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 Using Assay Designs' Testosterone EIA Kit With Bird Plasma

This protocol is an adaptation of the protocol that comes with the Assay Designs Testosterone EIA kit (# 901-065). It incorporates an ether extraction as well as dilution of plasma. It has been validated by Joe Casto for use in dark-eyed juncos, and the validations can be found in the dark-blue EIA notebook in the Ketterson lab. This version is a streamlined update incorporating the last several years of comments and suggestions, written by Danielle Whittaker in August 2007 (*updated May 2010*). Be sure to read the manufacturer's instructions that come with the kit.

This procedure takes 3 days to complete.
Day 1: Aliquotting samples and adding radioactive T to measure recoveries
Day 2: Extraction of the samples
Day 3: Making a standard curve and running the EIA

PRIOR TO START: Make sure you have the following materials:

1. Nitrogen Gas (Air Gas, 1-866-224-7427, extension *810, Customer # TFI 57)
2. Testosterone Kit (Assay Designs Inc. 800 Technology Dr., Ann Arbor, MI 48108 (800-833-8651) **Catalog No. # 901-065** 480 well (5 x 96 well) Kit)
3. Testosterone standard (refrigerator)
4. Labeled testosterone (refrigerator)
5. Ultima-Gold Scintillation Fluid (PerkinElmer, item # = 6013329, $225/2x5L Containers)
6. Scintillation vials (available in stockroom)
7. 13 x 100 test tubes (available in stockroom)
8. Conical Pyrex test tubes (in drawer)
9. Pipet tips
10. Anhydrous diethyl ether (available in chemistry stockroom)
11. Ethyl Alcohol (located in flammable cabinet)
12. Methanol (located in flammable cabinet; can re-use)

DAY 1: Setting up the samples

1. **How many samples?** For each 96-well plate, you will need room for the blanks, positive and negative controls, and the 9-point standard curve, all in duplicate, which use a total of 26 wells, leaving 70 wells. You may run **35 samples and standards** (run in duplicate) on each plate. Typically we use about 3 standards per plate, leaving room for **32 samples**.
2. Arrange your samples in a random order, and space your standards evenly throughout. Use the Testosterone Plate Layout to record your arrangement.
3. Number the 12 or 15 ml conical pyrex test tubes (in the drawer) accordingly.
4. Vortex each sample.
5. Using a Hamilton syringe or a pipette, aliquot a fixed volume of each plasma sample into a test tube.
    a. For male juncos in breeding condition, 20 µl
    b. Females: 40 µl
    c. If running both males and females, pick one volume (usually 30 µl)
    d. If you have a sample with less than the predetermined amount, record it in your notes and add distilled water to make up the difference.
6. For the standards: use the Ketterson lab "Wingfield standard." Use 12.5 µl of standard, and add enough water to equal the volume of the plasma samples.
7. Add 100 µl of distilled H_2O to all samples and standards. (Use the repeater pipette with a previously used tip.)
8. Now, add 20 µl labeled testosterone to all samples and standards using the repeater pipette and a brand-new 0.5 ml combitip. **Also add 20 µl of labeled T to a scintillation vial using the same tip.**
9. Vortex each test tube. Cover them all with parafilm and store them in refrigerator at 4ºC overnight.
10. Add 2.5 µl Ultima Gold Scintillation Fluid to the vial, cap and vortex, and store it in a drawer until day 3.

DAY 2: Extracting and Reconstituting the Samples

1. Make the working solutions of EIA wash buffer (1 in 20 solution) and Assay Buffer 3 (1 in 10 solution) following the manufacturer's instructions. Make only as much as you need. Reagents can be stored for up to one month in sealed bottles.
2. **EXTRACTION:**
    a. Using a 50 ml combitip and the repeater pipette, add 1 ml of diethyl ether to each sample. Vortex each sample for about 10 seconds.
    b. Let the samples sit for 20 minutes to allow complete separation.
    c. While waiting, prepare a snap-freezing bath of dry ice in methanol. Also label your 13x100 test tubes.
    d. Snap freeze each sample and pour off unfrozen supernatant into a labeled 13x100 test tube.
    e. Place the evaporack (leave the corners empty!) into a 40ºC water bath and evaporate with nitrogen.
    f. Repeat steps a-e. You may want to repeat a 3rd time.
    g. After final extraction, be sure the samples are completely dried down.
    h. The pyrex test tubes and their remaining contents can be put into a bucket of soapy water (use the radioactive wash).
3. **RECONSTITUTE THE SAMPLES:**
    a. Use the repeater pipette and a brand-new 0.5 ml combitip to add **50 µl of 100% EtOH** to each sample.
    b. Vortex thoroughly.
    c. Use repeater pipette and a brand new 5 ml combitip to add **300 µl of Assay Buffer 3** to each sample.
    d. Vortex at least 10 seconds.
    e. Cover tubes with parafilm and refrigerate at 4ºC overnight.

