

## *Preparation of Egg Yolk Samples for RIA*

### Preparation of yolk samples.

- Label an appropriate number of 1.5 ml Eppendorf tubes and pre-record their masses.
- Place a sample of homogenized yolk in each tube and re-record the masses. A total of 5-15 mg of yolk has been appropriate for the detection of steroids in the red-winged blackbird.
- Suspend the yolk sample in 500 ml of distilled water and vortex vigorously until the sample is homogenous (the addition of 2-3 small glass beads will facilitate suspension).

### Extraction of steroids from yolk samples (Part 1).

*(this differs slightly from the plasma extraction)*

- Transfer all samples into 12 ml conical glass tubes using an Eppendorf P1000 Pipetman. Rinse each sample with 500 microliters distilled water and add rinse to sample.
- Prepare the extraction solvent which consists of petroleum ether:diethyl ether in a ratio of 30:70. The diethyl ether must be freshly opened to avoid the formation of peroxides.
- Add 3 ml of extraction solvent to each sample. This can be done with an Eppendorf repeater pipette set on 4 with a 12.5 ml tip.
- Vortex each sample for approximately 10 seconds.
- Let the samples sit for 20 minutes to allow for complete phase separation. While waiting prepare a snap freezing bath of dry ice in methanol.
- Snap freeze each sample and pour off the unfrozen supernatant into a 12x75 test tube.
- Repeat the extraction and combine the extracts.
- Evaporate the samples under nitrogen gas.
- Add 1 ml 90% ethanol to each tube, vortex, and freeze covered with parafilm overnight at -20°C.

### Extraction of steroids from yolk samples (Part 2).

- Run the Beckman TJ-6 centrifuge for about 15 minutes to cool it to 0°C.
- Spin the samples at 2000 rpm for 5 minutes. This will pellet neutral lipids and proteins, which can interfere in the chromatography step.
- Pour the supernatant into a 13x100 test tube and dry under nitrogen gas.
- Resuspend the samples in 500 ml 10% ethyl acetate in isooctane. The Eppendorf repeater pipette may be used.

### *Yolk Sampling From Viable Eggs*

- Use a freshly-laid, unincubated egg. An unincubated egg is used to avoid the development of the embryo, which could potentially synthesize its own hormones.
- Place the egg over a fiberoptic light source. This allows illumination of the egg from underneath so the yolk is visible and avoids overheating of the egg. (It helps to secure a spongy material with a hole in it over the light. Make the hole large enough to just hold the egg in place.)
- Put on some sterile latex gloves.
- Clean the eggshell at the small pole with 70% ethanol and a cotton swab or Q-tip. This will reduce the risk of bacterial infection.
- Remove a sterile Butterfly 27 x 3/8, 8" Tubing Infusion Set (Owens and Minor, 9727 Bauer Dr. East, Indianapolis, IN 46280-1904. Item # 0208-4995-01-01 (\$98.67/120 needles = 1 case, other sizes are available depending on the size of the egg you are sampling)) from its package and attach it to a small syringe. A 1 ml syringe or smaller will work best.
- Use a ruler and a permanent marker to measure off a predetermined volume on the tubing. For this size tubing, 13 mm is equal to 10 ml.
- Penetrate the eggshell with the tip of the needle in the middle of the small pole of the egg. This will allow you to avoid puncturing the air sac, which is vital to the health of the developing embryo.
- Aim the tip of the needle to the middle of the yolk and penetrate the yolk membranes using a slight push. Too much of a push will force the needle completely through the yolk, while a weak push will not penetrate the yolk membranes. Therefore some practice is necessary. You can test to see if the needle is in the yolk by slightly moving the needle back and forth and looking for movement of the entire yolk.
- Apply suction to the syringe and gently draw the yolk to the mark on the tubing. Too much suction can destroy the yolk membranes and draw albumin, so do not rush.
- Gently withdraw the needle from the egg and place withdrawn yolk into an Eppendorf 1.5 ml tube.
- Add 500 µl distilled water, vortex vigorously until homogenous and freeze. The addition of two or three small glass beads prior to mixing will facilitate suspension.
- Cut two small patches of OpSite transparent wound dressing (Briggs Corporation, 7887 University Blvd., Des Moines, IA 50306. Item # = 16-4575 (\$1.05/2" x 3" strip)). Remove the paper backing and patch the hole in the shell crosswise. This flexible dressing will allow for the proper exchange of gases while reducing the probability of infection.
- Return the egg to the nest and allow it to develop.