by electroejaculation or by massage. With the latter two methods, volume and density may not be representative of a bull's normal capacity to produce sperm; however, with good technique, clean ejaculates with good concentration are often possible when the bulls are sexually rested and handled in a calm way.

## Motility

### Gross Motility

A 5 mm diameter drop of the semen is placed on a warm glass-slide and mass motion is observed under bright field microscopy at 40 X magnification with the field diaphragm closed. Factors that affect mass motion of the spermatozoa include concentration, percentage of progressively motile cells and the speed/vigor of sperm motion. If one or more of these factors is compromised the swirling of mass motion will be suppressed.

Descriptive assessment of gross motility:

- Very Good (VG): rapid dark swirls and eddies
- Good (Good): slower swirls and eddies



- Fair (F): no swirls, but prominent individual cell motion
- Poor (P): little or no individual cell motion

## Individual Progressive Motility

- A 5 - 7  $\mu$ l volume of the semen is placed on a new warm glass-slide creating a drop approximately 3-5 mm in diameter, which is then covered with a coverslip. The volume of semen used (5 vs. 7  $\mu$ l) for evaluation will depend on the size of the coverslip preferred (18x18 mm or 22x22 mm coverslips).
- The sample is observed under phase contrast microscopy at 200 - 400 x magnification and the percentage of sperm cells having progressive linear motion is determined. If the semen is too concentrated, the semen sample may be diluted with a buffered diluent or semen extender before coverslipping.
- Descriptors:
  - Very Good (VG): 80 - 100% motile
  - Good (G): 60 - 79% motile
  - Fair (F): 40 - 59% motile
  - Poor (P): <40% motile

Visual microscopic analysis of individual progressive motility is somewhat subjective even when performed by very skilled people and becomes tedious when large numbers of samples must be analyzed. Computer Assisted Semen Analysis (CASA) systems (Fig. 7 and Fig. 8) have the potential to increase objectivity of analysis and reduce worker fatigue. In addition, CASA systems should reduce variability in analytical results between AI centers.



Figure 7. SpermVision CASA system. (Courtesy of Minitube International).

