

US EPA ARCHIVE DOCUMENT

Appendix L

Protocols for Assays Included in Tier 1 Screening Battery

[NOTE TO THE READER: Many of the following draft protocols are undergoing internal validation within NHEERL laboratories and until completion of this process they should be considered preliminary. The final protocols will likely differ from what is currently presented.]

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In Vitro* Assays (presented as examples)*I. Rat Estrogen Receptor Equilibrium Exchange Assay****A. Purpose and Applicability**

The purpose of this protocol is to outline a procedure for the quantitation of estrogen receptor number and binding affinity in various adult female rat reproductive tissues. As tissue receptor number is finite, the binding of ligand to the receptor (i.e., specific binding) is a saturable process. Unsaturable binding of ligand is called nonspecific binding and is due to ligand binding to non-receptor proteins etc. Total binding is saturable binding + unsaturable binding. Total and nonspecific binding are determined empirically, while specific binding is calculated as their difference (i.e., total-nonspecific). The assay described below measures the binding of radiolabelled synthetic ligand (i.e., [³H]-E2) by cytosolic and/or

nuclear receptor extracts. Total [^3H]-E2 binding is determined by incubating the extracts with increasing concentrations of [^3H]-E2 during which time the labelled ligand binds to the unoccupied receptors in the cytosol extract or exchanges with endogenous hormone bound to the nuclear receptors. The total bound ligand (i.e., saturable + nonsaturable binding) is separated from free ligand via hydroxylapatite extraction, eluted from the receptor with ethanol and quantified using liquid scintillation counting. Nonspecific binding is determined exactly as above except that a 100-fold molar excess of radioinert E2 is included in each incubation together with the increasing concentrations of [^3H]-E2 (i.e., binding of [^3H]-E2 in the presence of a 100-fold molar excess of radioinert E2 represents unsaturable binding). Specific binding is calculated as total - nonspecific binding and is analyzed graphically via Scatchard analysis.

B. Safety and Operating Precautions

All procedures with radioisotopes should follow the regulations and procedures as described in the Hazardous Agent Protocol (HAP) and in the Radiation Safety Manual and Protocols.

C. Equipment and Materials

1. Equipment
 - Corning Stir/hot Plates
 - Digital Pipets
 - Balance
 - Polytron PT 35/10 Tissue Homogenizer
 - Vacuum Concentrator
 - Refrigerated General Laboratory Centrifuge
 - High-Speed Refrigerated Centrifuge (up to 105,000 x g)
 - pH Meter with Tris Compatible Electrode
 - Scintillation Counter
2. Chemicals
 - Tris HCL & Tris Base
 - Glycerol 99%+
 - Ethylenediaminetetraacetic acid (EDTA); Disodium salt
 - Dithiothreitol (DTT)
 - Potassium Chloride
 - Hydroxylapatite (BIO-RAD)
 - Scintillation Cocktail (Flow Scint III)
 - Ethyl Alcohol, anhydrous
 - [^3H]-Estradiol & Radioinert Estradiol (NEN)
 - Triamcinolone Acetonide
 - Steroids (Steraloids - recrystallized)
3. Supplies
 - 20 ml Polypropylene Scintillation Vials
 - 12 x 75 mm Borosilicate Glass Test Tubes
 - 1000 ml graduated cylinders
 - 500 ml Erlenmeyer flasks

-yellow (0-200 ml) pipet tips

D. Methods

1. Preparation of TEDG buffer

To make 250ml:

Add to 150ml distilled water:

.303g Tris base

.140g EDTA

25ml glycerol

Bring volume to 250 ml and stir.

pH to 7.6

Add .04g DTT before use

2. Preparation of 50 mM TRIS Buffer

Add 50.0 ml 1.0 M TRIS to 950 ml ddH₂O. Store at 4° C. Check pH of the final solution to make sure it is 7.4 at 4° C.

3. Preparation of 60% Hydroxylapatite (HAP) Slurry

Shake BIO-RAD HT-GEL until all the HAP is in suspension (i.e., looks like milk). The evening before the receptor extraction, pour 100 mls (or an appropriate volume) into a 100 ml graduated cylinder, parafilm seal the top and place in the refrigerator for at least 2 hours. Pour off the phosphate buffer supernatant, and bring the volume to 100 mls with 50 mM TRIS. Suspend the HAP by parafilm sealing the top of the graduated cylinder and inverting the cylinder several times. Place in the refrigerator overnight. The next morning, repeat the washing steps two times with fresh 50 mM TRIS buffer. After the last wash, add enough 50 mM TRIS to make the final solution a 60% slurry (i.e., if the volume of the settled HAP is 60 ml bring the final volume of the slurry to 100 mls with 50 mM TRIS). Store at 4° C until ready for use in the extraction.

4. Standard Curve Construction for Saturation and Scatchard Analysis

The first step is to pipet the radioactive ligand (i.e., [³H]-E2) with and without a 100-fold excess of radioinert E2 into each tube so that the final concentrations of [³H]-E2 are 6,3,1,.6,.3,.1,.03,.01 nM in a 300 ml total volume. To accomplish this, label tubes and pipet the following into separate 12 x 75 mm glass test tubes.

Total Binding Tubes

6 nM= 21 ml of 10⁻⁷ [³H]- E2
 3 nM= 10.5 ml of 10⁻⁷ [³H]-E2
 1 nM= 35 ml of 10⁻⁸ [³H]-E2 ±
 .6 nM= 21 ml of 10⁻⁸ mM [³H]-E2

Nonspecific Binding Tubes (+)

± 21 ml of 10⁻⁵ radioinert E2
 ± 10.5 ml of 10⁻⁵ radioinert E2
 35 ml of 10⁻⁶ radioinert E2
 ± 21 ml of 10⁻⁶ radioinert E2

.3 nM= 10.5 ml of 10-8 [³ H]-E2	±	10.5 ml of 10-6 radioinert E2
.1 nM= 35 ml of 10-9 [³ H]-E2	±	35 ml of 10-7 radioinert E2
.03 nM= 10.5 ml of 10-9 [³ H]-E2	±	10.5 ml of 10-7 radioinert E2
.01 nM = 3.5 l of 10-9 [3H]-E2		3.5 l of 10-7 radioinert E2

After the tubes have been pipetted as above, place them in the speed-vac and dry the tubes according to the instructions in the speed-vac SOP. When tubes are dry place them in an ice-water bath and cover with aluminum foil until ready to pipet the nuclear or cytosolic extract into each tube for incubation. (Total tubes have hot only and NSB tubes have hot + cold)

5. Estrogen Receptor Assay Procedure

a) Make TEDG buffer and place in an ice-water bucket.

b) Kill the rat and excise uterus. Tissues should be trimmed of fat, weighed and the weights recorded. Place on dry ice. Before assay, place tissues into a homogenizing tube in an ice-water bath and add TEDG buffer at 1.0 ml/50mg tissue. It is extremely important to keep the tissues/extracts at 4° C at all times.

c) Mince tissues with Metzenbaum scissors until all pieces are small 1-2mm cubes. Then homogenize the tissues at 4° C with a Polytron homogenizer using five second bursts of the Polytron. [Note: place probe of the Polytron in an ice-water bath with TEDG buffer to cool it down prior to its use for homogenization]

d) Transfer homogenates to pre-cooled centrifuge tubes, balance, and centrifuge at 105,000 x g for 60 minutes (i.e., 35,000 rpm using 90 Ti Beckman rotor). The supernatant is the low-salt unoccupied cytosolic receptors.

e) While these tubes are spinning, the tubes containing [³H]-E2 ± radioinert E2 for the saturation experiment can be pipetted as described in section 4.4 above. Dry these tubes in the speed-vac, and place in a rack in an ice-water bath as indicated.

f) Pipet 300 µl of the cytosolic extract into each of the total and nonspecific binding tubes containing the [³H]-E2 and [³H]-E2 + radioinert E2, respectively. Gently vortex the samples and place them in the refrigerator overnight in rotor covered with foil for 20 hours.

g) Before leaving for the day, prepare the first wash of the HAP slurry as described in section 4.3 above. Also, label the HAP tubes and the scintillation vials to be used the following day - see underlines below.

h) The following morning, wash the HAP as described above, dilute with 50 mM TRIS to yield a 60% slurry, and transfer contents to a 100 ml Erlenmeyer flask. Place a stir bar in the flask and place the flask into a beaker containing ice-water; stir the HAP slurry by placing the beaker on a magnetic stir plate.

i) While the HAP slurry is constantly being stirred, pipet 500 ml of the HAP slurry into clean pre-labelled 12 x 75 mm glass test tubes. These tubes should be prepared in duplicate - i.e., two tubes for every incubation tube. Place these tubes in a rack in an ice-water bath prior to pipetting the HAP slurry and keep them in the ice-water bath for the remainder of the assay.

j) Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipet 100 ml in duplicate from each of the incubation tubes into the appropriate pre-labelled tubes containing HAP. Repeat for all tubes. Quickly take each rack from the ice-water bath and vortex each rack of tubes using the whole-rack vortex unit. Place racks back into the ice-water bath and vortex as above every five minutes for 20 minutes.

k) During the above 20 minute HAP extraction, pipet 30 ml in duplicate from the remaining 100 ml in each incubation tube into pre-labelled 20 ml scintillation vials. These tubes will be used to estimate the concentration of total [^3H]-E2 used in the equilibrium assay and will be called the total counts tubes. The extraction tubes containing the remaining 40 ml of incubate can be discarded into the plastic radiation safety barrel together with some sawdust to adsorb the excess liquid.

l) Centrifuge the HAP tubes for two to three minutes at 4° C and 600 x g (1780 rpm in a Beckman GLC refrigerated centrifuge). Place the tubes back into the rack and into the ice-water bath.

m) While the tubes remain in the ice-water bath, aspirate the supernatant from each tube using a 9 inch pipet connected to an aspiration apparatus as per the radiation safety protocol.

n) Add 2 ml of 50 mM TRIS to each tube, vortex and centrifuge at 600 x g as above. Place the tubes into decanting racks in an ice-water bath and decant the supernatant TRIS wash into the radiation safety container. Gently tap the tube openings on a clean adsorbent diaper, place the rack back in the ice-water bath and add 2 mls of 50 mM TRIS. Repeat the TRIS washing procedure three or four times (to be determined empirically) keeping the tubes at 4°C at all times.

o) Following the last wash and decanting, add 2 mls of ethanol to each tube, vortex three times at five minute intervals and centrifuge the tubes at 600 x g for ten minutes. Decant the supernatants into pre-labelled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

II. Protocol to Determine Affinity of Compounds for the Rat AR Androgen Receptor By Equilibrium Exchange Assay

A. Purpose and Applicability

The purpose of this protocol is to outline a procedure for the quantitation of androgen receptor number and binding affinity in various adult male rat reproductive tissues. As tissue receptor number is finite, the binding of ligand to the receptor (i.e., specific binding) is a saturable process. Unsaturable binding of ligand is called nonspecific binding and is due to ligand binding to non-receptor proteins etc. Total binding is saturable binding + unsaturable binding. Total and nonspecific binding are determined empirically, while specific binding is calculated as their difference (i.e., total-nonspecific). The assay described below measures the binding of radiolabelled synthetic ligand (i.e., [^3H]-R1881) by cytosolic and/or nuclear receptor extracts. Total [^3H]-R1881 binding is determined by incubating the extracts with increasing concentrations of [^3H]-R1881 during which time the labelled ligand binds to the unoccupied receptors in the cytosol extract or exchanges with endogenous hormone bound to the nuclear receptors. The total bound ligand (i.e., saturable + nonsaturable binding) is separated from free ligand via hydroxylapatite extraction, eluted from the receptor with ethanol and quantified using liquid scintillation counting. Nonspecific binding is determined exactly as above except that a 100-fold molar excess of radioinert R1881 is included in each incubation together with the increasing concentrations of [^3H]-R1881 (i.e., binding of [^3H]-R1881 in the presence of a 100-fold molar excess of radioinert R1881 represents unsaturable binding). Specific binding is calculated as total - nonspecific binding and is analyzed graphically via Scatchard analysis.

B. Safety and Operating Precautions

All procedures with radioisotopes should follow the regulations and procedures as described in the Hazardous Agent Protocol (HAP) and in the Radiation Safety Manual and Protocols.

C. Equipment and Materials

1. Equipment

- Corning Stir/hot Plates
- Digital Pipets
- Balance
- Polytron PT 35/10 Tissue Homogenizer
- Hewlett-Packard HPLC with on-line Radiomatic Radiochromatograph
- Vacuum Concentrator
- Hamilton Syringes (50 ml)
- Refrigerated General Laboratory Centrifuge
- High-Speed Refrigerated Centrifuge (up to 30,000 x g)
- pH Meter with Tris Compatible Electrode
- Scintillation Counter

2. Chemicals

- Tris HCL & Tris Base

- Phenylmethylsulfonyl Fluoride (PMSF)
- Glycerol 99%+
- Sodium Molybdate
- Ethylenediaminetetraacetic acid (EDTA); Disodium salt
- Dithiothreitol (DTT)
- Potassium Chloride
- Hydroxylapatite (BIO-RAD)
- Scintillation Cocktail (Flow Scint III)
- Ethyl Alcohol, anhydrous
- [³H]-R1881 & Radioinert R1881 (NEN)
- Triamcinolone Acetonide
- Steroids (Steraloids - recrystallized)

3. Supplies

- 20 ml Polypropylene Scintillation Vials
- 12 x 75 mm Borosilicate Glass Test Tubes
- 1000 ml graduated cylinders
- 500 ml Erlenmeyer flasks
- yellow (0-200 ml) pipet tips

D. **Methods**

1. Preparation of TEDG Stock Solutions

- a) Add 7.444g disodium EDTA to 100 ml ddH₂O = 200mM. Store at 4° C. Use 750 ml/100ml TEDG buffer = 1.5 mM.
- b) Add 1.742 g PMSF to 100 ml ethanol = 100 mM. Store at 4° C. Use 1.00 ml/100ml TEDG buffer = 1.0 mM.
- c) Add 2.419 g sodium molybdate to 8.0 ml ddH₂O in a 10 ml volumetric flask; bring the total volume to 10 mls = 1.0 M. Store at 4° C. Use 100ml/100ml TEDG buffer = 1.0 mM.
- d) Add 15.4 mg DTT directly to 100 ml TEDG buffer the morning of the receptor isolation = 1.0 mM.
- e) Add 147.24 g Tris-HCL + 8.0 g Tris base to 800mls ddH₂O in a volumetric flask; bring the final volume to 1.0 liter. Refrigerate to 4° C and pH (using 4°C pH standardizing solutions) the cooled solution to 7.4. Store at 4° C. Use 1.0 ml/100 ml TEDG buffer = 10mM.
- f) Add 298.2 g KCL to 600 ml ddH₂O in a 1000 ml volumetric flask; bring the total volume to 1000 ml = 4.0 M. Store at room temperature. Use 10.0 ml per 100 ml high-salt TEDG buffer = 0.4M.

