Rooster Semen Cryopreservation Protocol

Semen samples are collected from roosters by abdominal massage (Burrows and Quinn, 1937) and observed to ensure they are free of urates and feces.

Cryopreservation with ***glycerol*** as the penetrating cryoprotectant

Immediately after collection samples are diluted 1:1 (vol/vol) with 5°C glycerol-free Lake's diluent (Lake and Stewart, 1978; recipe below), placed in a rack on ice and transported to the laboratory (<15 min). Samples are then diluted 1:2 (vol/vol; sample to cryopreservation medium) with 5°C Lake's diluent containing glycerol (11% final concentration, 5°C) in a 5°C room and loaded into 0.5-mL straws. Samples diluted with glycerol cryoprotectant are frozen in an extruded polystyrene foam box containing liquid nitrogen. A rack is placed in the polystyrene box so that the samples are in the vapor 6.4 cm above the liquid nitrogen. The samples are left in this position for 10 min (cooling rate: 10°C/min) and plunged into the liquid nitrogen for storage (Phillips et al., 1996). Samples cryopreserved using glycerol are thawed in a 5°C water bath for 5 min (Phillips et al., 1996).

Glycerol removal techniques

If the cryopreservation medium contains glycerol then it is necessary to remove the glycerol prior to insemination because the glycerol is contraceptive in chickens. Two methods of glycerol removal are described and both should be performed in a 5°C room.

Step-wise dilution
The following example assumes an initial sample volume of 1.5 mL. Glycerol-free Lake's diluent at 5°C is added to the every minute in the following increments: 10 × 100 µL (10 dilutions of 100 µL per dilution), 10 × 200 µL, 10 × 500 µL, and 5 × 1000 µL (Tajima et al., 1989; Phillips et al., 1996; Purdy et al., 2009) and then centrifuged at 300 × *g* for 25 min. After centrifugation, the supernatant is discarded and the pellets are measured to determine volume and sperm concentration.

Accudenz centrifugation
Discontinuous gradients are prepared in 15-mL conical centrifuge tubes by depositing 0.5 mL of 30% (wt/vol) Accudenz (Accudenz, Accurate Chemical & Scientific Corporation, Westbury, NY) under a 5-mL volume of 12% (wt/vol) Accudenz, using the stock solutions (recipe below) described by McLean et al. (1998) and Long and Kulkarni (2004). Glycerolized semen (≤ 1 mL) is layered on the discontinuous gradient and then the samples are centrifuged (1,250 × *g*; 4°C) for 25 min. A centrifuge brake should not be used so that the isolated sperm are not disrupted within the column. After centrifugation the sperm cells are present at the interface between the 12 and 30% layers. To recover the sperm the upper gradient layer is removed first then the 30% layer is aspirated using a new pipette tip so that only the sperm layer remains in the centrifuge tube.

Because of the viscosity of the sperm layer, up to 1 mL of extender, depending on the sperm volume, may be added and mixed prior to recovery of the sperm layer. See Figure 1, below.

Cryopreservation with ***dimethlyacetamide*** (DMA) as the penetrating cryoprotectant

Immediately after collection samples are diluted 1:1 (vol/vol) with 5°C glycerol-free Lake's diluent (Lake and Stewart, 1978; recipe below), placed in a rack on ice and transported to the laboratory (<15 min). Samples are then diluted 1:2 (vol/vol) with Lake's diluent containing DMA (6% final concentration; Blesbois et al., 2007) and loaded into 0.5-mL straws. Samples containing DMA are frozen in a similar manner to the samples treated with glycerol except the height of the rack is 1 cm and the time in the liquid nitrogen vapor prior to plunging and storage is 7 min (cooling rate: 59°C/min; Blesbois et al., 2007). The samples frozen using DMA are thawed in a 50°C water bath for 20 s (Blesbois et al., 2007).

Artificial insemination

Inseminations using 200 x 10^6 sperm are used for either vaginal or intramagnal inseminations.