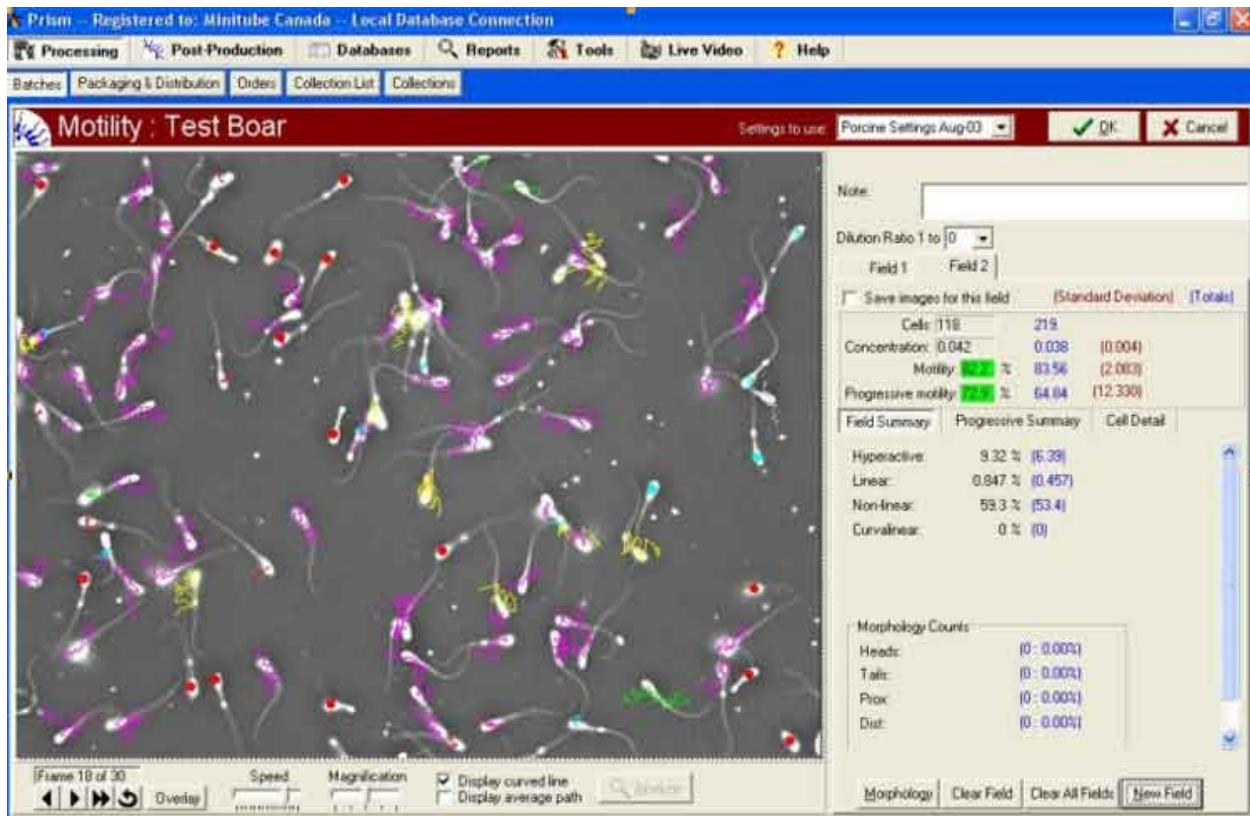


Figure 8. View of motility analysis by the SpermVision CASA system. (Courtesy of Minitube International).

## Sperm Morphology Evaluation

Sperm cells are translucent when observed with bright field microscopy; therefore, phase contrast microscopy or the use of sperm stains are needed for analysis of sperm morphology. Eosin-nigrosin stain is commonly used as a "live/dead" stain because in addition to providing background-contrast for sperm cells with the nigrosin component, sperm membrane penetration by eosin, or lack thereof, is an indicator of sperm membrane integrity and thus of sperm viability.

### Technique:

1. Put a glass slide on a warming plate (37°C) for 30 - 60 seconds.
2. Put a 5 - 6 mm droplet of eosin-nigrosin stain at one end of the glass slide.
3. Put a droplet of semen beside the droplet of stain (Fig. 9). The droplet's size depends on the density of the semen:
  - 3 mm for VG density
  - 4 mm for G density
  - 5 mm for F or P densities
4. Mix the stain and the semen on the slide.
5. Spread the mixture slowly on the slide from one end to the other using a wooden applicator stick or the edge of another glass slide (Fig. 10).
6. Dry the smear quickly by blowing air over it.
7. Perform the sperm morphology evaluation at 1000 x magnification using immersion oil, counting at least 100 sperm per sample (Fig. 11). If a high number of abnormalities are observed, a count of 300 or more sperm will give a more accurate differential count.

Eosin-nigrosin stain is very hypotonic and, therefore, may cause artifacts in sperm morphology. Drying stained smears quickly by using warm glass slides and blowing over them after spreading the stain will minimize formation of artifact.

The percentage of individual progressive motile sperm and the percent of sperm staining alive are highly correlated when semen quality is good.



Figure 9. Glass slide on a warming plate with droplets of stain and semen before making a smear for sperm morphology evaluation.



Figure 10. A second glass slide may be used to spread the semen - stain mixture.



Figure 11. A bovine semen smear stained with eosin-nigrosin at 1000 X magnification. The white cells are live sperms (6 cells) and the red ones are dead sperms (1 cell).

## Semen Concentration

Although a rough prediction of sperm concentration can be determined by visual evaluation of a semen sample, exact concentrations must be known to determine the number of doses of semen to be frozen. The following methods can be used to determine sperm concentration:

### 1. Electronic Counting

Photometers such as the Photometer SDM5 (Fig. 12) are in common use. A sample of raw semen is diluted in a cuvette with a predetermined volume of diluent and analyzed with the photometer. This analysis takes about 30 seconds. The volume, progressive motility, number of sperm per dose and the volume per dose is entered. The number of doses to be frozen and the final extender volume will be calculated.



