Ram Semen Processing, Cryopreservation and Non-surgical Insemination Protocol

**Semen Processing**

The concentration and motility of the semen sample are determined using spectrophotometry and a Hamilton Thorne motility analyzer (Beverly, MA), respectively (at least 5 fields of analysis and 500 cells). Samples are frozen with either skim milk egg yolk cryopreservation medium or Tris-egg yolk-glycerol freezing medium.

*If skim milk egg yolk cryopreservation medium is used, then:*

Semen samples are diluted to 1200 x $10^6$ sperm/mL in 37 °C skim milk-egg yolk cooling media (see recipe below). The tubes are then placed in a 37 °C water jacket and cooled to 5 °C over 45 minutes.

The samples are then diluted drop-wise over 5 min (1:1; volume to volume) with 5°C skim milk-egg yolk freezing media (see recipe below) resulting in a final sperm concentration of 600 x $10^6$ sperm/mL and finally loaded into 0.5 mL semen straws.

*If the Tris-egg yolk-glycerol freezing medium is used, then:*

The samples are diluted slowly, in one step, with the Tris-egg yolk-glycerol freezing medium so that the final sperm concentration is 400 x $10^6$ sperm/mL and cooled to 5 °C over 90-120 minutes. The samples are loaded into 0.5 mL straws and frozen.

**Cryopreservation**

Samples can be frozen one of two ways:

1) Box freezing: Samples are placed on a rack and frozen in liquid nitrogen vapor (4 cm above liquid nitrogen) for 10 min and plunged into the liquid nitrogen for storage.

2) Programmable freezer: The samples are frozen using the Cryo Bio System Mini Digitcool UJ400 (IMV Corporation, Minneapolis, MN) with the following curve: 5 °C to -10 °C at 5 °C/min; -10 °C to -130°C at 60 °C/min and then plunged into liquid nitrogen for storage.

Cryopreserved samples are thawed for 30 seconds in a 37°C water bath.

*Semen Cryopreservation Media Recipe from: Paulenz et al., 2007*
Skim milk-egg yolk cooling media:

Dilute 11 grams of non-fat dried skim milk into approximately 80 mL of distilled/deionized water and heat this solution to 95 °C for 10 min. Allow the solution to cool to room temperature and then add 5 mL of egg yolk, 1mg/ml streptomycin sulfate and sufficient water to bring the final volume to 100 mL. Mix the solution until homogeneous.

Skim milk-egg yolk freezing media:

This solution is created by adding glycerol to an aliquot of the skim milk-egg yolk cooling media so that the final glycerol concentration is 14% by volume. The solution is then mixed until it is homogeneous.

Both of these solutions can be frozen in aliquots until the day of use when they can be thawed and used as described.

Tris-egg yolk-glycerol freezing media:

This is a 1-step dilution medium and is also commercial available under the names Biladyl or Triladyl. This recipe makes 500 mL of ram semen cryopreservation diluent.

TRIS (MW 121)  12.112 g
Citric acid  6.8g
Glucose  5.0g
Glycerol  25.0 mL

Fill to 400 mL with distilled, deionized water
Add 100 mL egg yolk (20% by volume)
Add antibiotics (either CSS specifications or at least 500 mg streptomycin sulfate)

Ram semen evaluation medium:

200 mM Tris
65 mM citric acid monohydrate
55 mM glucose

Cervical Artificial Insemination
The estrous cycles of ewes are synchronized using either:

Sponges for 14 days (e.g. Chronogest CR containing 40 mg fluorogestone acetate, Intervet, Milton Keynes, UK) and then given PMSG (400 IU, i.m.; total volume = 4mL from an 18 gauge needle; single injection) 48 hours prior to sponge removal;

-or-

CIDRs for 12 days (e.g. 0.3 g progesterone in an inert silicone elastomer; Pfizer Animal Health, New York, NY). PMSG (400 IU i.m. as described previously) is administered 24 hour prior to CIDR removal.

The ewes are then inseminated twice at 53 and 57 hours following sponge/CIDR withdrawal with a single 0.5 mL straw (contains a minimum of 100 x 10⁶ motile sperm) and a sheep and goat AI gun (e.g. All-2-Mate Goat Gun, Continental Plastics, Delavan, WI). The labium are parted and the insemination gun is inserted upward at a 45° angle. When the gun touches the top of the vagina it is tilted into a horizontal position and gently inserted into the cervix without force. The insemination dose is placed as deep as possible in the cervix of the ewe without cervical manipulation or force. All inseminations are performed with the ewe restrained in a standing position in a sheep handling squeeze chute.

Reference:

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