

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>).

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Assay _____

Date _____

DAY 1: Setting up Samples (takes ~1.5 hours)

- Set up plate layout sheet. Each plate has space for 32 samples in duplicate plus 3 standards.
- Defrost samples. While this is happening, label 12-15 mL conical test tubes with Sharpie (1-32) and 6 red-stripe tubes WS1-WS6. Arrange them so that WS1-2, 1-16, WS3-4, 17-32, WS5-6.
- Vortex each sample, then use a Hamilton syringe to draw up the **target of 30 μ L** (this number is flexible between assays). Record sample number, assay number (1-32), and the sample volume (if you can't get 30) in lab notebook). Rinse syringe in dH₂O between each sample.
- Use Hamilton Syringe to add dH₂O to bring all samples up to a final volume of 30 μ L.
- Use Hamilton Syringe to add 20 μ L of Wingfield T Standard (T std 500 pg/100 μ L) to the WS tubes
- Use a **used** 5 mL combitip and the repeater pipette set to 1 to add 100 μ L of dH₂O to each sample and standard (every tube).
- Use a **new** 0.5 mL combitip and the repeater pipette set to 1 to add 10 μ L of dH₂O to each **standard** (red-tubes).
- Switch the repeater pipette to 2 and add 20 μ L of dH₂O to each **sample**.
- Using a **brand new** 0.5 mL combitip and the repeater pipette set to 2, add 20 μ L of labeled testosterone (from the radioactive fridge) to each red-tubed **standard**, as well as to a scintillation vial or two (label as Hot T [Date]).
- Vortex each tube for 10 sec, then cover all with parafilm and refrigerate overnight.
- Refreeze samples. (Empty sample tubes can be tossed.)
- Add 2.5 mL of scintillation fluid to the vial(s), and store in drawer.
- Clean up! Make up dish solution in bucket with 30 mL of ContRad soap and ~3 L water from the DW tap. Label your bucket! Leftover hot T can be poured back into bottle in the fridge. 0.5 mL Hot T combitip goes in the radioactive trash; other combitip and beakers go into wash bucket.

Assay _____

Date _____

DAY 2: Extractions and Reconstituting Samples (takes ~3-3.5 hours)

- Assay Buffer Prep: Dilute 4.4 mL of concentrate with 39.6 mL of dH₂O using graduated cylinder (enough for 2 plate with 64 samples).
- Wash Buffer Prep = 44 mL of concentrate 396 mL of dH₂O using graduated cylinder (enough for two plates plus).
- Store buffers in orange-lidded jars (250 mL for Assay Buffer 3, 500 mL for wash) at room temperature (label with initials, contents, date).

Extraction:

- Using a **used** 50 mL combitip and the repeater pipette set to 1, add 1 mL of anhydrous diethyl ether to each sample, then vortex for 10 seconds.
- Allow to sit for 20 minutes after ether has been added to last sample.
- Label enough 13 x 100 test tubes for all samples and standards; Turn hot water bath on.
- With 7 minutes left, get ~2 lbs of dry ice in ice bucket, pour methanol over top.
- Snap-freeze each sample by dipping it into ice bath for 20 seconds.
- Dry tube, then quickly pour supernatant into labeled 13 x 100 test tube.
- Place rack of 13x100 tubes in warm water bath for 20 minutes while doing 2nd extraction.
- Repeat extraction, freeze, and pour-off (2 times total).
- Evaporate liquid in 13x100 tubes in water bath with nitrogen gas manifold (not long).
- Using a **brand new** 0.5 mL combitip and the repeater pipette set to 5, add 50 μ L of 100% EtOH to each sample and vortex for ~10 sec.
- Using a **brand new** 5 mL combitip and the repeater pipette set to 3, add 300 μ L of Assay Buffer 3 to each sample and vortex for ~10 sec.
- Cover samples with parafilm and refrigerate overnight.

Assay _____

Date _____

DAY 3: Setting up the plates, Determining Recoveries (Takes ~2.5 hours)

- Allow reagents (plate, samples, blue, yellow, T, T standard) to warm to room temperature (about 30 min)

Serial Dilution of Testosterone:

- Label nine 13 x 100 test tubes (S1-9).
- Add 1 mL of Assay Buffer 3 to tube S1 and 500 μ L to Tubes S2-9
- Remove 40 μ L of buffer from tube S1
- Add 40 μ L of the testosterone standard from the kit to tube S1 and vortex
- Add 500 μ L of tube S1 to S2 and vortex, Repeat for S2-S9

Setting up the plates:

- Pipette 100 μ L of Assay Buffer 3 into the NSB and Bo wells
- Pipette 100 μ L of Standards S1-S9 into the appropriate wells
- Defrost aliquot of T standard and pipette 100 μ L into appropriate wells (3x2 duplicates)
- Pipette 100 μ L of samples into duplicate wells
- Randomly choose ~3 samples, and pipette last 100 μ L into a labeled scintillation vial for non-radioactive baseline counts.
- Pipette 100 μ L of Wingfield Standards 1-6 into labeled scintillation vials.
- Pipette 50 μ L of Assay Buffer 3 into NSB wells
- Add 50 μ L of Blue Conjugate into each well except the TA and Blank wells
- Add 50 μ L of the Yellow Antibody into each well except the TA, NSB, and Blank wells.
- Cover the plate with the sealing strip and incubate for 2 hours at ~500 rpm on plate shaker (Greg's lab) **OR** overnight (no shaking) in the refrigerator
- Add 2.5 mL of scintillation fluid to each scintillation vial. Vortex each vial.
- Count all vials (USER #7) including vials in drawer from day 1. (2 hot T, 6 WS, ~3 samples)

Assay _____

Date _____

DAY 4: Reading the Plates, Calculating Results

- Let pNpp, Blue conjugate, stop solution, and plate warm up for 30 mins.
- Get results from scintillation counter, calculate average extraction efficiency.
- Bring reagents, 2 tip boxes, wash solution, plate layout, and timer upstairs. Run plate washer – prime in Rinse first, than place tube into wash buffer and waste into radioactive bottle, then run EIA program (3 washes)
- Run rinse cycle twice with Rinse (not wash buffer), then change waste back
- Tap plate upside-down to remove any leftover buffer
- Add 5 μL of Blue Conjugate to the TA wells
- Using a multi-channel pipette, add 200 μL of the p-Npp substrate to every well.
- Cover with sealer strip and incubate at 37°C for 1 hour with no shaking (Greg’s Lab)
- Turn on plate reader; with a multi-channel pipette, add 50 μL of Stop Solution to every well.
- Set up the Plate reader: New Endpoint Protocol (dual mode) with the measurement filter at 405 nm, reference filter at 570 nm. 1st row of plate is B, then nothing, then neg, then pos.
- Print absolute absorbance readings
- Edit>Standard Concentrations: Concentrations of T are: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0.78125 pg/well in the standard curve.
- View>Standard Curve Report (set to curve, not linear). Print this. View>Unknown Concentration Report. Print this. Save everything.

DAY 5: DISHES!!! (Takes ~1 hour)

- Wash dishes in dish bucket, rinse into “NO SOAP” bucket, leave overnight in dish dryer
- Empty scintillation vials
- Fill tip boxes