Figure 12. Photometer SDM5 for determination of sperm concentration, and automatic calculation of extender volume required and number of doses that can be produced from an ejaculate. (Courtesy of Minitube International).

## 2. CASA Systems

Systems such as SpermVision (Fig. 7 & Fig. 8), use computer programmed digital analysis of microscope fields of moving sperm. Several characteristics of motility are quantified, and sperm concentration is also determined. These systems require appropriate dilution of semen samples before filling of one or more commercially supplied disposable counting chambers with a depth of 20 microns. A counting chamber is placed under a microscope and up to 7 microscope fields are analyzed in just seconds per field. Data can be stored and provided on customized printed forms.

## 3. Hemacytometer (Fig. 14)

Hemacytometers are used as the standard method for determination of cell concentrations and for calibration of electronic systems of cell counting. The method is very reliable and inexpensive, but will take about 10 minutes per sample and involves somewhat tedious visual counting of sperm. Sperm dilution prior to filling the 2 chambers of the hemacytometer can be done in a variety of ways. One method which facilitates accurate dilutions of semen is the Unopette® Microcollection system (Becton-Dickinson, Rutherford, New Jersey). Unopette® Microcollection systems (Fig. 13) are used in laboratories to count red or white blood cells, but

also work well for counting sperm. Unopettes utilize a plastic reservoir containing an exact volume of a diluent and a capillary pipette of exact volume.



Figure 13. Components of a Unopette® Microcollection system: plastic reservoir with a known volume of solution and a capillary pipette stored inside a sharp-ended cover to perforate the plastic reservoir.



Figure 14. Hemacytometer with the Neubauer chamber. It requires an exclusively designed cover slip that comes in the original hemacytometer packaging. (Courtesy of the Melissa Rouge).

### Technique:

The sharp-ended cover over the capillary pipette is used to puncture the top of the fluid container. The capillary pipette is then withdrawn from the sharp-ended cover to be filled with semen. The pipette is held almost horizontally and touched to the edge of a semen sample to allow filling by capillary action. The diluent vial is squeezed slightly before the pipette is inserted through the perforated top. Releasing the squeeze draws the semen from the capillary pipette into the vial. Several slight squeezing actions rinse the inside of the capillary tube. The collar end of the capillary tube is fitted onto the diluent vial and the capillary tube can then be used to fill both chambers of the cover slipped hemacytometer (Fig. 14). The solution present in the Unopette plastic reservoir inhibits sperm motility. The filled hemacytometer is then allowed to stand for 3 - 5 minutes to let the sperm settle to the bottom of the chambers. The full grid on each hemacytometer chamber has 9 main squares, each of which is 1 mm square (Fig. 15). Each of the main squares (1 mm<sup>2</sup> each) located on the four corners of the grid are divided into 16 smaller squares (Fig. 15). The main central square (1 mm<sup>2</sup>) is divided into 25 squares and each of these large squares is divided into 16 tiny squares (Fig. 16). With a phase contrast microscope using 400 x magnification, count the number of sperm in a minimum of 2 main squares (2 mm<sup>2</sup>) per grid, for a total of 4 main squares (4 mm<sup>2</sup>) per chamber, and determine the mean number of sperm per square mm (Fig. 15). Count sperm heads that are present inside or touching the left

sperm per square mm (Fig. 15). Count sperm heads that are present inside or touching the left and top side of each square mm (Fig. 16).

The mean number of sperm per square mm is multiplied by factors for chamber depth and dilution of semen and then converted to sperm per cubic centimeter, for example:

- Hemacytometer depth 0.1 mm x 10
- Dilution factor x 200
- Sperm/mm<sup>3</sup> x 1000 = sperm cm<sup>3</sup>
- 0.25 or 0.5 ml straw x 0.25 or 0.5 ml = sperm/dose
- Sperm/dose x % motile = motile sperm /dose