2. Preparation of Low-Salt TEDG Buffer (pH 7.4)

To make 100 mls of low-salt TEDG buffer add the following together in this order:

- 87.15 ml ddH₂O
- 1.0 ml 1M TRIS
- 10.0 ml glycerol
- 100 ml 1M sodium molybdate
- 750 ml 200mM EDTA
- 1.0 ml 100mM PMSF
- 15.4 mg DTT

Check pH of the final solution to make sure it is 7.4 at 4° C.

3. Preparation of High-Salt TEDG Buffer (pH 7.4)

To make 100 mls of high-salt TEDG buffer add the following together in this order:

- 77.15 ml ddH₂O
- 10 ml 4.0 M KCL
- 1.0 ml 1M TRIS
- 10.0 ml glycerol
- 100 ml 1M sodium molybdate
- 750 ml 200mM EDTA
- 1.0 ml 100mM PMSF
- 15.4 mg DTT

Check pH of the final solution to make sure it is 7.4 at 4° C.

4. Preparation of 50 mM TRIS Buffer

Add 50.0 ml 1.0 M TRIS to 950 ml ddH₂O. Store at 4° C. Check pH of the final solution to make sure it is 7.4 at 4° C.

5. Preparation of 60% Hydroxylapatite (HAP) Slurry

Shake BIO-RAD HT-GEL until all the HAP is in suspension (i.e., looks like milk). The evening before the receptor extraction, pour 100 mls (or an appropriate volume) into a 100 ml graduated cylinder, parafilm seal the top and place in the refrigerator for at least two hours. Pour off the phosphate buffer supernatant, and bring the volume to 100mls with 50 mM TRIS. Suspend the HAP by parafilm sealing the top of the graduated cylinder and inverting the cylinder several times. Place in the refrigerator overnight. The next morning, repeat the washing steps two times with fresh 50 mM TRIS buffer. After the last wash, add enough 50 mM TRIS to make the final solution a 60% slurry (i.e., if the volume of the settled HAP is 60 ml bring the final volume of the slurry to 100 mls with 50 mM TRIS). Store at 4° C until ready for use in the extraction.

6. Preparation of [³H-17a-Methyl]-R1881 Stock Solutions

Dilute the original 1.0 mCi/ml stock of [³H-17a-methyl]-R1881 to 0.1 mM (i.e., 1 x 10⁻⁷ M). This is most easily accomplished by pipeting 1 ml of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 mls with ethanol. Thus, if the specific activity of the stock vial is 86 Ci/mmol, then pipet 86.0 ml into an amber

colored vial (i.e., R1881 is photosensitive) and add 10.0 mls ethanol to the vial; this solution is $1 \times 10^{-7} \text{M}$.

Calculation Check

$$\begin{aligned} 86 \text{ ml} \times 1.0 \text{ mCi}/1000 \text{ ml} &= 86 \times 10^{-3} \text{ mCi R1881} = 86 \times 10^{-6} \text{ Ci R1881} \\ 86 \times 10^{-6} \text{ Ci} \times 86.0 \text{ Ci}/\text{mmol} &= 1 \times 10^{-6} \text{ mmol R1881} = 1 \times 10^{-9} \text{ moles R1881} \\ 1 \times 10^{-9} \text{ moles R1881} \times .010 \text{ liters} &= 1 \times 10^{-7} \text{ moles/liter} = 0.1 \text{ mM} \end{aligned}$$

To prepare the $1 \times 10^{-8} \text{M}$ stock simply make a 10-fold dilution of the $1 \times 10^{-7} \text{M}$ stock (i.e., pipet 1.0 ml of the $1 \times 10^{-7} \text{M}$ stock into a clean amber colored vial and add 9 mls ethanol = 0.01 mM).

7. Preparation of 100X Radioinert R1881 Solutions

The R1881 comes as a 5.00 mg quantity. Dilute the original stock to 5.0 ml with ethanol = 3.52 mM. Take 56.82 ml and dilute to 20 ml in an amber vial with ethanol = $1 \times 10^{-5} \text{M}$ R1881. This is the 10 mM radioinert R1881 stock. To make the 1.0 mM radioinert R1881 stock, pipet 2 ml of the 10 mM stock into an amber vial and dilute to 20 ml with ethanol = $1 \times 10^{-6} \text{M}$ = 1.0 mM radioinert R1881 stock.

8. Standard Curve Construction for Saturation and Scatchard Analysis

The first step is to pipet the radioactive ligand (i.e., [^3H]-R1881) with and without a 100-fold excess of radioinert R1881 into each tube so that the final concentrations of [^3H]-R1881 are 20, 16, 12, 8, 4, 2, 1, 0.5 nM in a 300 ml total volume. To accomplish this, label tubes and pipet the following into separate 12 x 75 mm glass test tubes.

<u>Total Binding Tubes</u>		<u>Nonspecific Binding Tubes</u>
a) 20 nM= 60 ml of 0.1 mM [^3H]-R1881	±	60 ml of 10 mM radioinert R1881
b) 16 nM= 48 ml of 0.1 mM [^3H]-R1881	±	48 ml of 10 mM radioinert R1881
c) 12 nM= 36 ml of 0.1 mM [^3H]-R1881	±	36 ml of 10 mM radioinert R1881
d) 8 nM= 24 ml of 0.1 mM [^3H]-R1881	±	24 ml of 10 mM radioinert R1881
e) 4 nM= 12 ml of 0.1 mM [^3H]-R1881	±	12 ml of 10 mM radioinert R1881
f) 2 nM= 60 ml of 0.01 mM [^3H]-R1881	±	60 ml of 1.0 mM radioinert R1881
g) 1 nM= 30 ml of 0.01 mM [^3H]-R1881	±	30 ml of 1.0 mM radioinert R1881
h) .5 nM= 15 ml of 0.01 mM [^3H]-R1881	±	15 ml of 1.0 mM radioinert R1881

After the tubes have been pipetted as above, place them in the speed-vac and dry the

tubes according to the instructions in the speed-vac SOP. When tubes are dry place then in an ice-water bath and cover with aluminum foil until ready to pipet the nuclear or cytosolic extract into each tube for incubation.

9. Androgen Receptor Assay Procedure

a) Make low- and high-salt TEDG buffer and place in an ice-water bucket.

b) Kill the rat and excise epididymis, ventral prostate, and/or seminal vesicles. Tissues should be trimmed of fat, weighed and the weights recorded. As quickly as possible, place the tissues into a homogenizing tube in an ice-water bath and add low-salt TEDG buffer at 10 ml/gm tissue. It is extremely important to keep the tissues/extracts at 4° C at all times.

c) Mince tissues with Metzenbaum scissors until all pieces are small 1-2mm cubes. Then homogenize the tissues at 4° C with a Polytron homogenizer using five second bursts of the Polytron. [Note: place probe of the Polytron in an ice-water bath with TEDG buffer to cool it down prior to its use for homogenization]

d) Transfer homogenates to pre-cooled centrifuge tubes, balance, and centrifuge at 30,000 x g for 30 minutes (i.e., 15,262 rpm using JA-17/JA-21 Beckman rotors).

e) The supernatant is the low-salt unoccupied cytosolic receptors and this material can be assayed directly or frozen at -70° C for future analysis.

f) Add high-salt TEDG at 10 ml/gm original tissue weight and homogenize the pellet thoroughly at 15 minute intervals for one hour (i.e., four times). This procedure extracts the occupied androgen receptor from the nucleus.

g) Balance the tubes and centrifuge at 30,000 x g for 30 minutes at 4° C. While these tubes are spinning, the tubes containing [³H]-R1881 ± radioinert R1881 for the saturation experiment can be pipetted as described in section 4.8 above. Dry these tubes in the speed-vac, and place in a rack in an ice-water bath as indicated.

h) Transfer the supernatant from the nuclear extract to a 16 x 100 mm polypropylene tube and place in an ice-water bath. Pipet 300 ml of the cytosolic or nuclear extract into each of the total and nonspecific binding tubes containing the [³H]-R1881 and [³H]-R1881 + radioinert R1881, respectively. Gently vortex the samples and place them in the refrigerator overnight for 20 hours.

i) Before leaving for the day, prepare the first wash of the HAP slurry as described in section 4.5 above. Also, label the HAP tubes and the scintillation vials to be used the following day - see underlines below.

j) The following morning, wash the HAP as described in section 4.5 above, dilute with 50 mM TRIS to yield a 60% slurry, and transfer contents to a 100 ml Erlenmeyer

flask. Place a stir bar in the flask and place the flask into a beaker containing ice-water; stir the HAP slurry by placing the beaker on a magnetic stir plate.

k) While the HAP slurry is constantly being stirred, pipet 500 ml of the HAP slurry into clean pre-labelled 12 x 75 mm glass test tubes. These tubes should be prepared in duplicate - i.e., two tubes for every incubation tube. Place these tubes in a rack in an ice-water bath prior to pipetting the HAP slurry and keep them in the ice-water bath for the remainder of the assay.

l) Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipet 100 ml in duplicate from each of the incubation tubes into the appropriate pre-labelled tubes containing HAP. Repeat for all tubes. Quickly take each rack from the ice-water bath and vortex each rack of tubes using the whole-rack vortex unit. Place racks back into the ice-water bath and vortex as above every five minutes for 20 minutes.

m) During the above 20 minute HAP extraction, pipet 30 ml in duplicate from the remaining 100 ml in each incubation tube into pre-labelled 20 ml scintillation vials. These tubes will be used to estimate the concentration of total [^3H]-R1881 used in the equilibrium assay and will be called the total counts tubes. The extraction tubes containing the remaining 40 ml of incubate can be discarded into the plastic radiation safety barrel together with some sawdust to adsorb the excess liquid.

n) Centrifuge the HAP tubes for two to three minutes at 4° C and 600 x g (1780 rpm in a Beckman GLC refrigerated centrifuge). Place the tubes back into the rack and into the ice-water bath.

o) While the tubes remain in the ice-water bath, aspirate the supernatant from each tube using a nine inch pipet connected to an aspiration apparatus as per the radiation safety protocol.

p) Add 2 ml of 50 mM TRIS to each tube, vortex and centrifuge at 600 x g as above. Place the tubes into decanting racks in an ice-water bath and decant the supernatant TRIS wash into the radiation safety container. Gently tap the tube openings on a clean adsorbent diaper, place the rack back in the ice-water bath and add 2 mls of 50 mM TRIS. Repeat the TRIS washing procedure three or four times (to be determined empirically) keeping the tubes at 4° C at all times.

q) Following the last wash and decanting, add 2 mls of ethanol to each tube, vortex three times at five minute intervals and centrifuge the tubes at 600 x g for ten minutes. Decant the supernatants into pre-labelled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

E. Data Processing

1. Free Concentration of [³H]-R1881

Multiply the DPM in the total counts tubes by 1.8047×10^{-5} . This value will yield the free concentration (i.e., nM) of [³H]-R1881 initially present in each incubation tube.

Calculation Check

$$\frac{\text{X DPM}}{2.22 \times 10^{12} \text{ dpm/Ci}} = \frac{4.5045 \times 10^{-13} \text{ Ci}}{83.2 \text{ Ci/mmol}} = \frac{5.4141 \times 10^{-15} \text{ mmol}}{1000 \text{ mmol/mole}} = \frac{5.4141 \times 10^{-18} \text{ moles}}{0.0003 \text{ liters}}$$

$$= \frac{1.8047 \times 10^{-14} \text{ moles/liter}}{1 \times 10^{-9} \text{ moles/nmol}} = \text{X } (1.8047 \times 10^{-5}) \text{ nM}$$

2. Calculation of Total, Nonspecific and Specific [³H]-R1881 Binding

•Total binding is calculated by multiplying the DPM from the tubes that contained only radiolabelled R1881 x (1.6242×10^{-2}) . This value will be total binding in fmoles.

•Nonspecific binding is calculated by multiplying the DPM from the tubes containing radiolabelled R1881 + 100-fold molar excess of radioinert R1881 x (1.6242×10^{-2}) . This value will be nonspecific binding in fmoles.

•Specific binding is calculated by subtracting nonspecific binding from total binding i.e., fmoles total binding - fmoles nonspecific binding = specific binding in fmoles.

Calculation Check

To get fmoles multiply the DPM values by 1.6242×10^{-2} . This is simply nM x 300, i.e.,

$$1.8047 \times 10^{-5} \text{ nM} \times \frac{0.0003 \text{ liter}}{1 \times 10^{-6} \text{ nmol/fmol}} = 1.6242 \times 10^{-2} \text{ fmoles}$$

3. Graphical Presentation of the Data

•An example of a typical saturation curve and the associated Scatchard analysis is illustrated in Figure 1. Maximal binding capacity (B_{max}) and association/dissociation constants (K_a / K_d) can be estimated using a number of commercially available iterative nonlinear regression analysis programs. One of the better programs was developed by Munson and Rodbard and is called LIGAND (Munson, P.J., and Rodbard, D. (1980) *Anal. Biochem.* 107, 220-239).

F. **References**

Nonneman, D.J., Ganjam, V.K., Welshons, W.V., and Vom Saal, F.S. (1992) *Biol. Reprod.* 47, 723-729

Segel, I.H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. 1st Ed, John Wiley and Sons, Inc., New York, NY

Tekpetey, F.R., and Amann, R.P. (1988) *Biol. Reprod.* 38, 1051-1060

III. MVLN Assay

A. Introduction

The MVLN estrogen specific transcription assay can be used to characterize estrogenic chemicals (Pons *et al.*, 1990). The assay utilizes an MCF-7 (Soule *et al.*, 1973) derivative that has been stably transfected with the Vit-Luc reporter gene (Pons *et al.*, 1990). Thus, the MVLN cell line expresses the endogenous estrogen receptor of MCF-7 and at the same time, contains an exogenous estrogen responsive reporter (luciferase). Therefore, the estrogen specific transcription activity of a test chemical is directly related to the activity of luciferase measured in the lysate of treated MVLN cells. The MVLN assay procedure presented here is a modified version of published methods (Gagne *et al.*, 1994; Pons *et al.*, 1990). While the protocol herein does utilize the MVLN subclone of MCF-7, all tissue culture materials, such as media and sera, are commercially available. In brief, MVLN cells are seeded into 12 well plates, fed media containing treatment compounds and then two days later, cell lysates are harvested and measured for luciferase activity. When cell counting is called for in this procedure, consult the method "Monolayer Cell Counting with a Coulter Counter" from this laboratory.

B. Maintenance of Cell Stocks

The MVLN cell line must be obtained from its source (Pons *et al.*, 1990). The MVLN clone has been shown to maintain a stable, estrogen responsive phenotype in this laboratory over many passages (at least 15). Stock cultures should be maintained in 10% fetal bovine sera (FBS) media under 5% CO₂ in a 37° C incubator. Such culture conditions will be "estrogen rich" and tend to favor cells that require estrogen for growth (MCF-7, MVLN). A regular schedule of passing stocks weekly (Monday into 8, T-25 flasks at a density of 5 x 10⁵ to 1 x 10⁶ cells/flask should provide enough cells for seeding stocks as well as experimentals (8, 12 well plates) seven days later. MVLN cells may grow slower than other MCF-7 derivatives. In addition, MVLN cells are very sensitive to seeding density. If seeded too light, MVLN cells will grow exceedingly slow and may not thrive. The common pH indicator phenol red has been shown to be estrogenic and therefore should not be used in cell cultures utilized for MVLN assays.

