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 RADIOIMMUNOASSAY OF PLASMA

This protocol has been copied and modified from protocols provided by Dr. John Wingfield at the University of Washington. This protocol can be used to measure the concentration of steroid hormones in plasma samples. It has been written for use in the laboratory of Dr. Ellen Ketterson at Indiana University, and therefore the details may require modification if the procedure is performed elsewhere. The general procedure, which takes 4-5 days to complete, is described below. Variations and additional information are provided in the appendices. Prepared January 1998 by Joe Casto, modified December 2005 by Jodie Jawor and May 2010 by Danielle Whittaker.

PRIOR TO START OF ASSAY

1. Distillation of ethyl acetate.

We purchase ACS quality ethyl acetate, which requires distillation no more than 24 hours prior to the start of the assay. 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 55-60o). 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.

2. Double-check of materials.

Before beginning an assay, make sure that all of the following materials are available for use.

Nitrogen Gas - Air Gas, 317-241-5097, Acct# TFI 57, PO# 20307-1085
Nanograde Quality 2,2,4-Trimethylpentane (Isooctane) - Fisher Scientific
Petroleum Ether - Chemistry Stockroom
Ethyl Ether - Chemistry Stockroom
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
Charcoal/Dextran Suspension - See Appendix C
Standard, Labeled Steroid, and Antibody for the particular hormone(s) being assayed - See Appendices E,F, and G
Ultima-Gold Scintillation Fluid - Packard Instrument Company, 800 Research Parkway, Meridian, CT 06450. (800-323-1891). Item # = 6013329 ($225/2x5L Containers)

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 1000oF (range of 950-1100oF) 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.

Preparation of plasma samples.

Set up plasma sample in glass centrifuge tubes (pointed bottoms, 12 ml). The first and last tubes are always blanks and contain only distilled H20. The second and second to last tubes are always standards into which measured amounts of the steroids being assayed are placed. This measures the accuracy of the current assay as well as the interassay variation, when samples may be spread over more than one assay. When all plasma sample have been measured, a small amount of labelled steroid (20 μl of each steroid being tested = 2000 cpm) is added to all tubes except the blanks. Total cpm is measured at the end to determine what percentage of labelled steroid is recovered, and assuming the unknown steroid behaves the same, the final dose of the unknown steroid is adjusted according to the recovery value.

1. Setting up plasma samples.

Set up a data sheet for plasma volumes numbering samples as follows: 1. blank; 2. standard; 3. ... samples; second to last = standard; last = blank. Mix plasma on a whirl mixer if you have more than needed. Measure 50 - 400 μl plasma using a Hamilton syringe for each sample and palce in centrifuge tube. 75-200 μl is ideal. Rinse syringe with distilled H20 and add to plasma. Bring all tubes to the same volume by rinsing the syringe (usually 100 or 200 μl). When all tubes are complete go back and bring volumes up to 400 μl using distilled water and an Ependorf Repeater. Note: If LH will be performed on the same samples, save 25 μl plasma and refreeze.

2. Preparation of blank tubes.

Blank samples provide a negative control for background radiation in the assay. The first and last tubes in the assay should be blanks. Add 400 μl of distilled water to two glass centrifuge tubes (pointed bottoms, 12 ml). Do not add hot steroid to the blanks

3. Preparation of standard tubes.

Standard samples consist of a known amount of the steroid being assayed. They are used to measure the accuracy of the assay and to provide a measure of interassay variation. The second and second-to-last tubes in the assay should be standards. Additional standard samples may be placed throughout the assay if desired.

The standard tubes should be prepared in glass centrifuge tubes (pointed bottoms, 12 ml). For each steroid being assayed, place 50 μl of cold (non-radioactive) steroid of each steroid being assayed in each standard tube using a Hamilton Syringe. This will be 500 pg for progesterone (P), 1000 pg for corticosterone (B), and 250 pg for testosterone (T), estradiol (E2), and dihydrotestosterone (DHT). Bring the volume up to 400 μl with distilled water.

4. Setting up the recoveries.

A small amount of labeled steroid is now added to each sample and standard tube. Total cpm for each sample will be measured at the end of the assay to determine the percentage of labeled steroid that is recovered (i.e. not left behind during the extraction and chromatography steps), and the final concentration of steroid in each sample will be adjusted accordingly.