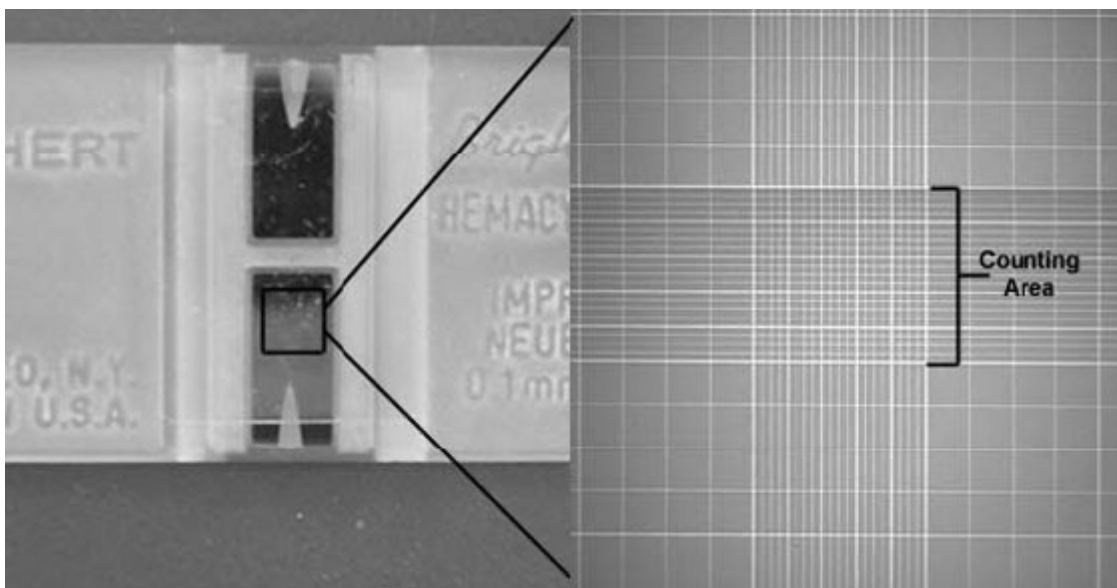


Figure 15. Left: Top view of the hemacytometer showing the location of the chamber's full grid in one of the two sides where the count is done. Right: Magnification of the hemacytometer full grid that contains nine main squares, each of which is 1 mm x 1 mm. Each corner main square has 16 small squares. (Courtesy of the Melissa Rouge).



