



# **Proper steps for bull semen dilution and freezing**

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## Introduction

Since Polge reported the first successful cryopreservation of spermatozoa in 1949, spermatozoa from many mammalian species have been successfully frozen. Since then, millions of calves have been produced after artificial insemination (AI) of females with cryopreserved sperm. In fact freezing and thawing of bull semen is necessary for widespread utilization of genetically superior sires. It allows thousands of cattle producers to access bulls/genetics, they could otherwise not afford to purchase.

A.I Bull studs are devoted to assure that maximum numbers of spermatozoa survive the freeze-thawing process, thus providing high numbers of viable sperm for insemination coming from disease-free bulls and in a proper package.

Semen cryopreservation implies storage of spermatozoa at subzero (i.e. frozen) temperatures. The cryogen normally used to accomplish this task is liquid nitrogen (LN -196°C). If spermatozoa withstand the freezing and thawing process, spermatozoa integrity may be maintained indefinitely in liquid nitrogen because metabolic activity of spermatozoa is considered to be negligible at this temperature.

Maintaining the highest fertilizing potential of semen through the freeze/thaw process requires the best available conditions for cryopreservation. Many studies over the past 50 years have attempted to optimize the freezing and thawing conditions for sperm from literally hundreds of species. However, sperm from each specie possesses its own unique characteristics including specific sperm size, shape, membrane lipid and protein composition and metabolic requirements, each of which affects the conditions required to cryopreserve the cells.

Regardless of the successful research to improve cryopreservation, some spermatozoa fail to survive freezing and thawing. The reasons for cell death due to freezing and thawing are not fully understood. Two known causes of sperm injury/death are: a) formation of ice crystals within spermatozoa (intracellular ice formation), when fast cooling rates are used resulting in irreparable damage to the sperm membrane and, b) development of regions of high solute concentrations (solution effects) that dehydrate the cell when slow rates of cooling or thawing are employed.

The objective of any freezing procedure is to optimize the cooling rate so that cell death due to formation of intracellular ice and hypertonic solutions is minimized.

The quality of the semen produced by a cryopreservation laboratory is controlled by numerous interactions between individual processing procedures including: cooling rate, equilibration time, equilibration time x thaw rate, thaw rate x freezing rate, freezing rate x glycerol level and glycerol level x thaw rate. Due to these interactions, evaluating the effects of individual components of the cryopreservation process on semen viability can be misleading. Thus, there is no clearly defined "best" semen extender or processing protocol. It is the responsibility of each A.I lab to consistently monitor the quality of their processing protocol using acceptable viability criteria and final thawing and handling procedures that are consistent with on-farm recommendations.

One of the main benefits of A.I is hygienic safety. Several studies on bacteriological quality of bull semen show however that semen is often far from being sterile and is often contaminated by numerous microbial agents like bacteria and fungi, which can be currently found in cattle environment. The level of microbial contamination depends highly on the conditions of semen collection as well as the microbial quality of the semen extender.

Since the development of cryopreserving techniques for mammalian spermatozoa, glycerol, egg yolk or milk were most commonly used as components of freezing extenders. Extenders used for cryopreservation of bovine spermatozoa generally contain 20 % egg yolk. Therefore, the risk of microbiological contamination from egg yolk might occur. IMV Technologies has introduced a new concept in bovine sperm cryopreservation: Bioxcell™, an egg yolk-free sterile extender, which has been widely used in artificial insemination centers. The aim of this paper is to describe the procedures to dilute and freeze bovine semen with Bioxcell™.

The ultimate aim of laboratory assessment of semen characteristics is prediction of fertilization and gestation results.

## **PROTOCOL OF BIOXCELL with frozen semen**

### **Extender preparation**

- 1) Place the bottle of 100ml concentrated extender in a water-bath at 32-34°C for 10 minutes.
- 2) Place an Erlenmeyer of 400ml bi-distilled apyrogen sterile water or equivalent in a water-bath at 32-34°C.



- 3) Put the 100ml concentrated extender into the 400ml bi-distilled apyrogen sterile water or equivalent. Rinse the bottle twice with the final solution.



- 4) Mix gently the solution; the extender is ready to use.
- 5) It is recommended to use the extender for semen dilution within 6 hours since time of preparation.

Once prepared, the BIOXCELL extender can be frozen at  $-20^{\circ}\text{C}$  (it is recommended to do that operation just after the extender preparation, should you desire to preserve it for a later use). If so, then split the solution in small 100ml aliquots. Thawing must be done rapidly (plunge the flasks directly into a water-bath at  $32-34^{\circ}\text{C}$  for 15 minutes). Mix once the thawed extender. Do not freeze again.

### **User's instructions**

#### **1) Protocol for a one-step dilution (at $32-34^{\circ}\text{C}$ )**

##### **1a) Semen packaging at room temperature ( $20^{\circ}\text{C}$ )**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at  $32-34^{\circ}\text{C}$ .



- Mix the tube once.
- Incubate the pre-diluted semen in a water-bath at 32-34°C for 10 minutes



- Do the final dilution by adding the pre-diluted semen into the extender. Do not mix.



- Leave the flasks of diluted semen at room temperature (20°C) for 10 to 15 minutes.



- Mix and pack semen at 20°C.



- Place the straws horizontally in a plastic container, then place the container in a cold cabinet at +4°C. The straws should be protected from cold so they go from 20°C down to +4°C in one hour to one and a half hour. The number of straws should be adjusted according to the type of container used. For example : 300 mini straws in a 65mm goblet.



- Equilibrate semen for 3 to 5 hours away from light.
- Place the straws on the freezing racks into the cold cabinet.

- Freeze. Remember that your freezer machine has to be close to the cold cabinet.



