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).

CORT EIA Protocol

based on Caymen Kit (#500655), modified by KAR, DGR, RH, and RS in Jan 2012.

Good assay parallelism ($R^2 = 0.996$).
Good efficiencies (~91%).
Good accuracy/recovery ($R^2 = 0.9957$)

This procedure takes 3 days to complete.
Day 1: Aliquotting samples and adding radioactive CORT to measure recoveries
Day 2: Extraction of the samples
Day 3: Making a standard curve and running the EIA
One day later: clean-up, run samples on scint counter, do wipe test.

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. Corticosterone EIA Kit (Cayman Chemical Company Inc. 1180 Ellsworth Rd, Ann Arbor, MI 48108) Catalog No. 500655 96 Well Kit
3. CORT standard (refrigerator), There is a JWA one and and RH one (top shelf, egg-area, RH's labeled "B wking solu RH 10/30/11")
4. Labeled CORT (refrigerator), B* made by RH
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, can get half-filled bottle)
11. Ethyl Alcohol (located in flammable cabinet)
12. Methanol (located in flammable cabinet; can re-use)
13. Ultra-pure water (can be obtained from CISAB lab; wear gloves while dispensing)
14. 1mL syringe for making buffer (stockroom)

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 8-point standard curve, all in duplicate, which use a total of 24 wells, leaving 72 wells. You may run **36 samples and standards** (run in duplicate) on each plate. We use about 3 standards per plate, plus one pooled sample to be run across plates, leaving room for **32 samples**. *Recall DR and KAR to use pooled extract on plate (On Day 3, use 1:4 dilution of pooled extract from test plate, in DR 2011 WYO freezerbox)*
2. Arrange your samples in a random order, and space your standards evenly throughout. Use the Plate Layout to record your arrangement.
3. Number the 12 or 15 ml conical pyrex test tubes (in the drawer) accordingly (or 13 x 100 if no conical tubes)
4. For the standards: use the Ketterson lab "CORT standard." Put it in a little beaker. Use 5µl of standard per vial, but MAKE A STOCK OF it for today. (wet tip), and add enough water to equal the volume of the plasma samples (water in a little beaker too). ***extra standard can be poured back. WS-CORT: ____***
    a. Pipette **20uL CORT-WS + 60uL of water into a 13x100, vortex.** Then pipette 20uL of that stock into each of your 3 WS conical tubes (same tip)
5. Vortex each sample.
6. Using a a pipette, aliquot a fixed volume of each plasma sample into a test tube. ***rinse syringe or use new tip between samples***
    a. We are starting with, 20 µl
    b. If running mixed samples, pick one volume. 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.
7. Add 200 µl of distilled H2O to all samples and standards. (Use the repeater pipette with a previously used tip, left drawer.) ***5mL tip, set to 2.**
8. Put some Hot CORT in a beaker**. HOT CORT: _____**
9. Get out 2 scint vials, and label them with Initials, hot B, plate #, date.
10. Using the repeater pipette and a brand-new 0.5 ml combitip ***set to 2,*** add 20 µl labeled CORT to all samples and standards **AND to both scint vials** using the same tip. Tip goes in radio trash, and leftover B* returned to fridge. (B*2 = 2600 CPMA on Jan 1 2012)
11. Vortex each test tube *for 10 sec.* Cover them all with parafilm and store them in refrigerator at 4ºC overnight.
12. Add 2.5 **m**l Ultima Gold Scintillation Fluid to the vial, cap and vortex, and store it in a drawer until day 3. Use pump (can estimate volume).
13. Make up dish solution, and label your dish bucket with your name (on lab tape) – 40mL ContRad, 4L DW H2O).