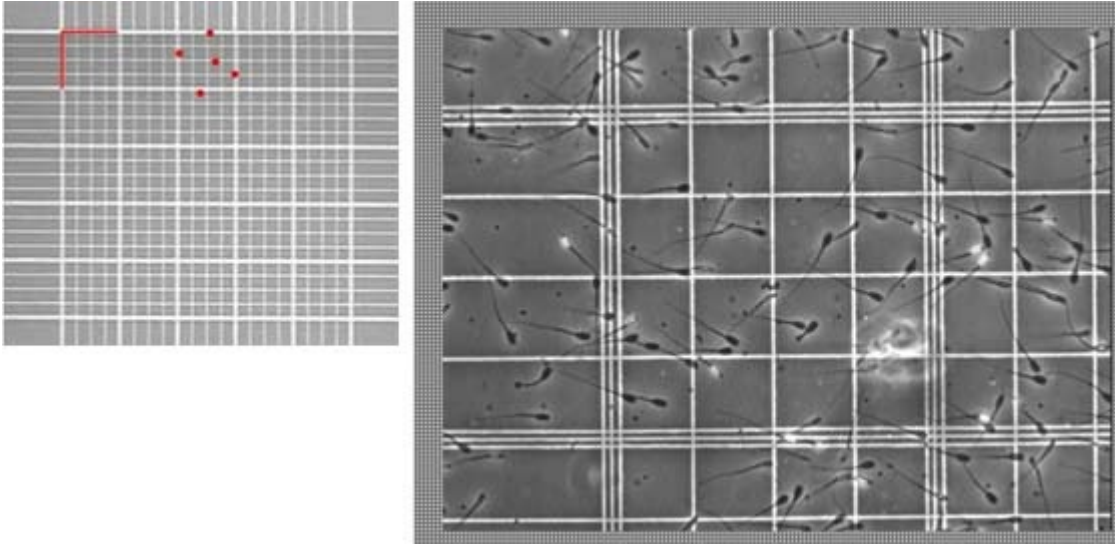


Figure 16. Left: Higher magnification (100 x) of the main central counting square area (1 mm<sup>2</sup>) of the hemacytometer grid showing the 25 squares. Each of these squares contains 16 tiny squares. The red lines are bordering one square where sperm are counted. If red dots represent sperm, one would count 3 sperm in the top middle large square. (Courtesy of the Melissa Rouge). Right: Higher magnification (200 x) of the main central counting square area showing the top- left large square with sperm cells inside.

## Progressively Motile Morphologically Normal Sperm Cells

After the semen quality evaluation has been performed and a calculation of the concentration of sperm is obtained, it is important to determine the number of Progressively Motile Morphologically Normal (PMMN) sperm in the ejaculate. PMMN is used to calculate the number of doses that can be frozen.

Example: in a 9.5 ml ejaculate with the following characteristics: 75% progressively motile sperm; 80% morphologically normal sperm; concentration of 620 million spermatozoa/ml

Next: Calculate the number of sperm in the ejaculate:  $9.5 \text{ ml} \times 620 \text{ million/ml} = 5.89 \text{ billion sperm}$

Then: Calculate the number of PMMN sperm:  $5.89 \text{ billion sperm} \times 75\% \text{ motile} \times 80\% \text{ normal} = 3.534 \text{ billion PMMN sperm in the ejaculate.}$

## Calculation of the Number of Doses and Dilution

The average number of sperm provided per dose in the last 5 decades in North America has been 10 million progressively motile sperm post-thaw. Since approximately 50% of sperm die in the freezing process, it is necessary to double the number of sperm per dose; i.e., if 10 million progressively motile sperm post-thaw were the goal, the number of sperm per dose would be 20 million progressively motile sperm.

To continue with the previous example, the number of doses that can be packaged with an ejaculate that has 3.534 billion PMMN sperm cells would be 176 straws after doing the following calculation.

$$\frac{3.534 \text{ billion PMMN}}{20 \times 10^6 \text{ PMMN/straw}} = 176 \text{ straws}$$

## Extenders for Freezing Semen

Certified Semen Services (CSS) is a voluntary non-profit organization that regulates many North American AI businesses. CSS establishes minimum requirements for health of bulls, establishes standards and rules to assure the authenticity of semen products, disseminates information concerning the handling and processing of semen, establishes standard procedures for the protection of the user, establishes uniform labeling of semen containers, facilitates the identification of semen products, and is engaged in other activities for improvement of animal agriculture.

CSS has approved two different protocols that can be used to extend bovine semen:

- A. The Standard CSS Protocol which is a 2-step method
- B. The Alternative CSS protocol which is a 1-step method

### A. The Standard CSS Protocol

The Standard CSS Protocol used in the USA is a 2-step method. In Europe however, 1-step protocols are more commonly used. The most common extenders used in the Standard CSS protocol have Tris (hydroxymethyl aminomethane) or sodium citrate as the buffer component, require egg yolk to protect sperm cells against cold-shock, and consist of two fractions (A and B) plus a cocktail of antibiotics. Fraction B is the same as A except it contains 14% of the cryoprotectant glycerol. New extender formulations which do not require egg yolk are now available and approved by CSS. The 2-step protocol will yield a final concentration of 20% egg yolk, 7% glycerol, glycerol, 50 µg of tylosin, 250 µg of gentamicin, 150 µg of lincomycin, and 300 µg of spectinomycin in each ml of extended frozen semen. Examples of commercially available extenders for the 2-step method are Biladyl® (Fig. 17) and AndroMed® CSS 2 step (no egg yolk) (Minitube Germany).