### **1b) Semen packaging at +4°C**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at 32-34°C.
- Mix the tube once.
- Incubate the pre-diluted semen in a water-bath at 32-34°C for 10 minutes.
- Do the final dilution by adding the pre-diluted semen into the extender. Do not mix.
- Place the flask of diluted semen in a plastic container with a constant level of water from the water-bath at 32°C (the level of water should not be above the level of diluted semen). Then place the container in a cold cabinet at +4°C. It is recommended to place a thermometer in the water. When the temperature reaches 12-14°C, you can add ice-cubes to the water. Anyway, the 4°C temperature should be reached within one hour – one and a half hour.



- Equilibrate diluted semen for 3 to 5 hours away from light.
- Pack semen into straws in the cold cabinet.



- Place straws on the racks.



- Freeze.

## **2) Protocol for a two-step dilution (+32-34°C and + 4°C)**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at 32-34°C.
- Mix the tube once.

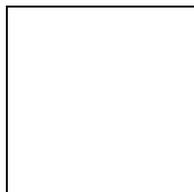
- Place the pre-diluted semen in a water-bath at 32-34°C for 10 minutes, and add it to 20 to 25 ml of Bioxcell at 32-34°C.



- Place the flasks of diluted semen in a plastic container filled with water from the water-bath at 32-34°C. It is recommended to place a thermometer in the water.
- Then, place the flasks in a cold cabinet at +4°C for one hour to one and a half hour (You can add ice cubes when the temperature reaches 12-14°C).



- Do the final dilution at +4°C.



- Equilibrate diluted semen for 3 to 5 hours away from light.
- Pack semen into straws at +4°C.
- Place straws on racks.
- Freeze.

## **PROTOCOL OF BIOXCELL with fresh semen**

### Extender preparation

- 6) Place the bottle of 100ml concentrated extender in a water-bath at 32-34°C for 10 minutes.
- 7) Place an Erlenmeyer of 400ml bi-distilled apyrogen sterile water or equivalent in a water-bath at 32-34°C.
- 8) Pour the 100ml concentrated extender into the 400ml bi-distilled apyrogen sterile water or equivalent. Rinse the bottle twice with the final solution.
- 9) Mix the solution; the extender is ready to use.
- 10) It is recommended to use the extender for semen dilution within 6 hours from time of preparation.

Once prepared, the BIOXCELL extender can be frozen at – 20°C (it is recommended to do that operation just after the extender preparation). Split the solution in small 100ml aliquots. Thawing must be done rapidly (plunge the flasks directly into a water-bath at 32-34°C for 15 minutes). Mix once once the extender is thawed. Do not freeze again.

### **User's instructions**

#### **2) Protocol for a one-step dilution (at 32-34°C)**

##### **1a) Semen packaging at room temperature (20°C)**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at 32-34°C.
- Mix the tube once.
- Incubate the pre-diluted semen in a water-bath at 32-34°C for 10 minutes
- Do the final dilution by adding the pre-diluted semen to the extender. Do not mix.
- Leave the flasks of diluted semen at room temperature (20°) for 10 to 15 minutes.
- Mix and pack semen at 20°C.
- Place the straws horizontally in a plastic container, then place the container in a cold cabinet at +4°C.

The straws should be protected from any cold shocks. They should go from 20°C down to +4°C in one hour to one and a half hour. The number of straws should be adjusted according to the type of container used. For example : 300 mini straws in a 65mm goblet.

- Equilibrate semen for 3 to 5 hours away from light.
- Store semen at +4°C in our thermostatic cabinet re f. 005485.  
Storage (time between semen dilution and A.I.) should not exceed 72 hours.

### **1b) Semen packaging at +4°C**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at 32-34°C.
- Mix the tube once.
- Incubate the pre-diluted semen in a water-bath at 32-34°C for 10 minutes.
- Do the final dilution by adding the pre-diluted semen to the extender. Do not mix.
- Place the flask of diluted semen in a plastic container with a constant level of water from the water-bath at 32°C (the level of water should not be above the level of diluted semen). Then place the container in a cold cabinet at +4°C. It is recommended to place a thermometer in the water. When the temperature reaches 12-14°C, you can add ice-cubes to the water. In any case, the 4°C temperature should be reached within one hour – one and a half hour.
- Equilibrate diluted semen for 3 to 5 hours away from light.
- Pack semen into straws in the cold cabinet.
- Store semen at +4°C in our thermostatic cabinet re f. 005485  
Storage (time between semen dilution and A.I.) should not exceed 72 hours.

## **2) Protocol for a two-step dilution (+32-34°C and + 4°C)**

- After semen examination, do a pre-dilution 1:1 with Bioxcell at 32-34°C.
- Mix the tube once.

- Place the pre-diluted semen in a water-bath at 32-34°C for 10 minutes, and add it to 20 to 25ml of Bioxcell at 32-34°C.
- Place the flasks of diluted semen in a plastic container filled with water from the water-bath at 32-34°C. It is recommended to place a thermometer in the water.
- Then, place the flasks in a cold cabinet at +4°C for one hour to one and a half hour (You can add ice cubes when the temperature reaches 12-14°C).
- Do the final dilution at +4°C.
- Equilibrate diluted semen for 3 to 5 hours away from light.
- Pack semen into straws at +4°C.
- Store semen at +4°C in our thermostatic cabinet ref. 005485  
Storage (time between semen dilution and A.I.) should not exceed 72 hours



### **IMPORTANT!**

- Fresh semen should be stored at +4°C in our thermostatic cabinet ref. 005485 until A.I.
- Time between semen dilution and A.I. should not exceed 72 hours
- Do not warm semen before A.I.
- Use IMV fresh semen straws