DAY 2: Extracting and Reconstituting the Samples

1. Turn on the water bath first
2. Take the kit out of the freezer. Move it to the fridge, but put Ellman's and CORT std. back in the freezer, labeled with your initials and the date.
3. Make the working solutions of EIA wash buffer (1 in 400 solution) and Assay Buffer (1 in 10 solution) following the manufacturer's instructions. Reagents can be stored for about <u>two months</u> in sealed bottles in the fridge.
    a. Assay buffer: Dilute contents of one vial of EIA buffer concentration (10x; #400060) with 90 mL ultrapure water. Be sure to rinse the vial to remove any salts that may have precipitated. **Lot/Date_____**
    b. Wash buffer: Add 5mL vial of concentrate (400062; **Lot/date: _____**) with 2L of ultrapure water and add 1 mL of polysorbate 20 (#400035; **Lot/date: _____**). Notes that polysorbate is a viscous liquid and can't be measured with regular pipette; use 1mL syringe.

EXTRACTION: *ether date*___

1. Using a **used** 50 ml combitip (with gray add-on) and the repeater pipette **set to 1**, add 1 ml of diethyl ether to each sample **Slowly to avoid splashback!!** Vortex each sample for about 10 seconds. ***can't pour back extra from beaker into bottle***.
2. Let the samples sit for 20 minutes to allow complete separation.
3. While waiting, prepare a snap-freezing bath of ~2 lbs dry ice in methanol. *don't forget the **wire rack**!* Acct: 66-273-00. Also label your 13x100 tubes and turn on bath. During second wait, make buffer if needed.
4. Snap freeze each sample (~20 sec) – dry tube quickly - and pour off unfrozen supernatant into a labeled 13x100 test tube.
5. Place the little tubes evaporack (leave the corners empty!) into a 40ºC water bath. Let them sit there **while doing 2nd extraction –** ie, while conical tubes are getting their ether*****. You will add supernatant from the next round to these same tubes. (so, no N2 during first bath)
6. Repeat steps a-e. You may want to repeat a 3rd time. *3 extractions for test plate, efficiency = 91.2%*
7. After final extraction, be sure the samples are completely dried down. Evaporate with N2.
8. The pyrex test tubes and their remaining contents can be put into a bucket of soapy water (use the radioactive wash) -- Recommended to let tubes dry fully in hood for ONE day before putting them in the bucket, so that fumes do not overcome room.

RECONSTITUTE THE SAMPLES: (once they are dry)

1. Use the repeater pipette and a brand-new 0.5 ml combitip (set to 3) to add **30 µl of 100% EtOH** to each sample. (extra can be poured back.)
2. Vortex thoroughly. (10 sec)
3. Use repeater pipette and a brand new 5 ml combitip (set to 2.5) to add **250 µl of Assay Buffer** to each sample, then use a new 0.5 tip and add 20uL uL (270uL buffer).
4. Vortex at least 10 seconds.
5. Cover tubes with parafilm (well!) 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 **plate, samples, tracer, antiserum, and CORT stock soln** warm to room temperature for about 30 minutes.
2. Serially dilute the B standard to make the standard curve:
    a. Make a CORT stock solution if you haven't already (good for 6 weeks in fridge).
    b. Label 8 13x100 test tubes (S1-S8).
    c. Equilibrate pipette tip in ethanol by repeatedly filling it with EtOH. Then, use that tip to transfer 100uL of the CORT EIA Standard (#400657) into clean tube labelled BULK (can use clean scint vial). Dilute by adding 900uL ultrapure **water (not buffer!)**. Bulk should be 50ng/mL.
    d. Add 900uL EIA buffer to S1 and 750uL buffer to S2-8.
    e. Transfer 100uL of the BULK to S1, mix. ***Switch tips each time***
    f. Add 500 µl of tube #1 to tube #2 and vortex,
    g. Add 500 µl of tube #2 to tube #3 and vortex.
    h. Continue for tubes #4-8.
    i. This prepares enough for 1-2 plates. Standards can be stored in the fridge for 24h
3. Reconstitute Tracer and Antiserum (careful with these; there is very little extra)
    a. Tracer: Add 6mL buffer to the 100dtn vial (good for 4 weeks in fridge)
    b. Antiserum: Add 6 mL buffer to the 100 dtn vial (good for 4 weeks in fridge)
4. Label scint vial lids, including WS1-3.
5. Make **1:4 dilutions** of each sample of extract into new 13x100 tubes, but NOT FOR THE WS. Use 40 uL of extract + 120 uL of assay buffer (if you started with less plasma on Day 1, use a different dilution)