### Technique of Semen Dilution:

- Antibiotics are added carefully to the neat semen in a proportion of 0.02 mL of antibiotic cocktail per mL of neat semen. Antibiotics should be allowed a 3 to 5 minute time period to be in contact with the neat semen.
- Dilute the semen slowly with a small volume of warm Fraction A extender in a 50 ml dilution tube. Depending on the calculated number of straws to be processed, the ejaculate will be then extended with Fraction A to 50% of the calculated final volume. Fraction A extender temperature and the semen temperature should be the same (aprox. 28-32°C) (Fig. 18).
- Cool the extended semen slowly to 4°C over a minimum of 2 hours by placing it first at ambient temperature, and later into a cool room or refrigerator at 4°C. Note: A 300 ml volume of liquid at a temperature of 30 to 35°C will cool to 4°C over a period of about 2 hours.
- The other half extender volume is the Fraction B containing the glycerol which must be pre-cooled at 4°C. Fraction B should be added gradually to the semen extended with Fraction A over a period of 30 minutes until a 1 to 1 ratio is reached. The final volume of extended semen will contain 7% glycerol concentration.
- The extended semen should be allowed to equilibrate at 4°C for at least 4 hours. During this time, the straws may be filled, sealed and placed on racks for freezing and counting



Figure 17. Two step extender Biladyl® with its components: Fraction A, Fraction B and Cocktail AB (antibiotics). (Courtesy of Minitube International).



Figure 18. Stainless steel water bath with temperature control. (Courtesy of Minitube International).

## B. The Alternative CSS Protocol

The Alternative CSS Protocol is a one-step method. This protocol is approved for a 20% Egg Yolk Tris (hydroxymethyl aminomethane) extender. The extender is not fractionated and contains 7% glycerol plus antibiotics. The 1-step protocol will yield a final concentration of 100  $\mu\text{g}$  of tylosin, 500  $\mu\text{g}$  of gentamicin, 300  $\mu\text{g}$  of lincomycin, and 600  $\mu\text{g}$  of spectinomycin in each ml of extended frozen semen. An example of a commercially available extender for the 1-step method is Triladyl® CSS (Minitube Germany) (Fig. 20) and the egg-yolk free extender AndroMed® CSS one step (Minitube Germany). As mentioned previously, 1-step protocols are more commonly used in Europe.

### Technique of Semen Dilution:

- Match extender temperature to semen temperature and maintain them in a water bath; usually this is at 28 - 32°C (Fig. 18). The antibiotics should also be maintained at the same temperature.
- Antibiotics are added to the neat semen in a proportion of 0.02 ml of antibiotic cocktail per ml of neat semen. Antibiotics should be allowed a 3 to 5 minute time period to be in contact with the neat semen.
- Dilute the semen slowly with a small volume of extender in a 50 ml dilution tube.
- Depending on the number of straws to be processed, extend the ejaculate to the final volume.
- A Smart Dispenser (Fig. 19) could be used to add the extender in a precise, electronically controlled automatic manner.

controlled, automatic manner.

- Cool the extended semen tube slowly to 4°C over a minimum of 2 hours by placing it at ambient temperature, and later into a cool room or refrigerator at 4°C. Note: A 300 ml volume of liquid at a temperature of 30 to 35°C will cool down to 4°C over a period of 2 hours.
- After the initial cooling period, the extended semen should be allowed to equilibrate at 4°C for at least 4 hours. During this time, the straws could be filled with extended semen, sealed and placed on racks for freezing and counting.



Figure 19. Smart dispenser to automatically and electronically add semen extender to an ejaculate. (Courtesy of Minitube International).



Figure 20. One step extender Triladyl®. (Courtesy of Minitube International).

A magnetic stirrer (Fig. 21) could be used in the preparation of extenders to ensure that all of the extender components are completely dissolved and mixed. After stirring the extender may be filtered.



Figure 21. Magnetic stirrer with heater and contact thermometer. (Courtesy of Minitube International).

## Packaging the Semen in Straws

At present, most AI companies throughout the world package and market frozen semen in straws which are available in two different sizes: mini straw (0.25 ml) and medium straws (0.5 ml) (Fig. 22).

The packaging of the extended semen can be done at room temperature or after cooling to 4°C. Straws can be labeled either manually or with computerized equipment (Fig. 23 & Fig. 24) before filling.