DAY 3: Running the EIA, Determining Recoveries, Calculating Results

Preparing:

1. Remove the EIA kit from the refrigerator and let the reagents warm to room temperature for about 30 minutes.
2. Serially dilute the testosterone standard to make the standard curve:
    a. Label 9 13x100 test tubes (#1-9).
    b. Add 1 ml of Assay Buffer 3 to tube #1 and 500 µl Assay Buffer 3 to tubes #2-9.
    c. Remove 40 µl of Assay Buffer 3 from tube #1 and discard. Add 40 µl of the testosterone supplied with the kit (50,000 pg/ml) to tube #1. Vortex.
    d. Add 500 µl of tube #1 to tube #2 and vortex.
    e. Add 500 µl of tube #2 to tube #3 and vortex.
    f. Continue for tubes #4-9.
    g. This prepares enough for 1-2 plates.

Running the EIA:

1. Refer to the Assay Layout Sheet. Pipet 100 µl of Assay Buffer 3 into the NSB and Bo (0 pg/ml standard) duplicate wells.
2. Pipet 100 µl of Standards #1 through #9 into the appropriate duplicate wells.
3. Pipet 100 µl of samples into the appropriate duplicate wells. At this time also pipet 100 µl of each sample into a scintillation vial in order to determine recovery values.
4. Pipet 50 µl of Assay Buffer 3 into the NSB duplicate wells.
5. With a multi-channel pipette add 50 µl of Blue Conjugate into each well except the Total Activity (TA) and Blank wells.
6. With a multi-channel pipette add 50 µl of Yellow Antibody into each well except the Total Activity (TA), NSB, and Blank wells. **NOTE:** Every well should be **GREEN** in color except the NSB wells which should be **BLUE**. The Blank and T wells are empty at this point and have no color.
7. Cover the plate with the adhesive plate sealing strip provided with the EIA kit. Incubate the plate at room temperature on a plate shaker for 2 hr at ~500 rpm or overnight at 4°C.
8. While waiting add 2.5 ml scintilation cocktail (Ultima Gold) to each scintilation vial. *This is easiest to do by pouring the amount of scint fluid needed into a beaker (keeping in mind that it is light sensitive – DO NOT pour more than you need into beaker!) and use a repeater pipette tip (50 mL) with the end cut off to enlarge the opening.* Cap, number, and vortex each vial. ***Let the vials sit for a day to settle out the bubbles before counting.*** Next, count all scintilation vials **including the total cpm vial from Day 1** on the scintillation counter.
9. Hook up the plate washer to the water container. Prime the plate washer with water. Now hook it up to the bottle containing the wash buffer.
10. Wash out the wells with the plate, using the setting TEST2.
11. Firmly tap the plate on lint free paper to remove any remaining wash buffer.
12. Add 5 µl of the Blue Conjugate to both of the TA wells.

13. With a multi-channel pipette add 200 µl of the p-Npp Substrate solution to every well. Recover the plate with the plate sealer strip. Incubate the plate at 37°C for 1 hr without shaking.
14. With a multi-channel pipette add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
15. Turn on the plate reader. The password is 00000. Also be sure the computer is on.
16. Put the plate in the reader, and close the cover.

READING THE PLATE

1. Open Microplate Manager
2. File – New Endpoint Protocol
3. Dual: Measurement filter 405, reference filter 580
4. Show template: Click "2" on pull-down menu and fill in template.
    a. B for blank
    b. Triangle with – for negative controls
    c. Triangle with + for positive controls
    d. Circle with S for standard curve
    e. Box with X for samples

Example:

B	**S1**	**S5**	**S9**	**4**	**8**						
B	**S1**	**S5**	**S9**	**4**	**8**						
	S2	**S6**	**1**	**5**	**ETC.**						
	S2	**S6**	**1**	**5**							
-	**S3**	**S7**	**2**	**6**							
-	**S3**	**S7**	**2**	**6**							
+	**S4**	**S8**	**3**	**7**							
+	**S4**	**S8**	**3**	**7**							

5. Run.
6. View Raw Data, Print.
7. Edit Concentration Report: fill in 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 pg/well.
8. View Standard Curve Report. Change to Logistic 5 Place (Cook). Print.
9. View unknown concentration report. Print.

And you're done!

Be sure to make a copy of the printouts for the lab EIA notebook.

Calculating the results

Use the blank worksheet available on the Ketterson lab protocols page to calculate recoveries and concentrations.

Note: if you have used RIA standard solution as a way to check for intra- and interassay variation you will want to use the pg/well measurement for determining your variation. If 12.5μl of RIA standard has been used then you would expect 18pg/well.

Cleanup

- 13 x 100 test tubes, pipette tips, and EIA plates can be disposed of in the yellow radioactive waste bucket.
- Scintillation vials should be put in the double-bagged, white radioactive waste bucket **after** pouring the fluid into the liquid waste container.
- All reusable items (pyrex test tubes, combitips, multi-channel filling wells, glassware, etc.) must be washed in a solution of Contrad 70 and distilled water. Prepare a plastic tub on the first day. After everything has soaked for at least a day, scrub, rinse with distilled water, and put in the drying oven overnight.