Ketterson / Nolan Research Group Collection

This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement
This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (https://scholarworks.iu.edu/dspace/handle/2022/7911).

PROTOCOL FOR STEROID LABEL CLEAN UP

PRIOR TO START
Double-check of materials.
Before beginning, make sure that all of the following materials are available.
Nitrogen Gas - Air Gas, 317-241-5097, Acct# TFI 57, PO# 20307-1085
Nanograde Quality 2,2,4-Trimethylpentane (Isooctane) - Fisher Scientific
Celite - NOTE as of 20 February 2004 Sigma's stock of Celite has been compromised, use Fisher Scientific's Celite Analytical Filter Aid #C211-500
Phosphate Buffer - See Appendix C
Ethylene Glycol - Sigma
Propylene Glycol - Sigma
10x75 Test Tubes - Fisher Scientific
Ultima-Gold Scintillation Fluid

All other materials that are needed throughout the assay can be purchased at the biology stockroom. Make sure that you have everything you need if you are going to work over a weekend when the stockroom is closed.

DAY 1
1. Desiccation of celite.
Celite (diatomaceous earth) should be heated in a crucible at 1000^{o}F (range of 950-1100^{o}F) for 24 hours prior to use. The temperature gauge on the oven should be set at about 1.9. Be sure to pack plenty of celite into the crucible so you do not run out while packing the columns.

2. Distillation of ethyl acetate.
We purchase ACS quality ethyl acetate, which requires distillation no more than 24 hours prior to starting. Pour the new or existing ethyl acetate (up to 1.5 liters) into the distillation apparatus and secure the stopper in place. Turn on the cold running water and the heating block (adjusted to 55-60^{o}). The first and last 50 ml of distillate should be discarded. Isooctane (2,2,4-trimethylpentane) is not distilled due to its extreme flammability, so nanograde quality is purchased.

DAY 2
1. Take 500ul of condensed label stock (in assay room fridge), dry down under N2 gas in hood. This is a very concentrated, hot solution so use extreme care when handling. Resuspend in 500ul 10% ethyl acetate in iso-octane. Cover resuspended stock to avoid evaporation.

2. Preparation of chromatography columns.
Set up an appropriate number of 5 ml pipets with rubber bands to hold them in the rack. It is a good idea to make 2-3 extra columns in case some are broken or made improperly. Place a glass bead in the bottom of each pipet. To make the water trap mix 9 grams of celite with 3 ml distilled water using a mortar and pestle. Nine grams of celite will make approximately 36 water traps. (The mortar

will hold up to 10 grams at a time if more is desired.) Mix until you are sure it is homogenous. Shake the celite mix into each pipet using a joint adapter/funnel. Fill to about the 3.5 ml mark, then pack firmly to the 4.5 ml mark using a glass rod. Generally, the celite mix packs to about 1/3 of the loose volume.

To make the glycol phase of the columns mix propylene glycol (1,2-propanediol) and ethylene glycol in equal amounts. Add 3 mls of glycol mix to 6 grams of celite and mix well with a mortar and pestle until homogenous. Six grams of celite will make approximately 9 long columns (27 short columns). Add the celite mix with the joint adapter/funnel to the 3.0 ml mark and pack down to the 4.0 ml mark. Add again to the 2.5 ml mark and pack to the 3.5 ml mark. Add once more to the 2.0 ml mark and pack to the 3.0 ml mark. The columns are now packed with 1.5 ml celite/glycol mixture on top of 0.5 ml celite/water mixture.

The columns should be wet with 4 ml isooctane so they do not dry out. An ependorf Repeater with a 50 ml tip may be used for this. Attach the hoses and turn on the nitrogen gas to push the isooctane through the columns. (The flow rate may be as great as desired at this time, but the columns should never be allowed to dry out. If this does occur, the columns must be thoroughly rewet with more isooctane.) Stop when the solvent drops to within one mark of the celite. Add another 4 ml of isooctane and run through columns, reconstituted stock can then be added to the best column.

3. Chromatography.

To add the stock to the columns, first vortex, then transfer it to its column with an Eppendorf P1000 Pipetman. Rinse the 13x100 test tube with another 0.5 ml of 10% ethyl acetate in isooctane, vortex, and add to the same column.

Attach the hose to the column and regulate the nitrogen pressure so that the column drips no faster than 1 drip every 6 seconds. Discard the eluate (by collecting in the gutter and evaporating in the hood). When the solvent reaches the top of the celite turn off that column and remove the hose. Do not allow any part of the celite to dry out.

All of the following fractions (prior to the last being collected) must be eluted, but T and E2 are unwanted. Fractions are collected in 13x100 test tubes placed under the columns. Discard the T and E2 fractions carefully as they contain hot molecules.

To collect testosterone: Add 4.5 mls 20% ethyl acetate in isooctane as above.
To collect estradiol: Add 4.5 mls 40% ethyl acetate in isooctane as above.
To collect corticosterone: Add 4 mls 50% ethyl acetate in isooctane. (Use 4.5 mls if the 40% fraction was skipped.)

Evaporate the CORT under nitrogen gas in the 40^{o}C water bath. Add 550 µl of buffer and vortex. Begin diluting resuspended CORT in order to achieve a count of ~10,000cpm/100ul (see Appendix A).

AFTER COMPLETION OF PURIFICATION

All glassware that is used should be cleaned in a solution of Radiacwash detergent. Prepare a plastic tub with distilled water and the appropriate dilution of the detergent on Day 1. Glassware can be added to the tub as it is used after it is first rinsed with distilled water in the sink in the hood room. Be sure that all solid particles are removed prior to the soaking, and that there are no air bubbles in the submerged glassware. After all glassware has been soaked for at least a day, rinse three times with the tap-distilled water, and then rinse once with double-distilled water. Place the glassware in the drying oven set at 40oC. Higher temperatures can cause adsorption sites to be formed on the glassware. If this happens the glassware should be rewashed.

Radioactive waste, including scintillation vials, should be disposed of properly. Ask either the radioactivity safety personnel or others in the lab to explain the correct procedures to you.

APPENDIX A - PREPARATION OF TRITIATED STEROID SOLUTIONS

Tritiated steroids are ordered in 250 µCi vials from Perkin Elmer Life Sciences. Prices and catalog numbers are old and have changed, consult the catalog in the Service Center

Testosterone [1,2,6,7,16,17-3H(N)]	NET-553	$493
Estradiol [2,4,6,7,16,17-3H(N)]	NET-517	$377
Corticosterone [1,2,6,7-3H(N)]	NET-399	$288
Progesterone [1,2,6,7,16,17-3H(N)]	NET-1112	$334
Dihydrotestosterone [1,2,4,5,6,7-3H(N)]	NET-453	$744

Using approved safety measure for handling radioactive substances, pipet some pure ethanol into the vial, withdraw the contents and place them into a 10 ml volumetric flask. Continue to rinse and pipet the solution from the vial to the flask several more times. Bring the solution up to 10 mls with ethanol. You now have 250 µCi in 10 mls. To make solutions for the assay you want 10,000 cpm/100 µl. This works out to be about 500 µl of this stock solution (cleaned) in ~100 mls PBSG. Remember to rinse the small beaker you pour the stock solution into for measuring as well as the pipet tip itself. Measure the cpm of the working solution on the scintillation counter and adjust accordingly. It is better to add less buffer at the start and then further dilute than to get it too weak and have to add label. Start with 1ml of cleaned, reconstituted stock in 90ml of PBSG and proceed from this point. All glassware should be soaked in Radiacwash detergent.