Preprinted straws may be filled manually with a micropipettor (Fig. 25) when the number of straws is low.

Semiautomatic filling equipment, such as the SFS 133, (Fig. 27) fills preprinted straws using a vacuum pump and seals them with glass or metal sealing balls.

Due to the high number of straws processed by AI companies, more sophisticated equipment such as the MPP Quatro CombiSystem can be used to label, fill, and seal straws in one step (Fig. 28).

When the straw filling is done at 4°C, spacious cold handling cabinets (Fig. 29) are used in order to maintain semen equilibration temperature while filling and sealing the straws.



Figure 22. Straws in two different sizes 0.25 ml & 0.5 ml. (Courtesy of Minitube International).



Figure 23. HDR manual straw printer for 0.5 and 0.25 ml straws. (Courtesy of Minitube International).



Figure 24. Easy Coder automatic printing machine for both sizes of straws. Capacity: 3,600 straws/hour. (Courtesy of Minitube International).



Figure 25. Micropipettor with a handling wheel for filling individual straws. (Courtesy of Minitube International).

After filling, the straws may be sealed manually with heat sealers or ultrasonic sealers (Fig. 26).





Figure 26. Ultra Seal 21, device that seals straws perfectly with ultrasound. No heat is produced at time of sealing. (Courtesy of Minitube International).



Figure 27. Semiautomatic Filling & Sealing Machine SFS 133 for both sizes of straws. Capacity: 2,000 straws/hour. (Courtesy of Minitube International).



Figure 28. Mpp Quatro CombiSystem, automatic integration of straw printing, filling, and sealing in one step. Maximum capacity: 15,000 straws/hour. (Courtesy of Minitube International).



Figure 29. Cold Handling Cabinet made of stainless steel for semen processing at working temperature of 4°C. (Courtesy of Minitube International).

## The Freezing Process

Sealed straws of extended semen may be frozen using a traditional vapor freezing method or by computerized programmable freezers.

- In the vapor freezing method, the straws are placed on racks (Fig. 30) and held horizontally 4 cm above the surface of liquid nitrogen for 10 minutes. The straws are then plunged into the liquid nitrogen. Specialized floating freezing racks are available that float for about 20 minutes and then descend automatically plunging the straws into the liquid nitrogen.
- Computerized programmable freezers (Fig. 31 & Fig. 32) have the following components: a freezing chamber; a computer with a screen and a source of liquid nitrogen.

Before the straws are placed into the freezing chamber, the freezer should be running with the chamber temperature already at 4°C. The straws are organized on racks and placed into the freezing chamber. The desired freezing curve is chosen and activated by computer. The computer controls the temperature inside the chamber and in the straw and decreasing it from 4°C to the lowest temperature chosen (e.g., -140°C) at the desired rate per minute (e.g., 10°C/min). The computer monitor displays the temperature curves for the freezing chamber and for the straws being frozen. Temperature is controlled by the computer by varying the amount of liquid nitrogen flowing to the freezing chamber from the nitrogen source (Fig. 32). A small number or thousands of straws can be frozen with confidence and accuracy. Computerized freezers have unlimited choices of freezing programs, freezing rates from +0.01 to 60°C/minute, at an accuracy of 0.01°C, and temperature ranges from 40°C to -180°C.

After the freezing process, the straws are transferred to a liquid nitrogen tank for storage, distribution and marketing (Fig. 33).



Figure 30. Floating freeze rack for vapor freezing of semen packed in straws. (Courtesy of Minitube International).



Figure 31. Ice cube 14S Computer Controlled freezing system with integrated PC. Capacity: 1,080 straws (0.25 ml & 0.5 ml straws). (Courtesy of Minitube International).



Figure 32. Semen Freezer MT with an automatic freezing curve from +5°C to -120°C with very low liquid nitrogen consumption. The dewar on the right has a capacity of 100 liters. (Courtesy of

Minitube International).



Figure 33. Liquid nitrogen semen storage tanks with different sizes, capacities and evaporation rates.