For routine passage, the MVLN cell monolayer is removed with trypsin/EDTA treatment, diluted to 10 ml with whole media and then dispersed into a suspension (mostly single cell) through the use of a 10 ml syringe and bent canula. This canula is a 14 gauge, blunt tip, Luer lock needle (Thomas Scientific) in which 1 cm at the tip is bent 30° to 45°. The 10 cm length can be used with T-25 flasks while the 15 cm will work with T-25 or T-75 flasks. First, count one duplicate flask. Second, remove media from flask(s) to be passed (monolayer of cells stays attached). Then, wash each flask three times with Ca⁺⁺ free HBSS, remove and then add 1-2 ml trypsin for three to five minutes @ 37° C. Be sure to disperse the trypsin evenly over the monolayer. After incubation, dilute trypsin to 10 ml with whole media. With the sterile canula-syringe, draw the 10 ml of media up into the syringe. Expel the media, with moderate force, through the bent canula, towards the cell monolayer (almost, but not touching the monolayer surface) with a circular motion covering the cell surface of the flask. Repeat for a total of three cycles making sure that all the monolayer has been removed from the flask (keep air bubbles to a minimum). After last cycle, leave the cell suspension in the flask. Then, with a 10 ml pipette, rinse down the inside of the flask three times with the cell solution. The cells will not be harmed by this procedure. This method disperses cells better than trypsin treatment alone. Note that since all MVLN cells can express the reporter gene in response to estrogen, it is essential that cells are dispersed evenly before seeding experimental plates.

An aliquot of this concentrated media-cell solution should then be diluted with media in a sterile vessel, mixed and used to seed flasks. For precise seeding, it is recommended that the entire volume of cells and media to seed all the flasks/plates desired is mixed in a single vessel. For example, to seed eight flasks with 7×10^5 cells each, make 50 ml of a seeding solution that is 1.16×10^5 cells/ml, mix well and then add 6 ml to each T-25 flask. Recall that you know how many cells are in each flask since a duplicate flask was counted previously. The goal of this method is to seed all flasks/plates the same (high precision). Since all MVLN cells can express the reporter gene in response to estrogen, precision in MVLN assays is completely dependent on uniform seeding of plates. It may be a good idea to practice seeding flasks and then count them the next day.

C. MVLN Assay Setup and Time Sequence

With the following exceptions, passing MVLN cells for assays should be done as described above. First, it is essential that cells used to seed experimental plates were withdrawn from estrogen six days prior to passage. A suggested way to withdraw cells from estrogen involves seeding stocks (eight flasks) in 10% FBS media one week before you plan on seeding experimentals. The day after seeding the stocks, label two flasks as "stock" and replace the media in these with 10% FBS media. These stock flasks should be fed 10% FBS media every other day until used to seed eight more stock flasks the following week. The remaining 6 flasks are then withdrawn from estrogen by rinsing three times with sterile PBS (all flask surfaces) and fed 10% dextran coated charcoal (DCC) FBS media. The 10% DCC FBS media is almost devoid of estrogens. These "withdrawn" flasks should be rinsed 3X and fed DCC media every other day until used for seeding a MVLN experiment. When seeding MVLN stocks or experiments, one of the duplicate flasks is always counted to determine the cells per flask count for that series (one of two stocks is counted, one of six withdrawn is

counted). Keep in mind that it takes a week to get cells ready for a MVLN experiment.

MVLN experiment 12 well plates are seeded on day -1 (Monday) with 1×10^5 cells/well in 10% DCC FBS media. To ensure constant seeding, mix the required cells and media in a sterile bottle and then seed aliquots (2 ml) into each well. The seeding mixture bottle should be mixed often during this procedure. The following day (day 0, Tuesday), the seeded cells are left alone and allowed to firmly attach to the plates.

Two days after seeding (day 1, Wednesday), cells are fed treatment media (2 ml/well). Treatment media is 5% DCC FBS into which treatments in ethanol carrier have been added. Treatments may be made up in 50 ml polyethylene tubes (do not use polycarbonate or polystyrene tubes! (Soto *et al.*, 1991)) and should be no more than 0.05% v/v ethanol carrier solvent. Higher levels of ethanol may have confounding effects on MVLN studies. Treatment carrier solvents such as DMSO and methanol should be avoided since they are toxic to cells and will have confounding effects on MVLN studies. Experimental cells should have new treatment media applied the next day (day 2, Thursday).

On day 3 (Friday), treated cell lysate should be harvested for luciferase assay. First remove treatment media from each well. Then, wash each well one time with 2 ml PBS. It is essential that all PBS is removed from each well at this step. Removal of the PBS by aspiration followed by a one minute bench top incubation with the plate tipped 45° and final aspiration of drained residue is recommended. To lyse cells, add 500 µl lysis buffer to each well and then incubate at room temperature on a rocker for at least 30 minutes. Transfer lysate from wells with micro-pipet to micro tubes. Make sure that all cell debris is mixed and removed uniformly between wells. Lysate can be left at room temperature for up to six hours, kept at 4° C over night or stored indefinitely at -70° C without loss of luciferase activity. Sample tubes should be equilibrated at room temperature, vortexed and then spun down prior to sampling (20 to 50 µl) for luciferase assay.

D. MVLN Assay Design

Properly designed MVLN assays can be utilized to answer only the following four questions. First, does the test compound stimulate estrogen receptor mediated transcription (what is the shape of the corresponding dose response curve)? Second, if the test compound stimulates transcription, is this response an estrogen receptor mediated mechanism (is the compound an estrogen receptor agonist)? Third, can the test compound block the agonist effects of E₂ (is the compound an antiestrogen)? Last, is the test compound toxic to MVLN cells? Attempts to obtain additional information from the MVLN assay may be misleading. Keep in mind that this assay does not necessarily determine if the test compound binds to the ER.

Each data point of the MVLN assay should be run in duplicate (minimum) or triplicate during a trial. Then, that same trial should be repeated at least two more times.

E. Example Experiment Setup:

Blank (no additions to DCC FBS media)

2 wells	
E ₂ (positive controls) 10 ⁻¹² , 10 ⁻¹¹ , 10 ⁻¹⁰ , 10 ⁻⁹ , 10 ⁻⁸ , 10 ⁻⁷ M	2
wells	
ICI-182,780 10 ⁻⁶ M (check of estrogen free conditions)	
2 wells	
E ₂ 10 ⁻¹⁰ M + ICI-182,780 10 ⁻⁶ M (Check of ICI).....	
2 wells	
Test compound A: 10 ⁻⁸ , 10 ⁻⁷ , 10 ⁻⁶ , 10 ⁻⁵ M	
8 wells	
Test compound B: etc.	
ICI 10 ⁻⁶ M + TC A 10 ⁻⁵ M (ER mechanism test)	
2 wells	
ICI 10 ⁻⁶ M + TC B 10 ⁻⁵ M etc.	
E ₂ 10 ⁻¹⁰ M + TC A 10 ⁻⁵ M (antiestrogen test)	
2 wells	
E ₂ 10 ⁻¹⁰ M + TC B 10 ⁻⁵ M etc.	
to a Total of	96 wells

Note: Test compound toxicity is determined by comparing Luc activity of test compound treatments with blank activity and/or test compound with ICI to ICI alone.

F. Example Stock and Experiment Schedule

Day -1 (Monday)	Pass stocks, experimentals plated.
Day 0 (Tuesday)	Cells left undisturbed to attach to flasks/plates.
Day 1 (Wednesday)	Feed stocks (2 flasks FBS, 6 flasks withdrawn in DCC FBS).
	Feed experimentals treatments.
Day 2 (Thursday)	Feed experimentals treatments.
Day 3 (Friday)	Feed/withdraw stocks.
	Experimentals harvested (morning).

G. Regarding an Estrogen Free Laboratory Environment

All glassware, caps, hoses, etc. that may contact media must be free of estrogens. Soap wash and 3X hot rinse followed by rinsing three times with ddH₂O, air dry, rinse with 95% ethanol (from glass bottle; cans and plastic may have estrogenic contamination!) followed by rinse

three times with ddH₂O water. Baking of foil covered glassware for 12 hours in a 250° C oven will sterilize and remove more organic compounds. Wash and rinse bottle caps as above, dry in low temp oven and autoclave. Alternatively, bottles and caps may be autoclaved together with the risk of contamination by estrogenic condensates. Your cell culture environment should be characterized for estrogen contamination by proliferation assay tests with and without added ICI-182, 780 (Wakeling and Bowler, 1992). If the "estrogen free" cells grow faster (> 10%) than the ICI treated cells, you have estrogen contamination. All experiments conducted in the presence of such contamination are useless since regardless of how they are set up, you are testing combinations of chemicals. We have found plastic vessels and implements to be the major source of estrogen contamination. Polystyrene and polycarbonate seem to be the big problems. Do not use culture flasks with "phenolic" caps. Filter units may add estrogenic substances to media. The Corning bottle top units (orange) are suspect. Zap Caps seem to add some kind of nonestrogenic mitogen which induces MCF-7 cells to grow at maximum rate, even in the presence of ICI. It is unclear what effect Zap Cap contamination has on MVLN assays. Also, it appears to be relatively easy to extract estrogens from gloves and/or hands when rinsing items with ethanol. Lastly, ethanol may confound MVLN assays. Ethanol rinsed vessels and implements must be thoroughly dry before use.

H. Media

1. DMEM powder for 10L (Gibco 13000-096).
2. 59.58 gm HEPES (Gibco 11344-033), media will be 20 mM.
3. 100 ml non-essential amino acids (Gibco 11140-019), media will be 0.1 mM.
4. 100 ml sodium pyruvate (Gibco 11360-070), media will be 1 mM.
5. 37 gm NaHCO₃.
6. 10 ml/L media of Antibiotic-Antimycotic solution (Gibco 15240-062).

In 3 L tissue culture grade water, add 1, 2, 3 & 4 above. Mix 45 minutes in 4 L beaker. Add 5, mix 15 minutes. pH to 7.3. Dilute to 10 L and mix 15 minutes. Check pH. Filter into 500 ml sterile bottles (Gelman VacuCap 4622 or Gelman Micro Culture Capsule 12158). Store media at 4° C. Add Antibiotic-Antimycotic solution to media bottle when used (made up with sera). To make one bottle of 10% sera media, add 50 ml sera to 500 ml of media.

I. Sera

FBS	Hyclone Characterized Fetal Bovine Sera (A-1115-L)
DCC FBS	Hyclone Charcoal/Dextran Fetal Bovine Sera (A-1120-L)

J. Buffers

Ca ⁺⁺ Free HBSS	Gibco 14185-052
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PBS	Gibco 14080-055
Lysis Buffer	Promega E153A

K. References

- Gagne, D., Balaguer, P., Demirpence, E., Chabret, C., Trousse, F., Nicolas, J. C., and Pons, M. (1994): Stable luciferase transfected cells for studying steroid receptor biological activity. *Journal of Bioluminescence & Chemiluminescence* 9, 201-9.
- Pons, M., Gagne, D., Nicolas, J. C., and Mehtali, M. (1990): A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. *Biotechniques* 9, 450-9.
- Soto, A. M., Justicia, H., Wray, J. W., and Sonnenschein, C. (1991): p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environmental Health Perspectives* 92, 167-73.
- Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. (1973): A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute* 51, 1409-16.
- Wakeling, A. E., and Bowler, J. (1992): ICI 182,780, a new antioestrogen with clinical potential. [Review]. *Journal of Steroid Biochemistry & Molecular Biology* 43, 173-7.

IV. CV1 Cell Transfections

Purpose and Applicability

The purpose of this protocol is to outline a procedure for the quantitation of AR-mediated alterations of transcriptional activation in CV1 cells.

MONDAY

- plate 0.2×10^6 CV1 cells / 6cm Corning TC dish in the following media
 - DMEM - (high glucose + L-glutamine + 25 mM Hepes + NaHCO_3) - Take a 0.5 liter bottle and add
 - 5 ml of 100X antibiotic/antimycotic (frozen aliquots)
 - 50 ml fetal bovine serum (frozen 50 ml aliquots)

NOTES:

- Grow cells up in T-150 culture flasks in 10% FCS / DMEM.
- When there are enough cells for the experiment, decant media from the flasks into funnel waste beaker layered with sterile gauze to prevent back splash.
- Wash cells twice with 20 ml of 1X HBSS and decant into waste funnel (this gets rid of the serum which inactivates trypsin).
- Add 1-2 ml of 1X Trypsin (from 1.0X frozen stock) - roll the trypsin over the cells and place cells in the incubator for five to ten minutes.
- After the cells detach (i.e., round up and float), thump the side of the flask with the palm of your hand and add 10 ml of 10% FCS / DMEM (the serum in the media inactivates the trypsin).
- Transfer the cells to a 50 ml centrifuge tube (be sure to get all the cells off the plates) and spin the cells down at 1000 rpm (250 x g).

- Aspirate or decant the media and thump pellet loose from the tube - add 10 ml of 10% FCS / DMEM and resuspend the cells by pipeting up and down with a 10 ml pipet.
- Count cells on a hemocytometer as follows:
Total cell # in all center squares $\times 0.01 = \# \text{ cells (in millions) / ml}$
- Dilute the cell suspension out to 50,000 cells/ml and add 4ml of this cell stock solution to each 6 cm plate using a 10 ml pipet.
- In general, the following numbers of CV1 cells are obtained:
 - T-150 culture flask of almost confluent CV1 cells will contain $8-10 \times 10^6$ cells
 - T-75 culture flask of almost confluent CV1 cells will contain $4-5 \times 10^6$ cells

**** HANDS ARE WASHED WELL WITH SOAP AND WATER (ESPECIALLY UNDER NAILS) AND BEFORE PLACING HANDS IN THE CULTURE HOOD, HANDS SHOULD BE SPRAYED WITH 70% ETOH.**

-THE HOOD SURFACES ARE SPRAYED WITH 70% ETOH AND WIPED CLEAN BEFORE USE.

-ANYTHING THAT GOES IN THE HOOD THAT IS NOT STERILE IS SPRAYED WITH 70% ETOH INCLUDING MEDIA BOTTLES, TUBE RACKS, PIPETMEN, ETC. CAN ALSO FLAME THE OUTSIDE OF GLASS CULTURE MEDIA BOTTLES

TUESDAY

-Prepare 2X HBS (100 ml) as follows:

- 90 ml ddH₂O
- 5.6 ml 5M NaCl (from sterile stock)
- 2.5 ml 2M HEPES (Na⁺ salt)
- 1.5 ml 0.1M Na₂HPO₄ **** pH to 7.12-7.13**** , then filter, sterilize and store in the refrigerator.

-Prepare CaPO₄ precipitates (listed below are the amounts for 14 dish batches) after all components have reached room temperature. Add the following to a sterile 5.0 ml tube in this order

- 1.53 ml ddH₂O
- 700 ng pCMVhAR (P₉; 50 ng/dish)
- 70 ug MMTV-LUC (5 ug/dish) **vortex to mix**
- 219 ul 2M CaCl₂ (from frozen stock made by adding 22.197g CaCl₂ to a 100 ml volumetric flask and bring the volume to 100 mls with ddH₂O, sterile filter).