For each steroid being assayed add 20 μl (2000 cpm) of labeled steroid to all samples and standard tubes (but not to the blanks). An Eppendorf repeater pipette set on 2 with a 0.5 ml tip can be used. A yellow pipette tip (designed for the Eppendorf P200 Pipetman) can be fit over the end of the 0.5 ml tip to reduce drips. Also, for each steroid add 20 μl of hot steroid to a scintillation vial (one vial for each steroid). Add 2.5 ml Ultima-Gold scintillation fluid to this scintillation vial, vortex, cap, label, and store in a dark place. The total cpm of these vials will be measured to help determine the percentage recovered in each sample. Whirl mix all tubes and refrigerate overnight to allow the hot steroids to equilibrate with the plasma lipids and binding proteins.

DAY 2 (Possibly days 2 and 3)

1. Extraction of steroids from plasma samples.

- The diethyl ether must be freshly opened to avoid the formation of peroxides.
-Add 4 ml of diethyl ether to each sample. This can be done with an Eppendorf repeater pipette .
-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 13 x 100 test tube.
-Wait to blow down and resuspend samples until colums are made, are wet, and are ready to be used (columns can be made one day prior to the extraction if many columns are needed make sure they are wet with isooctane for overnight storage. Extraction should be performed on the day that the chromatography is run)
-Evaporate the samples under nitrogen gas.
-Resuspend the samples in 500 μl 10% ethyl acetate in isooctane. The Eppendorf repeater pipette may be used.

2. Preparation of chromatography columns. (For short columns see Appendix A.)

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, then attach the hoses to avoid overnight evaporation. Make sure the valves are closed and the gas is off. (If chromatography is done on the same day that the columns are made this second volume of isooctane can be run through and samples can then be added to columns)

2. Chromatography. (For short-column chromatography see Appendix A.)

Run the remaining isooctane from the previous day (may be same day) out of each column. Stop the flow in each column when the solvent front just reaches the top of the celite. To add the samples to the columns, first vortex a sample, then transfer it to its respective 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. Leave the blue pipetman tip in the top of the column to mark your place and to avoid adding two samples to the same column. When all samples have been transferred remove and discard the blue tips.

Attach the hoses to the columns and regulate the nitrogen pressure so that no single column drips 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 any that are unwanted may be discarded. Fractions are collected in 13x100 test tubes placed under the columns.

To collect progesterone: Add 4 mls 2% ethyl acetate in isooctane and blow down under regulated pressure into collection tubes. If P will be discarded, use 4 ml pure isooctane.
To collect dihydrotestosterone: Add 4.5 mls 10% ethyl acetate in isooctane as above.
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. This step is optional and may be skipped if estradiol is to be discarded.
To collect corticosterone: Add 4 mls 50% ethyl acetate in isooctane. (Use 4.5 mls if the 40% fraction was skipped.)

Evaporate the eluates under nitrogen gas in the 40oC water bath. Add 550 μl of buffer (PBSG) to each tube for all hormones except B. To the B eluates add 1 ml buffer. The buffer can be added using a combination of tips on the Eppendorf repeater pipette. Whirl mix the racks of tubes, cover with parafilm, and refrigerate overnight. (To save a day, you may place the racks on the shaker for at least 45 minutes and proceed to set up the radioimmunoassay.)

DAY 3 (possibly day 4)

This day is devoted to setting up the competitive-binding radioimmunoassay. Begin by placing the appropriate number of 10x75 test tubes in racks. First arrange 12 pairs for the standard curve, with a space between the 3rd and 4th pairs. Follow with a pair of tubes for each sample. (When you get to the charcoal step on Day 5, only 120 tubes may be centrifuged at one time. This translates into the standard curve plus 48 samples including blanks and standards. If you have more than 48 samples, a second spin in the centrifuge is required. This spin should have B2 and B3 tubes in it also; so between spin comparisons can be made)

For all steroids except B:

1. Setting up the samples.

Vortex each tube before aliquoting. Using an Eppendorf P200, pipet 200 μl of each sample into duplicate assay tubes. With another Eppendorf P200, pipet 100 μl of the sample into a scintillation vial to count the recoveries. Add 2.5 mls Ultima-Gold scintillation fluid to these vials: *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 and vortex. . ***Let the vials sit for a day to settle out the bubbles before counting*.** Next count on the scintillation counter. (Remember to also count the 2000 cpm vial from Day 1.)