Intramagnal insemination may be performed according to Song and Silversides (2007). Hens are anaesthetized using an injection of 0.15 mL to 0.20 mL (depending on the size of the bird) of xylazine (20 mg/mL) into the brachial vein. The hen is placed on her right side, her left leg is drawn up and the feathers overlying the left abdominal wall are removed. The area around the site of the surgery is washed with 70% ethanol and an incision (approx. 2.5 cm) is made in the skin between the thigh and the breast close to the last rib. A second incision (approx. 1 cm) is made in the thinnest section of the underlying muscle. A retractor is then used to expose the magnum which lies alongside the body wall in this area of the abdomen. A section of the magnum is held with large forceps and the sperm suspension is injected using a 1-mL syringe equipped with a 20-gauge needle. The exposed loop of the magnum is then returned to the peritoneal cavity and the skin is closed using continuous sutures.

Recipes

Low Lakes Temperature (LLT) Medium *without* penetrating cryoprotectants
169.1mM Sodium glutamate-monosodium salt
33.3mM Fructose
4mM Magnesium Acetate (4H20)
62mM Sodium Acetate (Anhydrous)
3.7mM Potassium citrate
pH 7.5

LLT + *Glycerol*
Identical to the LLT medium except it includes 16% glycerol (by volume)

--OR--

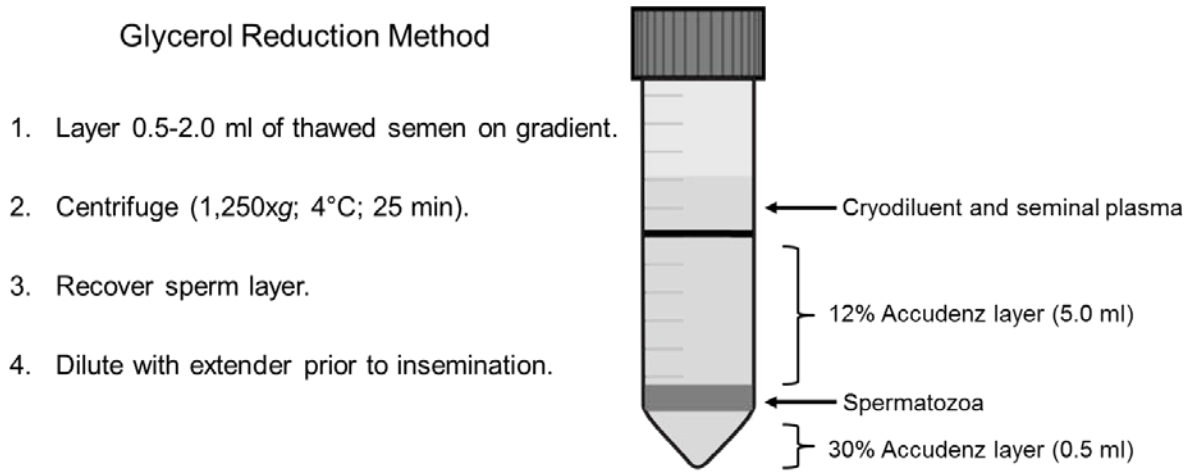
LLT + *DMA*
Identical to the LLT medium except it includes 9% DMA (by volume)

Accudenz Stock Solutions (from McLean et al., 1998) for glycerol removal

Four solutions must be prepared to create the Accudenz columns:

- 30% Accudenz solution: 30% wt/vol Accudenz dissolved in 3 mM KCl + 5 mM TES (pH 7.4)
- TES buffer: 50 mM TES + 130 mM NaCl (pH 7.4; 315 mOsm)
- Diluted TES buffer: TES buffer diluted to 275 mOsm with distilled, deionized water
- 12% Accudenz solution: dilute the 30% Accudenz solution 2.5x with the Diluted TES buffer.

Figure 1. Graphic representation of Accudenz discontinuous centrifugation gradient



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