****vortex to mix****

-While vortexing (setting 2.5) a 1.75 ml aliquot of the 2X HBS solution in a 15 ml screw top conical c-fuge tube add the contents of the 5.0 ml tube containing CaCl₂-DNA with a 5.0 ml plastic pipet.

****DROPWISE AND SLOWLY**.**

-Repeat for the remaining tubes then wait 10 minutes for ppt's to form.

-At ten minutes add 3.5 ml of media to each tube to stop the precipitation (the ppt will be

stable for at least 30 minutes)....repeat for the other tubes at their ten minute time periods. After all ppts have been stabilized, check the ppts to be sure they are small with individual grains. If particles are linked into chains or lumpy looking do not use it. Dilute the contents of each tube (now containing 7.0 mls) with 49 mls media and combine all in a sterile flask (i.e., for multiple 14 dish batches).

-Aspirate the media from the CV1 cell dishes and immediately add 4 ml of gently mixed DNA-ppt media to each of the dishes in the experiment using a 10 ml glass pipet.

-Return dishes to 37° C incubator for four to six hours.

-Aspirate the precipitate from each dish and wash each dish two times with 4 ml DPBS and add 4 ml 5%DCC-FBS DMEM media.

-Place all dishes in 37° C incubator overnight

WEDNESDAY

-Make up a 1000X concentrated stock solution for each treatment in ETOH (i.e., so won't have to sterilize) as follows: 10.0 mM, 1 mM, 0.5 mM, 0.2 mM, 0.05 mM.

-Make up 10X DHT working solution by adding 24 ul of 10 uM DHT (made fresh from frozen 10 mM stock) to a 5.0 ml sterile tube and combine with 2.4 mls of 5% DCC-media (this is enough for 60 dishes).

-Take plates out of incubator and add 4 ul of each treatment to appropriately labelled dishes (in duplicate).

Add treatments to the side of each dish and let run down into the media- rock plates after addition.

-Immediately add 40 ul of 10X DHT (to the side of each dish to receive DHT) using the positive displacement pipetting technique.

-Move plates back and forth to distribute the treatments evenly in the media.

-Return the dishes to the 37° C incubator overnight.

**Notes:Final []'s are 1nM DHT and 10 uM, 2 uM, 0.5 uM, 0.2 uM, 0.05 uM treatments.

--2X TBS Buffer (500 ml)----- For 4 liters

-8.18 g NaCl	-65.44 g
-0.23 g KCl	-1.84 g
-0.147 g CaCl ₂ - 2H ₂ O	-1.18 g
-0.1 g MgCl ₂ - 6 H ₂ O	-0.80 g
-0.128 g Na ₂ HPO ₄ - H ₂ O	-1.02 g
-3.03 g Tris -HCL	-24.24 g

pH the buffer to 7.4 and filter sterilize into a sterile bottle before use.

THURSDAY

MORNING - Aspirate media from all dishes and replce with 4.0 mls of fresh 5% DCC-media

using a 10 ml pipette. Repeat the above dosing protocol.

AFTERNOON - Harvest cells

-Aspirate media from all dishes; wash cells twice with 2 ml of 1X HBSS and aspirate the HBSS from all dishes.

-add 0.5 ml cell culture lysis buffer (at room temperature) to each plate and let rock at room temperature for 20-30 minutes.

-using a rubber scraper (if necessary), scrape the cells loose from the dish and then slowly pipette the lysis buffer and cells up and down. --Need to go slow because one does not want excessive bubbles.

** Can use the same rubber scraper for all plates, if the scraper is rinsed off with ddH₂O between dishes and go from high treatment dose to low treatment dose (i.e., low luciferase to high luciferase).

** Place all of the cell lysate into prelabelled 1.0 ml microcentrifuge tubes and store at -70° C until assay (i.e., Friday morning).

FRIDAY - LUCIFERASE ASSAY

-Prepare 20 mls of rxn buffer in a 50 ml centrifuge tube as follows (20 mls is enough for 50 samples):

-Add 18 mL ddH₂O to a 50 ml centrifuge tube

-Add 500 uL of 1 M Glycylglycine (make a 50 ml stock by adding 6.605g gly-gly (MW 132.1) to 50 ml ddH₂O, pH to 7.8, sterile filter and store at 4° C).

-Add 300 uL 1M MgCl₂ (from Sigma also stored at 4° C)

-Add 1.0 mL of 100 mM ATP (make a 50 ml stock by adding 2.755g of ATP (MW 551.1 g/mole) to 50 ml ddH₂O, pH the soln to 7.8, aliquot to 1.0 mL and store at -80° C).

-Add 200 uL of 50 mg/ml BSA (make a 10 ml stock by adding 500 mg BSA to 10 ml ddH₂O, aliquot to 200 uL and store at -20° C).

-Place the tube at room temperature

** NOTE: IT IS CRITICAL THAT THE pH OF THE RXN BUFFER IS 7.8 AT ROOM TEMPERATURE. IF THE GLY-GLY AND ATP SOLUTIONS ARE CAREFULLY pH'ED, THEN THE FINAL MIX WILL BE pH 7.8 - CHECK TO BE SURE. **

-Take cell lysates out of the freezer and allow to thaw at room temperature.

-Fill brown luminometer bottle with ddH₂O and connect to automatic injector in the front panel of the luminometer. Make sure a cuvette is in the chamber and flush the luminometer lines with water (Service 14). Empty the cuvette and replace in the chamber.

**NOTE: NEVER PLACE WET CUVETTES IN THE INSTRUMENT AND ALWAYS KEEP A CUVETTE IN THE INSTRUMENT WHEN NOT IN USE. **

- Flush the lines with air (i.e. disconnect the brown bottle) again using Service 14.
- Add the luciferin to brown bottle #1 and fill the bottle approximately to the shoulder (should be enough for about 100 samples). Add rxn buffer to the brown bottle #2 and fill the bottle approximately to the shoulder (should be enough for about 100 samples). Place a new cuvette in the chamber and flush the lines with the luciferin/rxn solution.
- The 1mM D-luciferin stock solution is made and stored as follows:
- Add 159.21 mg D-luciferin (K^+ salt; MW 318.41 g/mole) to 500 ml ddH₂O. Make 10 ml aliquots in 15 ml centrifuge tubes. Wrap tubes with aluminum foil (i.e., luciferin is light sensitive) and store at -20° C.

NOTE: Can also use the Na^+ salt of D-luciferin but it sometimes turns yellow - its probably still ok but better to use the K^+ salt to avoid any potential problems.

- Set-up luminometer (20 sec read, continuous measurements, raw data, no duplicates) so will get raw data (i.e., relative light units) for each sample.
- Vortex the cell lysate and spin tubes for 30 seconds to isolate cell debris.
- Add 50 ul of vortexed cell lysate to a new cuvette, place in the luminometer and press start. While the sample is being read get another tube ready (this usually works out so that by the time the machine is ready the next sample is also ready to go).
- Use a new cuvette for each sample, do not reuse cuvettes.
- When finished, exit and press service 13 to back the contents of each line back into the respective brown bottles, decant to stock solution and place the rxn buffer in the refrigerator and the luciferin in -70C freezer.
- Wash the brown bottles out with ddH₂O and place ddH₂O in each bottle and place back in-line. Place an empty cuvette in the machine and flush the lines (service 14) first with ddH₂O then with air. Leave the lines dry, shut off the luminometer and always keep an empty cuvette in the sample chamber.

V. Procedures to Assess Individual Steroidogenic Enzyme Activities in the Rat

A. Purpose and Applicability

The purpose of this protocol is to outline a procedure for the radiometric quantitation of steroidogenic enzyme activity. The principle of this procedure is to quantitate the conversion of radiolabelled substrate to radiolabelled product; data is usually expressed as pmoles product formed/(unit time x mg protein). To this end, a tissue homogenate or cell preparation is incubated under carefully controlled conditions (temperature, pH, time, substrate concentration, cofactor concentration, and osmolality) with radiolabelled substrate. Following the incubation the reaction is terminated and the steroids (androgens, progestins, etc.) are extracted from the biological matrix by solid phase extraction (SPE; see SPE SOP for this procedure). The extracted steroids are dried *in vacuo* using a vacuum concentrator (see vacuum concentrator SOP for correct operation of this equipment) and injected into the HPLC with on-line radiometric quantitation (see HPLC SOP's). The HPLC system

chromatographically separates the substrate from product(s), mixes the HPLC effluent with scintillation fluid and quantitates the amount of radioactivity in each substrate/product(s) peaks. See the references listed below for additional clarification. Please note the specifics for the radiometric quantitation of the following enzyme activities:

1. 5 α -Reductase: The substrate is testosterone (5.0 mM) and the products of the reaction are 5 α -dihydrotestosterone + 5 α -androstane-3 α /17 β -diols. β -NADPH (0.5 mM) is the cofactor.
2. 17 α -Hydroxylase: The substrate is progesterone (5.0 mM) and the products of the reaction are 17 α -hydroxyprogesterone + androstenedione + testosterone. β -NADPH (0.5 mM) is the cofactor.
3. C₁₇₋₂₀-Lyase: The substrate is 17 α -hydroxyprogesterone (5.0 mM) and the products of the reaction are androstenedione and testosterone. β -NADPH (0.5 mM) is the cofactor.
4. 17-Ketosteroid Reductase: The substrate for the reaction is androstenedione (5.0 mM) and the product is testosterone. β -NADPH (0.5 mM) is the cofactor.
5. 5-Ene-3 β -hydroxysteroid Dehydrogenase/Isomerase: The substrate for the reaction is pregnenolone (5.0 mM) and the product is progesterone. β -NAD⁺ (0.5 mM) is the cofactor.

B. Safety and Operating Precautions

All procedures with radioisotopes should follow the regulations and procedures as described in the Hazardous Agent Protocol (HAP) and in the Radiation Safety Manual and Protocols.

C. Equipment and Materials

1. Equipment

- Corning Stir/hot Plate
- Digital Pipets
- Balance
- Polytron PT 35/10 Tissue Homogenizer
- Hewlett-Packard HPLC with on-line Radiomatic Radiochromatograph
- Heated Shaking Water Bath
 - Vacuum Concentrator
- Hamilton Syringes (50 ml)

2. Chemicals

- Sodium Phosphate, Monobasic and Dibasic
- Sodium Chloride
- Magnesium Chloride
- Calcium Chloride

- Potassium chloride
- double-distilled de-ionized water
- Recrystallized Pregnenolone, Progesterone, 17a-Hydroxyprogesterone, Androstenedione or Testosterone
- ^3H -[X,X,X]-Pregnenolone, Progesterone, 17a-Hydroxyprogesterone, Androstenedione or Testosterone (New England Nuclear)
- Scintillation Cocktail (Flow Scint III)
- β -NAD $^+$ or β -NADPH cofactor
- Ethyl Alcohol, anhydrous

3. Supplies

- 20 ml Glass Scintillation Vials
- 12 x 75 mm Glass Test Tubes
- 100 ml, 1000 ml volumetric flasks
- 100 ml Erlenmeyer flasks

D. Methods

1. Preparation of Krebs-Ringer Phosphate Buffer (KRPB) pH 6.9

Add each of the following to individual 100 ml volumetric flasks and fill flasks with sodium phosphate buffer (prepared as described below) to 100 ml:

- 6.779 g Sodium Chloride
- 0.264 g Magnesium Chloride
- 0.336 g Potassium Chloride
- 0.386 g Calcium Chloride

Store the above glass-stoppered solutions in the refrigerator. To prepare the sodium phosphate buffer, place 2.5811 g of sodium phosphate in a 1000 ml and add distilled water to the volume line and then add a stir bar. Put the beaker and contents on a Corning stir plate and stir the solution until dissolved on a stir setting of 3.5. To prepare the KRPB add 10 mls of each of the above four salt solutions to a 100 ml Erlenmeyer flask. Add 55 ml of phosphate buffer and 5.0 ml of glycerol to the salt solutions in the Erlenmeyer flask. The final volume of KRPB should be 100 ml. Adjust the pH of the solution with 5N HCL to pH 6.9. Store the KRPB buffer in the refrigerator. This buffer is stable for approximately one week.

2. Preparation of Radioinert Steroid Standards

Prepare 1.0 mM stock solutions in ethanol. Weigh out the following amounts of steroids and place in 100 ml ethanol.

- Pregnenolone = 31.65 mg/100 ml
- Progesterone = 31.45 mg/100 ml
- 17a-Hydroxyprogesterone = 33.05 mg/100 ml
- Androstenedione = 28.64 mg/100 ml
- Testosterone = 28.84 mg/100 ml

For 5 mM concentrations in 1.0 ml incubations add 5.0 ml to each incubation vial as directed below.

3. Preparation of ^3H -Steroid Solutions

Dilute a 10 ml aliquot of the ^3H -steroid stock with ethanol so that 100 ml yields approximately 200,000 dpm. *** Remember to count the sample in 15 ml of scintillation fluid on channel 1 (i.e., 0-400) of the scintillation counter. ***

4. Preparation of Nicotinamide Cofactor Solutions

Prepare 5 mM stock solution immediately prior to the initiation of the reaction. ***Note that the addition of the cofactor should be the final step in the reaction commencement - see specific instructions below. *** Take the appropriate cofactor out of the freezer and immediately weigh as follows:

NAD⁺ = 3.32 mg/ml KRPB

NADPH = 4.17 mg/ml KRPB

For 0.5 mM concentrations use 100 μl /ml incubation volume as described below.

5. Steroidogenic Enzyme Assay Procedure

The first step is to pipet the radioinert and radioactive substrate into each vial. Assays of steroidogenic enzyme activity always are completed in duplicate; thus, label two 20 ml glass scintillation vials for each tissue sample, plus four control vials. Pipet 5 ml of the appropriate radioinert steroid into the bottom of each scintillation vial. Next pipet 100 μl of the radioactive steroid into each tube. For example, if the objective of the experiment was to quantitate 17 α -hydroxylase activity then add 5 ml of 1 mM progesterone and 100 μl of ^3H -progesterone (200,000 dpm) to each vial. Take the rotor out of the vacuum concentrator and place the vials inside the vacuum concentrator. Evaporate the ethanol vehicle *in vacuo* by turning on the vacuum switch of the vacuum concentrator. Allow ethanol to evaporate while the tissues are being prepared as described below.

Harvest and weigh the appropriate tissues from the control and/or treated animals. Record the weights and place each separate tissue in a 50 ml polypropylene homogenization tube. Place the tubes in an ice-water bucket and add 10 ml KRPB/gm tissue wet weight. Homogenize the tissues with 15 second bursts with the polytron separated in time by at least 30 seconds. This procedure is to keep the tissues at 0-4°C at all times. Filter the homogenates through 100 μm nylon mesh filters into clean 12 x 75 mm test tubes and place each tube on ice.