2. Setting up the standard curve.

The standard curve is used to determine the concentration of steroid in the unknown samples (See Appendix B for an explanation of the calculations.) The first three pairs of tubes in the standard curve are the B1-3. B1 measures the total cpm in 100 μl of the labeled steroid solution. B2 measures non-specific binding (background), and B3 measures the maximum binding of the labeled steroid with the antibody. The remaining 9 pairs of tubes (S1-9) generate the curve.

Add 200 μl buffer to the B1 and B2 tubes and 100 μl buffer to the B3 and S2-S9 tubes using the Eppendorf repeater pipette with a 5 ml tip and set on 1. (At this point do not add buffer to the S1 tubes.) Add 100 μl of cold steroid to each of the S1 and S2 tubes using a Hamilton Syringe. This will be 1000 pg for P, 2000 pg for B, and 500 pg for T, E2, and DHT. Vortex one of the S2 tubes, remove 100 μl, and add this to an S3 tube. By continuing this serial dilution the concentration will be halved each time. Discard the last 100 μl that is removed from the S9 tube. Repeat the serial dilution for the second half of

the pairs. Go back and add 100 μl of buffer to all B and S tubes to bring them up to the same volume as the samples.

3. Setting up the radioimmunoassay.

Gently whirl mix (do not shake) the labeled steroid and antibody solutions prior to use. Using the Eppendorf repeater pipette, add 100 μl of labeled steroid to all of the tubes (standard curve plus samples). Add 100 μl antiserum to all tubes starting with the B3 tubes (do not add antiserum to the B1 or B2 tubes). Cover with parafilm, whirl mix, and refrigerate overnight. Curves may be left in the refrigerator longer before the charcoal step is done, but overnight is the minimum time required.

For B:

The procedure for B is the same as that described above except that different amounts are used.

1. Setting up the samples.

Aliquot 100 μl of unknown sample into replicate test tubes, and place 200 μl in the scintillation vials for the measurement of the recoveries.

2. Setting up the standard curve.

The procedure is identical as above, but do not go back and add 100 μl of buffer to the B and S tubes following the serial dilution. This is because the unknown aliquots have a volume of only 100 μl, so the volumes in the curve and in the samples are already identical.

3. Setting up the radioimmunoassay.

Follow the same procedure as described above for the other steroids.

DAY 4 (possibly day 5)

1. Separation of bound and free counts.

Dextran-coated charcoal is added to all of the tubes except the B1's to adsorb any unbound steroid. The tubes are then centrifuged to force the charcoal-dextran to the bottom, and the supernatant, which contains all steroids (hot and cold) bound to antibody, is decanted. The total cpm's in the supernatant are counted. High radioactivity in the supernatant indicates a low concentration of endogenous steroid, while low counts of tritiated steroid indicate high levels of endogenous steroid. The exact concentrations of steroids in the unknown samples are calculated by comparing them to the standard curve (see Appendix B).

-Run the Beckman TJ-6 centrifuge through one complete cycle to cool it to 4oC.

-Add 0.5 mls distilled water to the B1 tubes using an Eppendorf repeater pipette set on 2 with a 12.5 ml tip.

-Place some dextran-coated charcoal in a beaker, add a magnetic stir bar, and place on a stirring plate. The charcoal mix should be stirred continuously while adding

0.5 mls of it to all of the remaining tubes. This step should be done as quickly as possible to avoid heating of the samples and/or the charcoal mix and to ensure that all samples will be subjected to the charcoal mix for the approximately the same amount of time. It may be helpful to cool the beaker and the repeater pipette tips prior to use or to place the charcoal/dextran beaker in an ice bath while aliquoting.

-Whirl mix the racks of tubes and let stand for 12 minutes. Start the timer as soon as the charcoal mix is added to the last sample. This is a critical step, so set a timer and do not get distracted. Load the centrifuge while waiting, being sure to balance the tubes both within and between buckets.

-After the 12 minutes is up centrifuge at 2000 rpm for 10 minutes.