Running the EIA:

1. Refer to the Assay Layout Sheet. Pipet 100 µl of Assay Buffer into the NSB well.
2. Pipet 50 uL of Assay Buffer into Bo (0 pg/ml standard) duplicate wells.
3. Pipet 50 µl of Standards #1 through #8 into the appropriate duplicate wells. **New tips each time. Tubes can go into normal trash bucket.**
4. Vortex each sample before: Pipet 50 µl of samples into the appropriate duplicate wells. (well, well, vial, repeat.) You can pipette 50 uL of the WS into the scint vials now, but wait to put samples into scint until plate is on shaker.
5. With a multi-channel pipette add 50 µl of **Tracer** into each well except the Total Activity (TA) and Blank wells. *Extra can be pipetted back into Tracer container; keep in fridge. Troughs can be rinsed and reused.*
6. With a multi-channel pipette add 50 µl of **Antiserum** into each well except the Total Activity (TA), NSB, and Blank wells.
7. Cover the plate with the clear adhesive plate sealer provided with the EIA kit. Incubate the plate at room temperature on a **plate shaker for 2 hr** [speed 5]. **Time start: _____**
8. Pipet 50 µl of each **undiluted sample** into a scintillation vial in order to determine recovery values.

9. While waiting, add 2.5 ml scintilation cocktail (Ultima Gold) to each scintilation vial. Cap, number, and vortex each vial. Let them settle overnight. (extra Ulima gold can go down sink; viscous, so pipette slowly)

Afternoon of Day 3: Reading the plates, calculating results.

1. (After incubation) Let **tracer** warm up for 30 min.
2. Just before your plate is ready, reconstitute Ellman's reagent (must be done immediately before use):
    a. 100 dtn vial + 20 mL ultrapure water
3. Before putting plate in machine, hook up the plate washer to the water container, and the waste tube into the radioactive waste. Prime ("rinse") the plate washer with water. Now hook it up to the bottle containing the wash buffer.
4. Wash out the wells with the plate (VERTICAL ORIENTATION), using CAYMAN (5 washes) (under RUN)
5. Firmly tap the plate on lint free paper to remove any remaining wash buffer. Switch tube back to rinse, and run rinse cycle twice. Then switch waste tube out of radio-waste.
6. With a multi-channel pipette add 200 µl of Ellman's solution to every well.
7. Add 5 µl of the Tracer to both of the TA wells.
8. Recover the plate with the plate sealer strip (or DARK strip from Rose). Incubate the plate at room temp, on shaker, for 40 min. **Time start: _____** *Want Bo to read at 0.3 to 1 (minus blank). Goal OD >0.5 (this happens at 40 min, took ~5 min from removal of shaker to actual read)*
9. Turn on the computer and plate reader. The password is 00000.
10. Wipe the bottom of the plate clean with a kimwipe.
11. No stop solution needed, just carefully carry plate to reader and read.
12. Put the plate in the reader and close the cover.

READING THE PLATE

1. Open Microplate Manager
2. File – New Endpoint Protocol
3. Single: Measurement filter 415.
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**									
B	**S1**	**S5**									
nsb	**S2**	**S6**									
nsb	**S2**	**S6**									
-bo	**S3**	**S7**									
-bo	**S3**	**S7**									
+ ta	**S4**	**S8**									
+ ta	**S4**	**S8**									

1. Run.
2. View Raw Data.
3. Edit Concentration Report: manually fill in 5000, 2000, 800, 320, 128, 51.2, 20.5, 8.2 pg/well.
4. View Standard Curve Report. Change to Logistic 4 Point.
5. View unknown concentration report.
6. Save everything, then print everything.

And you're done!

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

DAY 4

Count all scintilation vials **including the total cpm vial from Day 1** on the scintillation counter: Protocol #7 – flag on back-most row, left. F2 to start. Later, get results from scint counter. Be sure to write down the order on the printout before removing the samples from the counter.

Calculating the results

Use the blank worksheet available on the Ketterson lab protocols page to calculate recoveries and concentrations. Don't forget to correct for your extract dilutions (1:4 typically)

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 12.5ml 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.