Turn off the vacuum switch and allow the pressure on the inside of the vacuum concentrator to equilibrate to atmospheric pressure. Remove the scintillation vials, label them and place them in a 34°C water bath (Dubanoff -

with metal inserts to hold each vial). Add 700 ml of KRPB to each tube and allow the tubes to shake for ten minutes at 120 cycles/min in order to resuspend the steroid substrate. Next, add 200 ml of tissue homogenate (20 mg tissue) to each labelled tube. Allow the tissues to shake at 120 cycles/min for five minutes then add 100 ml of the appropriate cofactor solution to begin the reaction. *** Note that control vials will contain 800 ml of KRPB and 200 ml of tissue homogenate without NADPH. *** Time the incubation duration from the time the cofactor was added. At the end of the appropriate incubation time (need to determine the appropriate incubation time for the tissue being examined) stop the reaction and extract the steroids from the biological matrix in each incubation vial as described in the solid-phase extraction SOP. Submit the samples to Dr. Kelce for subsequent HPLC analysis.

E. Data Processing

Data will be processed by the software analysis package of the HPLC. The detection of eluted radioactive substrate and product peaks is reported in dpm and % of total radioactivity in each analysis. Data is presented as enzyme specific activity as described in the references listed below.

F. References

Kelce, W.R., Krause, W.J., and Ganjam, V.K.: Unique Regional Distribution of Delta-4-3-Ketosteroid 5a-Oxidoreductase and Associated Epididymal Morphology in the Marsupial, Didelphis virginiana. Biology of Reproduction 37:403-420, 1987.

Kelce, W.R. and Ganjam, V.K.: Radiometric Quantitation of Delta-4-3-Ketosteroid 5a-Oxidoreductase Utilizing High Performance Liquid Chromatography. Steroids 52:217-235, 1988.

Kelce, W.R., Rudeen, P.K., and Ganjam, V.K.: Prenatal Ethanol Exposure Alters Testicular Steroidogenic Enzyme Activity in the Newborn Rat Testis. Alcoholism: Clinical and Experimental Research 13:617-621, 1989.

Kelce, W.R., Raisbeck, M.F., and Ganjam, V.K.: Gonadotoxic Effects of 2-Hexanone and 1,2-Dibromo-3-chloropropane on the Enzymatic Activity of Rat Testicular 17a-Hydroxylase/C17,20-Lyase. Toxicology Letters 52:331-338, 1990.

Kelce, W.R., Ganjam, V.K., and Rudeen, P.K.: Inhibition of Testicular Steroidogenesis in the Neonatal Rat Following Acute Ethanol Exposure. Alcohol 7:75-80, 1990.

Kelce, W.R., Ganjam, V.K., and Rudeen, P.K.: Effects of Fetal Alcohol Exposure on Brain 5 α -Reductase/Aromatase Activity. J. Steroid Biochem. 35:103-106, 1990.

Kelce, W.R., Lubis, A.M., Braun, W.F., Youngquist, R.S. and Ganjam, V.K.: Influence of Rete Testis Fluid Deprivation on the Kinetic Parameters of Goat Epididymal 5 α -Reductase. Steroids 55:27-31, 1990.

VI. Research Protocol For Assessment Of Steroidogenesis Using In Vitro Testes Culture

Purpose and Applicability

The purpose of this protocol is to outline a procedure for the quantitation of steroidogenic hormone production from minced rat testicular tissue, as altered hormone production is indicative of altered gonadal enzyme activity.

A. 50 Mg Pieces (Rat or Other Species)

1. Each testis is cut into pieces that weigh approximately 50 mg, placed in a 1.5 ml microcentrifuge tube on ice and media is added to all of them at the same time. They are incubated in 1.0 ml of media and are assigned to various treatment as dictated by the protocol.
2. The tubes are capped and placed into a microcentrifuge tube water bath rack (USA/Scientific Products). The cover of the rack is fastened down in order to hold the caps of the tubes closed. These racks are then placed in a reciprocating shaker set at 72 cycles per minute. The shaker is set in an incubator at 36° C with 5% filtered carbon dioxide.
3. At the end of the appropriate amount of time the tubes removed from the incubator and are spun in a refrigerated centrifuge at 1500 RPM (430 x g) for three minutes. The supernatant is poured into a 1.5 ml storage tube and frozen at -50° C until assayed.

B. Pieces and 1/4 Testes (Rat And Other Species)

1. The testes are removed from the animal and put into cold Dulbecco's phosphate buffered saline.
2. The testes are decapsulated and the main blood vessels are removed.

3. For the 1/4 testes culture each testis is cut in half through the long axis and then in half through the short axis. Each piece is weighed (approximately 400 mg), put into a glass 20 ml scintillation vial and incubated in 5 ml of media 199 (Gibco, catalog # 400-1100EB). Each piece of the same testis is assigned to + or -hCG treatment.
4. The scintillation vials are capped and placed in racks that will hold them securely. They are placed in the shaker in the incubator at 34° C and 5% filtered carbon dioxide and shaken gently (72 cycles/minute) for one hour.
5. At the end of this time the vials are removed from the incubator. A 200-500 µl sample is taken from each one and placed in a storage vial to be frozen for later assays.
6. hCG is added to the appropriate vial and placed back into the incubator. The process is repeated every hour for the next three hours.

C. Stock Solutions and Media Preparation

1. PREGNENOLONE: 5-Pregnen-3 β -ol-20-one M.W.= 316.5
Sigma #P 9129

STORE STOCK IN REFRIGERATOR--STABLE FOR ONE MONTH

STOCK: 0.03165 grams + 10 ml ETOH = 10,000 µM/ml

STOCK 1: For 10 µM Pregnenolone:
Make a 1:10 dilution of the original stock = 1,000 µM/ml
ADD 10 µl to 1 ml of M-199 culture = 10 µM Pregnenolone

STOCK 2: For 2 µM Pregnenolone:
Make a 1:10 dilution of STOCK 1 = 100 µM/ml
ADD 20 µl to 1 ml of M-199 culture = 2 µM Pregnenolone

STOCK 3: For 0.1 µM Pregnenolone:
Make a 1:10 dilution of STOCK 2 = 10 µM/ml
ADD 10 µl to 1 ml of M-199 culture = 0.1 µM Pregnenolone

2. PROGESTERONE: 4-Pregnene-3,20-Dione M.W.= 314.5
Sigma # P 0103

STORE STOCK IN REFRIGERATOR--STABLE FOR TWO MONTHS

STOCK: 0.03145 grams + 10 ml ETOH = 10,000 µM/ml

STOCK 1: For 10 μ M Progesterone:
 Make a 1:10 dilution of the original stock = 1,000 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 10 μ M Progesterone

STOCK 2: For 2 μ M Progesterone:
 Make a 1:10 dilution of STOCK 1 = 100 μ M/ml
 ADD 20 μ l to 1 ml of M-199 culture = 2 μ M Progesterone

STOCK 3: For 0.1 μ M Progesterone
 Make a 1:10 dilution of STOCK 2 = 10 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 0.1 μ M Progesterone

3. CHOLESTEROL: 20 a-Hydroxycholesterol M.W.= 402.5
 Sigma # H 6378

STORE STOCK IN REFRIGERATOR--STABLE FOR TWO MONTHS

STOCK: 0.020 grams + 5ml ETOH = 10,000 μ M/ml

STOCK 1: For 10 μ M Cholesterol:
 Make a 1:10 dilution of the original stock = 1,000 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 10 μ M Cholesterol

STOCK 2: For 1 μ M Cholesterol:
 Make a 1:10 dilution of STOCK 1 = 100 μ M/ml
 ADD 20 μ l to 1 ml of M-199 culture = 1 μ M Cholesterol

STOCK 3: For 0.1 μ M Cholesterol:
 Make a 1:10 dilution of STOCK 2 = 10 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 0.1 μ M Cholesterol

4. ANDROSTENEDIONE: 4-Androstene-3,17-Dione M.W.= 286.4
 Sigma # A 9630

STORE STOCK IN REFRIGERATOR--STABLE FOR TWO MONTHS

STOCK: 0.02864 grams + 10ml ETOH = 10,000 μ M/ml

STOCK 1: For 10 μ M Androstenedione:
 Make a 1:10 dilution of the original stock = 1,000 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 10 μ M Androstenedione

STOCK 2: For 2 μ M Androstenedione:
 Make a 1:10 dilution of STOCK 1 = 100 μ M/ml
 ADD 20 μ l to 1 ml of M-199 culture = 2 μ M Androstenedione

STOCK 3: For 0.1 μ M Androstenedione:
 Make a 1:10 dilution of STOCK 2 = 10 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 0.1 μ M Androstenedione

5. hCG: Sigma # CG 10 (p. 258) = 10,000 IU/ml

STOCK: 10,000 IU/vial in 1 ml of distilled water = 10,000 mIU, aliquot into 100 μ l aliquots and freeze.

WORKING STOCK: 10 μ l of original stock into 10 ml of M-199
 This will = 10,000 mIU/ml.
 ADD 10 μ l of working stock to each ml of culture media.

6. db cAMP: N6,O21 Dibutyl Adenosine 3':5' Cyclic Monophosphate
 Sigma # D 0627, M.W. 491.4

PREPARE FRESH DAILY

STOCK: 0.04914 GRAMS + 1 ml M-199 = 100 mM/ml

10 μ l STOCK + 1ml M-199 = 1 mM
 ADD 10 μ l to 1ml M-199
 20 μ l STOCK + 1ml M-199 = 2 mM
 ADD 20 μ l to 1ml M-199
 40 μ l STOCK + 1ml M-199 = 4 mM
 ADD 40 μ l to 1ml M-199

7. 17 a OH PROGESTERONE 4-PREGNEN-17a,20b-DIOL-3-ONE
 Sigma # P 6285, M.W. 332.5

STORE STOCK IN REFRIGERATOR STABLE TWO MONTHS

STOCK = 0.3325 grams + 10 ml ETOH

STOCK 1: For 10 μ M 17 a OH Progesterone:
 Make a 1:10 dilution of the original stock = 1,000 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 10 μ M Progesterone

STOCK 2: For a 2 μ M 17 a OH Progesterone:
 Make a 1:10 dilution of STOCK 1 = 100 μ M/ml
 ADD 20 μ l to 1 ml of M-199 culture = 2 μ M Progesterone

STOCK 3: For a 0.1 μ M 17 a OH Progesterone
 Make a 1:10 dilution of STOCK 2 = 10 μ M/ml
 ADD 10 μ l to 1 ml of M-199 culture = 0.1 μ M Progesterone

Medium 199 powder is purchased from Gibco Laboratories (Catalog # 400-1122EB). One liter of liquid medium is prepared as follows:

- a. Add powder to a 1000 ml graduated cylinder. Rinse the packet three times with distilled water and bring the volume up to 1000 ml.
- b. Put a magnetic stir bar into the cylinder and place on a stirrer. Stir until all powder is off the bottom of cylinder and incorporated into liquid. Pour into 1000 ml beaker (for ease in mixing and determining pH). Position pH electrode in beaker.
- c. Add while stirring:
 - 0.71 g Sodium Bicarbonate (Sigma, S-8875)
 - 2.1 g HEPES (Sigma, H-3375)
 - 1.0 g BSA (Swartz-Mann, # 802247)
 - 0.025 g Trypsin Inhibitor (Sigma, T-9003)
- d. Adjust pH of medium, while stirring, to pH 7.1-7.3 (0.2 to 0.3 pH units below desired final pH 7.4) using 1 N NaOH (Sigma, S-5881) or 1 N HCL (Sigma, 920-1).
- e. Sterilize immediately by membrane filtration (Krackler Scientific, cat.# 161-0026, pore size, 0.2 microns) using negative pressure.

Preparation of Media containing hCG (100 mIU/ml)

One vial of hCG (Sigma, CG-10) containing 10,000 IU is

diluted in 1 ml of distilled water. This is separated into 100ml aliquots and stored at -50°C. When ready for use a vial is thawed and 10 µl are added to 10 ml of media 199. This is then added to the culture at 10 µl per ml of media to give a concentration of 100 mIU's per ml.

D. Additional Language to Consider in Developing Steroidogenesis Assay Procedure

An in vitro testis culture can be used as a screening assay to evaluate effects on the mammalian steroid hormone synthesis pathway. The procedure outlined below is a modification of that used by Lasky et al.(1994). Testes are removed from ten week-old untreated rats, and decapsulated. Approximately 50 mg of testicular parenchyma is then placed into a 20 ml scintillation vial containing 5 ml of culture media (RMPI-1640 media, 10% fetal calf serum, 50 ug/ml soybean trypsin inhibitor). The test materials are dissolved in DMSO and then added at an appropriate concentration to each scintillation vial; the final concentration of the solvent (DMSO) in the culture media should not exceed 0.2% (v/v). (Guidance on selection of "appropriate concentrations" needs to be added.) Finally, either 10 ul of hCG (500 IU/ml) stock solution, or 10 ul of distilled water (for non-hCG stimulated controls) is added. The vials are then capped, vortexed, and incubated vertically for three hours at 34° C under vigorous shaking (175 rpm). At the end of the incubation period, 1.4 ml of the culture media is removed and centrifuged at 14,000 x g for five minutes (4° C) to pellet all remaining testicular parenchyma. The resulting supernatant is then assayed for testosterone or estradiol concentration by RIA.

In Vivo Assays

VII. Research Protocol for Assessment of Uterotropic Activity in the Adult Ovariectomized and Juvenile (21 Day Old) Female Rat:

Purpose and Applicability

The purpose of this protocol is to outline procedures for the quantitation of the effects of potentially estrogenic compounds on the uterus of the adult ovariectomized female rat.

Required endpoints.

- Uterine Weight

- Uterine Histology

Optional endpoints

- Bodyweight change

- Vaginal cytology and histology

- Serum hormones (T4, T3, TSH, LH, E2 and Prolactin)

- Liver Weight and Histology

A. General Conditions

Typically, prior to treatment, female rats are housed in groups of 2 or 3 per cage. The following describes the housing conditions under which our animals are housed. Reasonable variations of this portion of the protocol should be tolerated. Rats are housed in clear plastic cages (20x25x47 cm) with heat treated (to eliminate resins that induce liver enzymes) laboratory grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals are maintained on Purina Rat Chow (5001) and tap water ad libitum, in a room with a 14:10 hour photoperiod (L/D, lights off at 11:00 EST) and temperature of 20-24° C with a relative humidity of 40-50 %.

B. Subjects. The Stwg Selected Option 2a as the Preferred Method

1. **OVARECTOMIZED ADULT FEMALE RATS.** Adult SD (or LE) female rat can be ovariectomized in the laboratory under appropriate conditions of anesthesia, and sterility or purchased from the supplier (about \$6 per rat). Upon receipt animals are examined and weighed, and allowed to acclimate from surgery for at least three weeks. This should provide the animals sufficient time to recover from the surgery and allows for regression of the reproductive tract. For example, upon examination, the vaginal opening should be small and difficult to lavage. Vaginal smears, if collected would lack cellularity, especially cornified or nucleated epithelial cells. Animals that displayed any characteristics of continued ovarian hormonal exposure, as indicated above, should be deleted from the study. Forty females of uniform weight are selected for the study and randomly assigned to treatment in a manner that provides similar means and variances in body weight.