-Decant the supernatant into scintillation vials, add 4.5 mls Ultima-Gold scintillation fluid, cap, vortex and count on the scintillation counter. The centrifuge tubes with the charcoal pellets should be discarded into the radioactive waste.

-To calculate the steroid concentrations of the samples see Appendix B.

AFTER COMPLETION OF ASSAY

All glassware that is used during the assay 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 - SHORT COLUMN CHROMATOGRAPHY

Short chromatography columns may be used if you are not assaying for both B and E2. The differences from the normal protocol are as follows:

-To make the water trap add celite/water mix to the 3.5 ml mark on the column and pack to about 1 mark below the 4.5 ml mark.
-To make the glycol phase add celite/glycol mix to the 3.0 ml mark on the column and pack to the 4.0 ml mark.
-Wet the columns once with 4 ml isooctane. The columns can be allowed to sit overnight at this stage as long as the hoses are attached to the columns and the nitrogen gas is off.
-Add the samples to columns with 0.5 mls of 10% ethyl acetate in isooctane. There is no rinsing step.
-When the samples have been added to the columns and blown down into the celite with nitrogen gas:

Add 2 mls isooctane, blow down, and discard the eluate. (This is the P fraction, but P is better separated and collected on long columns.)

Add 1.5 mls 10% ethyl acetate in isooctane. This is the DHT fraction.

Add 2.0 mls 20% ethyl acetate in isooctane. This is the T fraction.

Add 2.5 mls 50% ethyl acetate in isooctane. This is the B fraction.

-If E2 will be collected instead of B, Add 2.5 mls 40% ethyl acetate in isooctane after the T fraction and skip the B fraction.
-Continue with the normal protocol.

APPENDIX B - CALCULATIONS

Both Mac and DOS-based spreadsheets are available in the lab for the calculation of steroid concentrations in samples. These are self explanatory and are based on the calculations presented below. It is beneficial to calculate sample concentrations by hand and on the computer when learning the assay so that you can understand the logic behind the calculations. A plot of a standard curve (% bound versus log standard concentration) is first created. The amount of steroid in each of the unknown samples is estimated using this curve and the known % bound values for each sample. The concentration of the original plasma sample is then calculated taking into account the recovery values and the dilutions that occurred during the assay.

To draw the curve and calculate the amount of steroid in each unknown sample:

Average the cpm values from the B1-3 and S1-9 pairs. Calculate the % bound of each S sample with the following formula:

$$\% \text{ bound} = \frac{Sx-B2}{B3-B2}$$

Make a plot of % bound versus steroid concentration (in pg) in each of the S samples. Average the cpm values from each of the unknown samples and calculate the % bound using the formula above. Plug this value into the curve to estimate the pg of steroid in each sample.

To calculate the original plasma steroid concentrations of P, DHT, T, and E2:

$$\text{Plasma Concentration (pg/mg)} = \text{Value from curve (pg)} \times 2.75 \times \frac{1000}{\% \text{ recovery (decimal)} \quad \mu\text{l plasma}}$$

To calculate the percent recovery, divide the total cpm (taken from the original scintillation vial with 20 μl of hot steroid) by 5.5. Divide the cpm values from all recoveries by this value.

To calculate the original plasma steroid concentrations of B:

$$\text{Plasma Concentration (pg/mg)} = \text{Value from curve (pg)} \times 10 \times 1000$$

% recovery (decimal) μl plasma

To calculate the percent recovery, divide the total cpm (taken from the original scintillation vial with 20 μl of hot steroid) by 5.5. Divide the cpm values from all recoveries by this value.

APPENDIX C - REAGENTS

Steroid Assay Buffer - PBSG (0.1 M, pH 7.0)

NaH2PO4 : H2O	16.14 g
Na2HPO4 : 7H2O	49.05 g
NaCl	27.00 g
Gelatin	3.00 g
NaN3	3.00 g
Distilled Water	3 L

Mix in a 4 L jug and place on a heated, magnetic stirring plate. Heat over low heat overnight while stirring until gelatin is dissolved. A temperature of 50oC is adequate. Refrigerate.