2. **JUVENILE FEMALE RATS.** At 21 days of age female SD (or LE) rats will be weaned from their litters. These litters are derived from pregnant females that were generated in house by matings or purchased from a supplier as "timed pregnant" on days seven to ten of gestation. Upon birth, the litters are culled to eight to ten pups in order to assure normal growth rates in all pups. Growth is monitored on at least a weekly basis and any unthrifty litters or runt pups should be discarded from the study. Enough litters should be used to assure that about 55 pups are available at weaning. Pups are weaned at 21 days of age and weighed, weight ranked. A population of forty female rats that is as homogeneous as possible is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups). In this regard, one nuisance variable, i.e. body weight at weaning, is experimentally controlled. In addition, body weight at weaning is also statistically controlled, by assigning the forty females to four treatment groups in a manner that provides each group with similar means and variances in weaning weight. This source of variance is included in the data analysis as a blocking factor or by using weaning weight as a covariate. In addition, it is imperative that treatments should be initiated no later than 22 days of age, as waiting just a few days longer can result in failure of the study as control/untreated female rats will begin to show dramatic fluctuations in uterine size as they approach puberty.

3. **EXPERIMENTAL DESIGN.** The design is a randomized complete block design with ten female rats in each of four treatment groups. The treatment conditions are 1) Vehicle-injected control, 2) Xenobiotic-injected, 3) Estradiol treated and 4) Estradiol plus xenobiotic

injected. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should contain all treatment conditions and be balanced with respect to numbers of animals in each block (i.e. two blocks with four treatment conditions, with five females/treatment/block).

4. TREATMENT. Treatments are administered by sc injections on the dorsal surface, caudal to the nape of the neck, but anterior to the base of the tail, with a 21 gauge 1.0 inch needle, using a 1 cc glass tuberculin syringe for each treatment condition, in corn oil (1-2.5 ml/kg) at 0700-1000 for three consecutive days. The estradiol-treated groups should be dosed with 1.0 mg/rat of free estradiol per day simultaneously with the xenobiotic treatment. The xenobiotic should be administered on a mg/kg body weight basis, adjusted daily for weight changes and body weight and volume of the dose administered should be recorded each day.

5. NECROPSY. On the third day, approximately 6 hours after the final treatment the females are anesthetized in CO₂ and body weight is recorded to the nearest 0.1 g. The rat is subsequently euthanized by decapitation, and serum collected for optional hormonal analysis. During necropsy care must be taken to remove mesenteric fat with small surgical iris scissors from the uterine horns such that the uterine fluid is retained. Once free from the fat and adnexa the uterus and cervixes are separated from the vagina and the weight of the uterus with fluid is recorded to the nearest mg. Following this observation, the uterus is placed on a paper towel, slit to allow the fluid contents to leak out, gently blotted dry and reweighed. The uterus and vagina also can be examined histologically for "estrogen-like" alterations. The tissues should be placed in Bouins for 24 hours, after which they are rinsed and stored in 70 % alcohol, until being embedded in paraffin, stained with H and E, and examined for histological alterations (increased endometrial epithelial cell height, increased glandularity, increased vaginal cornification). In addition, as estrogens reduce food consumption, induce vaginal cornification, reduce serum LH and increase prolactin, these endpoints could be examined at this time.

6. STATISTICAL ANALYSIS. Uterine weight data are analyzed as one-way ANOVAs (Treatment), using PROC GLM the SAS version 6.08 on the USEPA IBM mainframe. The regression model should include bodyweight at weaning as a covariate. If the study was conducted in blocks, then the analysis is a two-way ANOVA with Block and Treatment as main effects, again, bodyweight at weaning is used as a covariate. Statistically significant effects ($p < 0.05$, F statistic) should be examined using the LSMEANS procedure on SAS (two-tailed t-test) to compare group 1) vehicle-treated to group 2) xenobiotic-treated and group 3) estradiol-treated to group 4) xenobiotic- plus estradiol-treatments.

7. DATA SUMMARIZATION. Data should be summarized in tabular form containing the mean, standard error of the mean and sample size for each group in the table. Individual data tables should also be included. The mean, SE and CV values for the control data should be examined to determine if they meet acceptable QA criteria based upon normal control values. Data presented should include at least, uterine weight with and without fluid, and

body weight at necropsy and body weight change from day 21 to 24. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data.

VIII. Research Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile (21 Day Old) Female Rats

Purpose and Applicability

The purpose of this protocol is to outline procedures for the quantitation of the effects of compounds on pubertal development and thyroid function in the intact juvenile female rat. This assay detects compounds that display antithyroid, estrogenic, antiestrogenic (ER or steroid enzyme mediated) activity, or alter FSH, LH, prolactin, Growth Hormone or hypothalamic function.

Required Endpoints

- Growth
- Age and Weight at Vaginal Opening
- Serum T4 and TSH
- Thyroid Histology
- Uterine and Ovarian Weights and Histology

Optional Endpoints

- Serum T3, E2 and prolactin
- Thyroid Weight
- Vaginal cytology and histology
- Liver, Kidney, pituitary, and adrenal Weights and Histology
- Ex vivo ovarian and pituitary hormone production
- Hypothalamic neurotransmitter levels
- Onset of estrous cyclicity and cycle length (requires extension of dosing)

A. General Conditions

Typically, prior to the onset of the study, pregnant female rats are housed individually. After assignment to treatments, they should be housed in pairs of similarly treated females. The following describes the housing conditions under which our animals are housed. Reasonable variations of this portion of the protocol should be tolerated. Rats are housed in clear plastic cages (20x25x47 cm) with heat treated (to eliminate resins that induce liver enzymes) laboratory grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals are maintained on Purina Rat Chow (5001) and tap water ad libitum, in a room with a 14:10 hour photoperiod (L/D, lights off at 11:00 EST) and temperature of 20-24° C with a relative humidity of 40-50 %.

B. Subjects - Juvenile Female Rats

At 21 days of age female SD (or LE rats will be weaned from their litters. These litters are derived from individually housed pregnant females that were generated in house by matings or purchased from a supplier as "timed pregnant" on days seven to ten of gestation. Upon birth, the litters are culled to eight to ten pups in order to assure normal growth rates in all pups. Growth is monitored on at least a weekly basis and any unthrifty litters or runt pups should be discarded from the study. Enough litters should be used to assure that about 45 pups are available at weaning. Pups are weaned at 21 days of age and weighed to the nearest 0.1 g, weight ranked. A population of thirty female rats that is as homogeneous as possible is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups). In this regard, one nuisance variable, i.e. body weight at weaning, is experimentally controlled. In addition, body weight at weaning is also statistically controlled, by assigning the thirty females to two treatment groups in a manner that provides each group with similar means and variances in weaning weight. This source of variance is included in the data analysis as a blocking factor or by using weaning weight as a covariate. In addition, it is imperative that treatments should be initiated no later than 22 days of age, as waiting just a few days longer can result in failure of the study as control/untreated female rats will begin to display "puberty" (i.e. vaginal opening) within a few days.

C. Experimental Design

The design is a randomized complete block (bodyweight at weaning is the blocking factor) design with fifteen female rats in each of two treatment groups. The treatment conditions are 1) Vehicle-treated and 2) Xenobiotic-treated. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should contain all treatment conditions and be balanced with respect to numbers of animals in each block (i.e. two blocks with two treatment conditions, with eight females/treatment/block). Varying dosage levels of the xenobiotic can be employed, although only one high dosage level (at or just below the MTD or limit dose) is required.

D. Treatment

Treatments are administered daily by oral gavage in 2.5 to 5.0 ml/kg bodyweight corn oil from 22 days of age for 20 days. This duration of treatment is unnecessary to detect estrogenic chemicals, but is required for the detection of pubertal delays and antithyroid effects. Using a #18 gavage needle and a 1 cc glass tuberculin syringe for each treatment. Xenobiotics are administered in corn oil at 2.5 ml/kg body weight at 0700-1000 daily. The treatments should be administered on a mg/kg body weight basis, adjusted daily for weight changes and body weight and volume of the dose administered should be recorded each day.

E. Vaginal Opening

Females are examined daily for vaginal opening. The appearance of complete vaginal opening, a small "pin hole" or a vaginal thread should all be noted if and when they occur. In addition, the weight at complete vaginal opening should be noted. Additional, optional measures could be taken that would facilitate interpretation of changes in vaginal opening

could be taken on these females prior to necropsy. The collection of daily vaginal lavages to identify the age at onset of estrous cyclicity, and the age at first estrous would enable one to distinguish pseudoprecocious puberty from true precocious puberty.

F. Necropsy

On the last day of treatment, the females are anesthetized in CO₂ and body weight is recorded. The rat is subsequently euthanized by decapitation, and serum collected for optional hormonal analysis. At necropsy, the paired ovarian, uterine, liver, adrenal and body weights should be recorded. During necropsy care must be taken to remove mesenteric fat with small surgical iris scissors from the uterine horns such that the uterine fluid is retained (organ weights to the nearest mg). Once free from the fat and adnexa the uterus and cervixes are separated from the vagina and the weight of the uterus with fluid is recorded. Following this observation, the uterus is placed on a paper towel, slit to allow the fluid contents to leak out, gently blotted dry and reweighed. For the ovaries, attached fat and the oviducts should be carefully removed prior to weighing. The thyroid, ovaries, uterus and vagina also can be examined histologically. These tissues should be placed in Bouins for 24 hours, after which they are rinsed and stored in 70 % alcohol, until being embedded in paraffin, stained with H and E, and examined for histological alterations. In addition, serum thyroxine (T4) and TSH should be measured.

G. Statistical Analysis

All data (age at vaginal opening, weight at vaginal opening, body and organ weights at necropsy, and serum hormones) are analyzed as one-way ANOVAs (Control versus Treatment), using PROC GLM the SAS version 6.08 on the USEPA IBM mainframe. The regression model should include bodyweight at weaning as a covariate. If the study was conducted in blocks, then the analysis is a two-way ANOVA with Block and Treatment as main effects, and again, bodyweight at weaning is used as a covariate. Statistically significant effects ($p < 0.05$, F/t statistic) should be examined using the LSMEANS procedure on SAS (two-tailed t-test) to compare group 1) vehicle-treated to the 2) xenobiotic-treated group. For organ weight data, bodyweight at necropsy could be used as a covariate in the model, although this is rarely useful for endocrine-related endpoints. If treatment reduces growth and delays vaginal opening, the mechanism responsible for the delay is always in question. In this regard, body weight change from day 22 until the average age of vaginal opening in the control group could be used as a covariate in the regression model, however, this is not the best use of ANOCOVA. If serum hormone levels, or any other data, display heterogeneity of variance, then appropriate data transformations should be employed. Often log transformation of serum hormone data is required because the variance is proportional to the mean.

H. Data Summarization

Data should be summarized in tabular form containing the mean, standard error of the mean and sample size for each group in the table. Individual data tables should also be included. The mean, SE and CV values for the control data should be examined to determine if they

meet acceptable QA criteria for consistency with normal values. Data presented should include at least, age and weight at vaginal opening, ovarian, uterine (with and without fluid), adrenal, liver and body weights at necropsy, body weight change from day 21 to necropsy and serum T4 and TSH. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data.

IX. Research Protocol for Assessment of (Anti)Androgenic Activity in the Immature Male Rat: The "Hershberger" Assay

Purpose and Applicability

The purpose of this protocol is to outline procedures for the quantitation of the effects of potentially antiandrogenic and androgenic compounds on the hormone dependent tissues in the immature male rat. Adapted from Hershberger et al., 1953, Proc Soc Exp Biol Med 83:175.

Required Endpoints

- Growth

- Seminal vesicle plus coagulating gland weight (with and without fluid)

- Ventral Prostate Weight

- Levator ani plus bulbocavernosus weight

Optional Measures

- Serum testosterone, estradiol, LH, prolactin, T4, TSH and T3

- Liver, kidney, thyroid, adrenal and pituitary weights and histology

- Hypothalamic neurotransmitter levels

A. General Conditions.

Typically, prior to and during treatment, male rats are housed in groups of two per cage. The following describes the housing conditions under which our animals are housed. Reasonable variations of this portion of the protocol should be tolerated. Rats are housed in clear plastic cages (20x25x47 cm) with heat treated (to eliminate resins that induce liver enzymes) laboratory grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals are maintained on Purina Rat Chow (5001) and tap water ad libitum, in a room with a 14:10 hour photoperiod (L/D, lights off at 11:00 EST) and temperature of 20-24° C with a relative humidity of 40-50 %.

B. Subjects - Castrated Immature Male Rats

Castrated immature animals can be purchased from a supplier or produced in house. For in house efforts, 21 days of age male SD (or LE) rats will be weaned from their litters. These litters are derived from individually housed pregnant females that were generated in house by matings or purchased from a supplier as "timed pregnant" on days seven to ten of gestation.

Upon birth, the litters are culled to eight to ten pups in order to assure normal growth rates in all pups. Growth is monitored on at least a weekly basis and any unthrifty litters or runt pups should be discarded from the study. Enough litters should be used to assure that about 55 pups are available at weaning. At 21 days of age males are weaned and castrated under appropriate conditions of anesthesia and sterility and allowed to recover for one week being housed in cages with three to four males per group. Alternatively, castrated 21 day old male rats can be purchased from a supplier. At 27 days of age pups are weighed to the nearest 0.1 g, weight ranked and a homogeneous population of forty male rats is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups). In this regard, one nuisance variable, i.e. body weight at the start of the study, is experimentally controlled. In addition, body weight is also statistically controlled, by assigning the forty males to one of four treatment groups in a manner that provides each group with similar means and variances in weaning weight. This source of variance is included in the data analysis as a blocking factor or by using the initial body weight as a covariate.

C. Experimental Design

The design is a randomized complete block (initial body weight is the blocking factor) design with ten 28 day old male rats in each of four treatment groups. The treatment conditions to detect androgenicity are 1) Oral Vehicle-treated and 2) Oral Xenobiotic-treated, while antiandrogens are detected by comparing group 3) sc Testosterone propionate (50 mg/d) plus oral vehicle treated versus 4) sc TP and oral xenobiotic-treatment. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should be contain all treatment conditions and balanced with respect to numbers of animals in each block (i.e. two blocks with two treatment conditions, with five males/treatment/block).

D. Treatment

Vehicle (groups 1 and 3) and xenobiotic (groups 2 and 4) oral treatments are administered daily for seven (to ten) days by gavage from 28 days of age to 37 days of age. Treatments are administered using a separate #18 gavage needle and a 1 cc glass tuberculin syringe for each treatment. Xenobiotics are administered in corn oil at 2.5 ml/kg body weight at 0700-1000 daily. The oral treatments should be administered on a mg/kg body weight basis, adjusted daily for weight changes and body weight and volume of the dose administered should be recorded each day. Sc injections of TP (50 mg/d in 0.2 ml oil to groups 3 and 4) are administered at the same time of day on the dorsal surface, caudal to the nape of the neck, but anterior to the base of the tail, with a 21 gauge 1.0 inch needle, using a 1 cc glass tuberculin syringe for each treatment condition.