Dextran-Coated Charcoal (2x)

Charcoal	2.50 g
Dextran T-70	0.25 g
PBSG	0.5 L

Place charcoal and dextran in bottle. Add PBSG and stir on a magnetic plate and refrigerate.

APPENDIX D - PREPARATION OF ANTIBODY SOLUTIONS

Testosterone/Dihydrotestoterone: Antibody is purchased from Research Diagnostics, Inc., Pleasant Hill Rd., Flanders, NJ 07836 (800-631-9384). The catalog number is WLI-T3003 and it costs $35.00 per vial. Each vial makes 100 ml. The vials should be stored frozen at -20oC.

To prepare the antibody solution first thaw the vial. Measure 100 ml PBSG. Pipet a small amount of the buffer into the vial of antiserum and swirl gently. Pipet this into a bottle. Continue this several more times until the vial is completely emptied and rinsed. Add the remainder of the PBSG to the bottle and swirl to mix. Never shake antisera vigorously. Maximum binding is usually around 35%.

Progesterone: Antibody is also purchased from Wien Laboratories. The catalog number is P-1604 and it costs $30/vial. Each vial makes 50 ml. Store frozen.

Measure 50 ml PBSG and follow the procedure for the T/DHT antibody. Maximum binding is 40-45%.

Estradiol: Antibody is purchased from Arnel, 119 Washington Place, Suite B, New York, NY 10014. (212-620-4622). The catalog number is 1702 and it costs $195/vial. Each vial makes 1000 ml. Store frozen.

Thaw the vial and add 1 ml distilled water to the vial and swirl to mix. Using a disposable pipet tip, pipet 100 μl of this solution into a volumetric flask. Bring up to 100 ml with PBSG. Swirl gently to mix. Maximum binding is 40-50%. If it is higher dilute accordingly. Take the remainder of the concentrated antiserum and freeze in 100μl alliquots in ependorf tubes for use at later dates.

Corticosterone: Antibody is purchased from Fitzgerald Antibodies & Antigens. (1.800.370.2222). The catalog number is 20-CR45 and it costs $200/vial. Dilute the entire 450ul of antibody in 50ml PBSG. Maximum binding is 45-50%.

APPENDIX E - PREPARATION OF STANDARD STEROID SOLUTIONS

Stock solutions of steroids are made and kept for further dilution in the assay. These steroids are all purchased from Sigma. They are weighed out in a balance away from the assay area and which will not be used to weigh anything that comes into contact with the assay. Weigh 100 mg of steroid onto a small piece of aluminum foil. Carefully fold this up and drop it into a 100 ml volumetric flask. Add 100 ml ethanol, seal with the stopper and parafilm, and swirl to mix. This is very concentrated in terms of the assay and must be handled carefully or contamination can occur.

For DHT, T, and E2: We want a concentration of 500 pg/100 μl, which is the same as 125 ng/25 ml. In an area away from the assay bench, take 100 μl of the stock solution (= 10 μg) and dilute it into 100 mls of distilled water. Discard the pipet tip and all other disposables into a plastic bag for disposal. Mix this solution well. It is now at 1 μg /ml and contains a total of 1000 ng. Put 125 μl of this solution into a 25 ml volumetric flask and fill with PBSG. You now have a 500 pg/100 μl solution. Discard the intermediate solution into a sink outside of the lab, rinse the container several times, and flush the drain with water. The container should be soaked in Radiacwash detergent.

For B: We want a dilution of 2000 pg/100 μl, which is the same as 500 ng/25 ml. Using the same methods as above, put 100 μl of the stock solution into 100 ml distilled water and mix thoroughly. This solution is at 1 μg/ml and contains a total of 1000 ng. Take

500 µl of this solution and mix with 25 ml PBSG in a volumetric flask. You now have a 2000 pg/100 µl solution. Clean up as described above.

For P: You need 250 µl of the intermediate solution in 25 mls of PBSG to obtain a 1000 pg/100 µl solution. Clean up as described above.

APPENDIX F - PREPARATION OF TRITIATED STEROID SOLUTIONS

Tritiated steroids are ordered in 250 µCi vials from PerkinElmer Life Sciences. Prices and catalog numbers should be checked on prior to ordering as they have changed: The ones listed below are not current.

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 in 200 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. All glassware should be soaked in Radiacwash detergent.