E. Necropsy

On the day after the last treatment, males are anesthetized in CO₂ and body weight is recorded. The rat is subsequently euthanized by decapitation, and serum collected for optional hormonal analysis. At necropsy, the paired testicular, paired epididymal, liver, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani plus bulbocavernosus muscles, to the nearest mg and body weights (nearest 0.1 g) should be

recorded. During necropsy care must be taken to remove mesenteric fat with small surgical iris scissors from these tissues such that the fluid in the sex accessory glands is retained. Once free from the fat and adnexa the weight with fluid is recorded and these tissues, and the thyroid, also can be examined histologically. Tissues should be placed in Bouins for 24 hours, after which they are rinsed and stored in 70 % alcohol, until being embedded in paraffin, stained with H and E, and examined for histological alterations. In addition, serum thyroxine (T4) and TSH should be measured. Serum LH and androgen levels are optional.

F. Statistical Analysis

All data (body and organ weights at necropsy, and serum hormones) are analyzed as one-way ANOVAs for two orthogonal contrasts. The first is between groups 1 and 2 identifies androgens, while the comparison between groups 3 and 4 detects antiandrogenicity. Data are analyzed on PROC GLM the SAS version 6.08 on the USEPA IBM mainframe. The regression model should include initial bodyweight at weaning as a covariate. If the study was conducted in blocks, then the analysis is a two-way ANOVA with Block and Treatment as main effects, again, initial bodyweight is used as a covariate. Statistically significant effects ($p < 0.05$, F/t statistic) should be examined using the LSMEANS procedure on SAS (two-tailed t-test) to compare group 1) vehicle-treated to the 2) xenobiotic-treated group. For organ weight data, bodyweight at necropsy could be used as a covariate in the model, although this is rarely useful for endocrine-related endpoints. If serum hormone levels, or any other data, display heterogeneity of variance, then appropriate data transformations should be employed. Often log transformation of serum hormone data is required because the variance is proportional to the mean.

G. Data Summarization

Data should be summarized in tabular form containing the mean, standard error of the mean and sample size for each group in the table. Individual data tables should also be included. The mean, SE and CV values for the control data should be examined to determine if they meet acceptable QA criteria for consistency with normal values. Data presented should include at least, levator ani, testicular, epididymal, ventral prostate, seminal vesicle (with coagulating glands and fluid), liver and body weights at necropsy, body weight change from day 28 to necropsy and serum T4 and TSH. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data.

X. Fish Gonadal Recrudescence Assay

A. Scope

1. Applicability. This guideline is intended to describe a method to screen for endocrine disrupting effects by exposing intact fish to a test substance and observing gonadal maturation from the regressed position (recrudescence) and other endocrine related endpoints.

2. Background. This assay is based on recommendations from the Workshop on Screening Methods for Endocrine Disruptors in Wildlife held in Kansas City, MO march 17-19, 1997 (Ankley et al., 1998).

B. Introduction

(1) Fish are the most phylogenetically distant class of vertebrates from mammals so the degree of homology with this latter group is uncertain. Assays with fish to screen for potential endocrine disruptive activity are important to adequately assess this group of important vertebrates.

C. Definitions

LOEC (Lowest-observed-effect-concentration) is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control.

NOEC (No-observed-effect-concentration) is the test concentration immediately below the LOEC.

D. Principle of the Test

Fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions, or where appropriate, semistatic conditions. Effects are assessed and compared with control values to determine the LOEC and the NOEC for the endpoints observed. For poorly soluble materials, intraperitoneal injection may be considered.

E. Information on the Test Substance

1. Results of an acute toxicity test, preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapor pressure of the test substance are known and a reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

2. Useful information includes the structural formula, purity of the substance, stability in water and light, pKa, Pow, and results of a test for ready biodegradability.

F. Validity of the Test

For a test to be valid the following conditions apply:

1. The dissolved oxygen concentration must be between 60 and 100 percent of the air saturation value throughout the test.
2. The water temperature must not differ by more than 1.0°C between test chambers and should be within the temperature prescribed regime specified for the test species.
3. Evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ± 20 percent of the mean measured values.
4. When a solubilizing agent is used it must have no significant effect on survival nor produce any other adverse effects as revealed by a solvent-only control.

G. Description of the Method

1. Test chambers. Any glass, stainless steel, or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomized block design with each treatment being present in each block is preferable to a completely randomized design. The test chambers should be shielded from unwanted disturbance.

2. Selection of species.

a) Recommended fish species -- fathead minnow, *Pimephales promelas*.

b) Feeding and handling requirements of test animals, test conditions, duration, and survival criteria

3. Holding of the brood fish.

a) Details on holding the brood stock under satisfactory conditions may be found in the references cited under paragraphs (j)(1), (j)(2), and (j)(3) of this guideline.

b) Test fish should be in "winter" condition, brought about by holding under an 8 hour light:16 hour dark photoperiod at $15 \pm 1^\circ\text{C}$ for a minimum of 30 days prior to the start of the test.

4. Water. Any water in which the test species shows control survival and good reproductive viability. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of test substance) or adversely affect the performance of the fish, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, sulfate), pesticides, total organic carbon, and suspended solids should be made, for example, every 3 months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in the following Table 1:

Table 1.--Some Chemical Characteristics of an Acceptable Dilution Water

Substance	Maximum Concentration
Particulate matter	< 20 mg/L
Total organic carbon	< 2 mg/L
Un-ionized ammonia	< 1 g/L
Residual chlorine	< 10 g/L
Total organophosphorus pesticides	< 50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/L
Total organic chlorine	< 25 ng/L

6. Test solutions.

a) For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10 percent throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 h has been found suitable.

b) The use of solvents or dispersants (solubilizing agents) may be required in some cases in order to produce a suitably concentrated stock solution.

c) For the semistatic technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels while a proportion (at least two-thirds) of the test water is changed.

H. Procedure

1. Conditions of exposure--

a) Duration. The test should start as soon as possible after appropriately conditioned fish are placed into exposure chambers. Test duration will be 21 days from the start.

b) Loading. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60 percent of the air saturation value (ASV) can be maintained without aeration. For flow-through tests, a loading rate not exceeding 0.5 g/L/24 h and not exceeding 5 g/L of solution at any time has been recommended.

c) Light and temperature. The test begins with a 12 hour light:12 hour dark photoperiod and a gradual temperature adjustment from 15° C at the start to 20° C at 24 hours. On day seven the photoperiod is changed to 16 hour light:8 hour dark and the temperature adjusted gradually from 20° C to 25° C by day eight. Day eight through day 21 will be maintained at a photoperiod of 16 hour light:8 hour dark and a temperature of 25° C.

d) Feeding. Fish should be fed brine shrimp (*Artemia*) larvae. Feeding should be *ad libitum* while minimizing the surplus. Surplus food and feces should be removed as necessary to avoid accumulation of waste.

e) Test concentrations.

(A) A single limit concentration identified from an appropriate range-finding test or five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC50 to period of exposure in the acute study should be considered when selecting the test concentration or range. The use of fewer than five concentrations and a narrower concentration interval may be appropriate in some circumstances. Concentrations of the substance higher than the 96-h LC50 not be tested.

(B) Where a solubilizing agent is used, its concentration should not be greater than 0.1 mL/L and should be the same in all test vessels. However, every effort should be made to avoid the use of such materials.

f) Controls. One dilution-water control and also, if relevant, one control containing the solubilizing agent should be run in addition to the test series.

g) Fish. Fish which are mature and have been through one reproductive cycle are used. A minimum of 10 male and 10 female fish, physically separated and divided into 2 replicates are used per test level.

2. Frequency of analytical determinations and measurements.

a) During the assay, the concentrations of the test substance are determined at regular intervals to check compliance with the validity criteria. A minimum of five determinations is necessary. Samples may need to be filtered (e.g. using a 0.45m pore size) or centrifuged to ensure that the determinations are made on the substance in true solution.

b) During the test, dissolved oxygen, pH, total hardness and salinity (if relevant), and temperature should be measured in all test vessels. Temperature should preferably be monitored continuously in at least one test vessel.

3. Observations--

a) Gonadosomatic index (GSI). Defined as the blotted wet weight of the gonad divided by the blotted wet weight of the intact fish.

b) Secondary sex characteristics. Presence and extent of tubercles on male fish is quantified.

c) Final oocyte maturation (FOM)/ovulation/spermiation. Maturity and production of gametes is quantified.

d) Plasma sex steroids and vitellogenin. Estradiol, testosterone/11-ketotestosterone, and vitellogenin may be quantified by appropriate ELISA or RIA.

e) Abnormal appearance. The number of fish showing abnormality of body form should be

recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several percent in the controls in some species. Abnormal animals should only be removed from the test vessels on death.

f) Abnormal behavior. Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence, and atypical feeding behavior should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data and influence a decision to extend the exposure period beyond the recommended duration.

g) Weight. At the end of the test all surviving fish must be weighed individually as wet weights (blotted dry).

h) Length. At the end of the test, measurement of individual lengths is recommended: Standard, fork, or total length may be used. If however, caudal fin rot or fin erosion occurs, standard lengths should be used.

Data for statistical analysis. These observations will result in some or all of the following data being available for statistical analysis:

Cumulative mortality.

Numbers of healthy fish at end of test.

GSI of males and of females.

Extent of tubercles on males.

Length and weight of surviving animals.

Gamete production and maturity.

Numbers of fish exhibiting abnormal behavior.

Plasma titers of sex steroids and vitellogenin (optional).

j) Data and reporting--

1. Treatment of results

a) It is recommended that a statistician be involved in both the design and analysis of the test results since this test guideline allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilized eggs, and number of parameters measured.

b) In view of the options available in test design, specific guidance on statistical procedures is not given here. However, it will be necessary for variations to be analyzed within each set of replicates using analysis of variance or contingency table procedures. To make a multiple comparison between the results at the individual concentrations and those for the controls, Dunnett's method might be found useful. However, care must be taken where applying such a method to ensure that chamber-to-chamber variability is estimated and is acceptably low. Other useful methods are also available.

2. Interpretation of results. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.

3. Test report. The test report must include the following information:

a) Test substance.

Physical nature and, where relevant, physicochemical properties.

Chemical identification data.

b) Test species. Scientific name, strain, source and method of collection of the fertilized eggs, and subsequent handling.

c) Test conditions.

Test procedure used (e.g. semistatic or flow-through design).

Photoperiods.

Test design (e.g. number of test chambers and replicates, number of embryos per replicate).

Method of preparation of stock solutions and frequency of renewal (the solubilizing agent and its concentration must be given, when used).

Nominal test concentrations, means of the measured values, their standard deviations in the test vessels, and the method by which these were attained, and evidence that measurements refer to concentrations of the test substance in true solution.

Dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured), and any other measurements made.

Water quality within test vessels: pH, hardness, temperature, and dissolved oxygen concentration.

Detailed information on feeding (e.g. type of feed, source, amount given, and frequency).

d) Results.

Evidence that controls met the overall survival acceptability.

Data on mortality/survival.

Data on the observational endpoints.

Data for length and weight.

Incidence and description of morphological abnormalities, if any.

Incidence and description of behavioral effects, if any.

Statistical analysis and treatment of data.

NOEC for each response assessed.

LOEC (at $p = 0.05$) for each response assessed.

Any concentration-response data and curves available.

e) Discussion of the results.[Reserved]

I. References

The following references should be consulted for additional background material on this test guideline.

- 4th Aquatic Toxicology Symposium, ASTM, Philadelphia, PA (1980).
- American Society for Testing and Materials (ASTM). Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. ASTM E 1241-92, p. 180-207, Philadelphia, PA (1992).
- Brauhn, J.L. and Schoettger, R.A., Acquisition and Culture of Research Fish: Rainbow trout, Fathead minnows, Channel catfish and Bluegills. p. 54, Ecological Research Series, EPA-660/3-75-011, Duluth, MN (1975).
- Brungs, W.A. and Jones, B.R., Temperature Criteria for Freshwater Fish: Protocol and Procedures. p. 128, Ecological Research Series EPA-600/3-77-061, Duluth, MN (1977).
- Dunnett, C.W., A multiple comparisons procedure for comparing several treatments with a control. Journal of the American Statistical Association 50: 1096-1121 (1955).
- Dunnett, C.W., New tables for multiple comparisons with a control. Biometrics 20:482-491 (1964).
- McClave, J.T. et al., Statistical Analysis of Fish Chronic Toxicity Test Data. Proceedings of McKim, J.M. et al., Metal toxicity to embryos and larvae of eight species of freshwater fish-II: Copper. Bulletin of Environmental and Contamination Toxicology 19:608-616 (1978).
- Rand, G.M. and Petrocelli, S.R., Fundamentals of Aquatic Toxicology. Hemisphere Publication Corporation, NY (1985).
- USEPA, Recommended Bioassay Procedure for Fathead Minnows, *Pimephales promelas* (Rafinesque), Chronic Tests. p. 13, National Water Quality Laboratory, Duluth, MN (1972).

Alternative *In Vivo* Assays

XI. Development Of An *In Vivo* Battery For Identifying Endocrine Modulators In Male Crl:CD¹Br Rats Protocol

A. Introduction

The primary purpose of the male *in vivo* battery is to identify compounds that have the potential to act as agonists or antagonists to the estrogen, androgen, progesterone, or dopamine receptor, 5 α -reductase inhibitors, steroid biosynthesis inhibitors (aromatase and testosterone biosynthesis), or compounds that alter thyroid function. This approach is based on our previous work (Cook *et al.*, 1992, Cook *et al.*, 1993; Biegel *et al.*, 1995; O'Connor *et al.*, 1996) as well as our experiences with two other proprietary compounds.

B. Study Design

All experiments will use sexually mature male rats approximately ten weeks of age. Each experiment will test a single compound. The daily dosage for each compound will be administered at approximately 8:00 a.m. daily.

Dosing by intraperitoneal injection will be performed for 15 consecutive days following release from quarantine. Rats will be sacrificed on the morning of test day +15. All animals (15/group) will be evaluated for gross observations of toxicity, organ weights (testes, prostate, seminal vesicles, epididymides, accessory sex gland unit), and serum hormone concentrations (testosterone, estradiol, dihydrotestosterone (DHT), luteinizing hormone (LH), thyroid stimulating hormone (TSH), thyroxine (T4)). Epididymal sperm concentration and motility will be evaluated. Histology of one testis and epididymis and the thyroid gland will be performed.

C. Materials and Methods

1. Test Species

Adult male Crl:CD⁰BR rats, approximately ten weeks of age and weighing between 260 and 300 grams, will be acquired from Charles River Laboratories, Raleigh, North Carolina. The Crl:CD⁰BR rat has been selected on the basis of extensive experience with this strain and its suitability with respect to sensitivity to endocrine modulators.

2. Animal Husbandry

All rats will be housed in stainless steel, wire-mesh cages suspended above cage boards. Animal rooms will be targeted at a temperature of $23 \pm 1^{\circ}$ C and a relative humidity of $50 \pm 10\%$. Animal rooms will be artificially illuminated (fluorescent light) on a 12-hour light/dark cycle. All rats will be provided tap water and Purina^o Certified Rodent Chow^o #5002 *ad libitum*. The feed is guaranteed by the manufacturer to meet specified nutritional requirements and to be free of a list of specified contaminants.

3. Pretest Period

Upon arrival, all rats will be removed from shipping cartons and housed one per cage in a quarantine room. The rats will be

- quarantined for approximately one week,
- identified with cage card identification,
- weighed three times, and
- observed with respect to weight gain and any gross signs of disease or injury.

The rats will be released from quarantine by the laboratory veterinarian on the bases of body weights and freedom from clinical signs.

All rats accidentally killed during the pretest period will be discarded without necropsy. All

rats found dead or sacrificed *in extremis* during the pretest will be necropsied but tissues will not be examined microscopically.

4. Assignment to Groups

During the pretest period, male rats will be divided by computerized, stratified randomization into groups as specified in a protocol amendment so that there are no statistically significant differences among group body weight means. For each experiment, a single compound will be tested. Each rat will be housed individually.

5. Dosage Preparation and Administration

All dosing solutions will be made within three days of study start and will be prepared weekly for the duration of the study. Solutions will be stored in the refrigerator when not in use. The dose volume will ideally be 2 mL/kg, but can be up to 10 mL/kg. The route of administration will be intraperitoneal injection. This route was selected to enhance the sensitivity of the assay and to facilitate potency comparisons. Intraperitoneal administration reduces variability associated with uptake which would occur with other routes of administration (i.e. gavage, subcutaneous). The dosages will be specified in a protocol amendment. The same volume of vehicle will be given to the control group. Individual rat dose volumes will be based on the daily body weight except on test day +15 which will use the previous day's weight.

6. Body Weights

All rats will be weighed daily.

7. Food Consumption and Food Efficiency

Individual food consumption data will be collected weekly. The amount of food consumed by each group will be determined. From these determinations, as well as body weight data, mean daily food consumption and mean food efficiency will be calculated for each group.

8. Clinical Observations and Mortality

Cage-site examinations to detect moribund or dead rats and abnormal behavior and appearance among rats will be conducted at least once daily throughout the study. Moribund rats will be sacrificed. Moribund and dead rats will be given a gross pathological evaluation. At every weighing, each rat will be individually handled and examined for abnormal behavior and appearance.

9. Pathological Evaluation

All rats accidentally killed during the pretest period will be discarded without necropsy. All rats found dead or sacrificed *in extremis* during the pretest will be necropsied but tissues will not be examined microscopically. After study start, all rats found dead, accidentally killed,

sacrificed *in extremis*, or sacrificed by design will be necropsied. Rats sacrificed *in extremis* or sacrificed by design will be euthanized by carbon dioxide (CO₂) anesthesia and exsanguination. Blood will be collected from the inferior *vena cava* for preparation of serum (Section K). Time of death will be recorded for all animals.

Final body, testes, accessory sex gland unit, prostate, epididymides, and seminal vesicles (with fluid) weights will be measured. Relative organ weights (% of final body weight) will be calculated. Blood will be collected in a serum separator tube and placed on ice until serum is prepared. One epididymis will be processed immediately for evaluation of sperm concentration and motility according to procedures recommended in the EPA reproductive toxicity testing guidelines (870.3800). The other epididymis and thyroid from each rat will be placed in formalin fixative and the testes will be placed in Bouin's fixative. The testes, epididymides, and thyroid will be evaluated microscopically. Microscopic evaluations will be performed on control and high dose animals for all compounds. Only compounds which show effects in the high dose group will have the remaining groups evaluated.

10. Hormonal Evaluation

Blood will be collected at the time of sacrifice from all animals. The blood will be placed in a serum separator tube on ice until the serum is prepared. Serum will be stored between -65° C and -85° C until analyzed. Serum testosterone, estradiol, DHT, LH, TSH, T4 levels will be measured by commercially available radioimmunoassays (RIAs). If serum is limiting, priority of analysis will be determined by the study director. Any remaining serum will be discarded after the report is issued.

D. Statistical Analyses

Mean final body weights and organ weights will be analyzed by a one-way analysis of variance (ANOVA). When the corresponding *F* test for differences among test group means is significant, pairwise comparisons between test and control groups will be made with Dunnett's test. Bartlett's test for homogeneity of variances will be performed and, when significant ($p < 0.005$), will be followed by nonparametric procedures (Dunn's test). Serum hormone levels will be analyzed using Jonckheere's test for trend. If a significant dose-response trend is detected, data from the top dose group will be excluded and the test repeated until no significant trend is detected. Except for Bartlett's test, all other significance will be judged at $p < 0.05$.

E. Safety And Housekeeping

Appropriate handling precautions will be used for each compound. Good housekeeping procedures will be practiced to avoid contamination of the dose preparation facilities and potential health hazards. To avoid skin contact, gloves will be worn when handling the test material. In addition, the neat test material will be handled in a chemical hood. Animal carcasses, feces, and unused dosing solutions will be incinerated.

F. References

- Biegel, L.B., Cook, J.C., O'Connor, J.C., Aschiero, M., Arduengo III, A.J., and Slone, T.W. (1995). Subchronic toxicity study in rats with 1-methyl-3-propylimidazole-2-thione (PTI): effects on the thyroid. *Fund. Appl. Toxicol.* 27, 185-194.
- Cook, J.C., Mullin, L.S., Frame, S.R., and Biegel, L.B. (1993). Investigation of a mechanism for Leydig cell tumorigenesis by linuron in rats. *Toxicol. Appl. Pharmacol.* 119, 195-204.
- Cook, J.C., Murray, S.M., Frame, S.R., and Hurtt, M.E. (1992). Induction of Leydig cell adenomas by ammonium perfluorooctanoate: A possible endocrine-related mechanism. *Toxicol. Appl. Pharmacol.* 113, 209-217.
- Laskey, J.W., Klinefelter, G.R., Kelce, W.R., and Ewing, L.L. (1994) Effects of ethane dimethanesulfonate (EDS) on adult and immature rabbit Leydig cells: comparison with EDS-treated rat Leydig cells. *Biol. Reproduction.* 50, 1151-1160.
- O'Connor, J.C., Cook, J.C., Craven, S.C., Van Pelt, C.S., and Obourn, J.D. (1996). An *in vivo* battery for identifying endocrine modulators that are estrogenic or dopamine regulators. *Fund. Appl. Toxicol.*, in press.
- EPA/FIFRA Good Laboratory Practice Standards, (40CFR Part 160). (1989).
- OECD Principles of Good Laboratory Practice (C(81)30(Final), Annex 2). (1987).
- MAFF Japan Good Laboratory Standards (59 NohSan No. 3850). (1985).

XII. Development Of An *In Vivo* Battery For Identifying Endocrine Modulators In Female Crl:CD®Br Rats Protocol

A. Introduction

The primary purpose of the female *in vivo* battery is to identify compounds that have the potential to be agonists or antagonists to the estrogen receptor. This approach is based on our previous work (O'Connor *et al.*, 1996).

B. Study Design

All experiments will use ovariectomized female rats approximately seven weeks of age. Each experiment will test a single compound. The groups and dosages for each compound will be described in protocol amendments. The daily dosage for each compound will be administered at approximately 8:00 a.m. daily.

Rats will be implanted with osmotic minipumps on test day-one for evaluation of cell proliferation. Dosing by intraperitoneal injection will be performed for four consecutive days following release from quarantine. Rats will be sacrificed on the morning of test day-five.

There will be ten animals per group.

The rats will be evaluated for vaginal cytology, organ weights (uterus and liver), gross observations. The uterus will be saved in formalin fixative for possible future epithelial cell height and cell proliferation analysis. Analyses will be performed at the discretion of the study director.

C. Materials And Methods

1. Test Substances

Test substances will be prepared in appropriate concentrations and solvents to deliver no more than 10 ml/kg dose volume.

2. Test Species

Adult ovariectomized female Crl:CD⁰BR rats, approximately 42 days of age and weighing between 115 and 160 grams, will be acquired from Charles River. The female rats will be ovariectomized on the day of shipment. The Crl:CD⁰BR rat has been selected on the basis of extensive experience with this strain and its suitability with respect to sensitivity to endocrine modulators.

3. Animal Husbandry

All rats will be housed in stainless steel, wire-mesh cages suspended above cage boards. Animal rooms will be targeted at a temperature of $23 \pm 1^{\circ}$ C and a relative humidity of $50 \pm 10\%$. Animal rooms will be artificially illuminated (fluorescent light) on a 12-hour light/dark cycle. All rats will be provided tap water and Purina[®] Certified Rodent Chow[®] #5002 *ad libitum*. The feed is guaranteed by the manufacturer to meet specified nutritional requirements and to be free of a list of specified contaminants.

4. Pretest Period

Upon arrival, all rats will be removed from shipping cartons and housed one per cage in a quarantine room. The rats will be

- quarantined for approximately one week,
- identified with cage card identification,
- weighed three times, and
- observed with respect to weight gain and any gross signs of disease or injury.

The rats will be released from quarantine by the laboratory veterinarian on the bases of body weights and freedom from clinical signs.

All rats accidentally killed during the pretest period will be discarded without necropsy. All rats found dead or sacrificed *in extremis* during the pretest will be necropsied but tissues will not be examined microscopically.

5. Assignment to Groups

During the pretest period, female rats will be divided by computerized, stratified randomization into treatment groups so that there are no statistically significant differences among group body weight means. For each experiment, a single compound will be tested. Each rat will be housed individually.

6. Dosage Preparation and Administration

All dosing solutions will be made within three days of study start. Solutions will be stored in the refrigerator when not in use. The dose volume will ideally be 2 mL/kg, but can be as high as 10 mL/kg. Route of administration will be intraperitoneal injection. This route was selected to enhance the sensitivity of the assay and to facilitate potency comparisons. Intraperitoneal administration reduces variability associated with uptake which would occur with other routes of administration (i.e. gavage, subcutaneous). The dosages will be specified in a protocol amendment. The same volume of vehicle will be given to the control group. Individual rat dose volumes will be based on the daily body weight.

7. Body Weights

All rats will be weighed daily.

8. Food Consumption and Food Efficiency

Individual food consumption data will be collected for the treatment period. The amount of food consumed by each group will be determined. From these determinations, as well as body weight data, mean daily food consumption and mean food efficiency will be calculated for each group.

9. Clinical Observations and Mortality

Cage-site examinations to detect moribund or dead rats and abnormal behavior and appearance among rats will be conducted at least once daily throughout the study. Moribund rats will be sacrificed. Moribund and dead rats will be given a gross pathological evaluation. At every weighing, each rat will be individually handled and examined for abnormal behavior and appearance.

10. Estrous Cycle Evaluation

Rats assigned to the biochemical subset will be evaluated for vaginal cytology on test days one through four. Vaginal washes will be collected and evaluated using established

cytological markers for evidence of conversion out of diestrus.

11. Pathological Evaluation

All rats accidentally killed during the pretest period will be discarded without necropsy. All rats found dead or sacrificed *in extremis* during the pretest will be necropsied but tissues will not be examined microscopically. After study start, all rats found dead, accidentally killed, sacrificed *in extremis*, or sacrificed by design will be necropsied. Rats sacrificed *in extremis* or sacrificed by design will be euthanized by carbon dioxide (CO₂) anesthesia and exsanguination.

Rats assigned for necropsy will be sacrificed using CO₂. Time of death will be recorded for all animals. Final body, liver, and uterine weights will be measured. The entire uterus from ovarian stump to cervix will be removed, quickly dissected free of fat and connective tissue, nicked, blotted to express luminal fluid, and weighed. The presence of fluid in the uterine horns will be recorded as a gross observation. Ovarian stumps will be collected from all animals, placed in 10% neutral-buffered formalin and processed to confirm the absence of ovarian tissue at the discretion of the pathologist.

Uteri will be collected and preserved in Bouin's fixative for cell proliferation evaluation and morphometry. Analyses will be performed at the discretion of the study director.

12. Cell Proliferation Evaluation

The uterus will be collected and placed in Bouin's fixative. The uterus will be cut into sections and mitotic index will be counted or PCNA in the nuclei will be visualized immunohistochemically using an avidin-biotin-peroxidase complex method with a monoclonal antibody against PCNA. Cell proliferation analysis will be performed at the discretion of the study director.

D. Statistical Analyses

Mean final body weights and organ weights will be analyzed by a one-way analysis of variance (ANOVA). When the corresponding *F* test for differences among test group means is significant, pairwise comparisons between test and control groups will be made with Dunnett's test. Bartlett's test for homogeneity of variances will be performed and, when significant ($p < 0.005$), will be followed by nonparametric procedures (Dunn's test). Cell proliferation indices, and uterine morphometry measurements will be analyzed using Jonckheere's test for trend. If a significant dose-response trend is detected, data from the top dose group will be excluded and the test repeated until no significant trend is detected. Uterine fluid imbibition and estrus conversion data will be analyzed by Fisher's test. Except for Bartlett's test, all other significance will be judged at $p < 0.05$.

E. Safety And Housekeeping

Appropriate handling precautions will be used for each compound. Good housekeeping

procedures will be practiced to avoid contamination of the dose preparation facilities and potential health hazards. To avoid skin contact, gloves will be worn when handling the test material. In addition, the neat test material will be handled in a chemical hood. Animal carcasses, feces, and unused dosing solutions will be incinerated.

XIII. Research Protocol for Assessment of Pubertal Development and Thyroid Function in Immature (33-53 Day Old) Male Rats

Purpose and Applicability

The purpose of this protocol is to outline procedures for the quantitation of the effects of compounds on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. This assay is detects compounds that display antithyroid, estrogenic, androgenic, antiandrogenic (AR or steroid enzyme mediated) activity, or alter FSH, LH, prolactin, Growth Hormone or hypothalamic function.

Required Endpoints

- Growth
- Age and Weight at Preputial Separation
- Serum T4 and TSH
- Thyroid Histology
- Seminal vesicle plus coagulating gland weight (with and without fluid)
- Ventral Prostate Weight
- Levator ani plus bulbocavernosus weight
- Epididymal and Testis Weights and Histology

Optional Measures

- Serum testosterone, estradiol, LH, prolactin and T3
- Liver, kidney, adrenal and pituitary weights and histology
- Ex vivo testis and pituitary hormone production

Hypothalamic neurotransmitter levels

A. General Conditions

Typically, prior to the onset of the study, pregnant female rats are housed individually. After assignment to treatments, subjects are housed in pairs of similarly treated males. The following describes the housing conditions under which our animals are housed. Reasonable variations of this portion of the protocol should be tolerated. Rats are housed in clear plastic cages (20x25x47 cm) with heat treated (to eliminate resins that induce liver enzymes) laboratory grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals are maintained on Purina Rat Chow (5001) and tap water ad libitum, in a room with a 14:10 hours to period (L/D, lights off at 11:00 EST) and temperature of 20-24° C with a relative humidity of 40-50%.

B. Subjects - Peripubertal Male Rats

At 21 days of age male SD or LE rats will be weaned from their litters. These litters are derived from individually housed pregnant females that were generated in house by matings or purchased from a supplier as "timed pregnant" on days seven to ten of gestation. Upon birth, the litters are culled to eight to ten pups in order to assure normal growth rates in all pups. Growth is monitored on at least a weekly basis and any unthrifty litters or runt pups should be discarded from the study. Enough litters should be used to assure that about 45 pups are available at weaning. Pups are weaned at 21 days of age and weighed to the nearest 0.1 g, weight ranked. A population of thirty male weanling rats that is as homogeneous as possible is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups). In this regard, one nuisance variable, i.e. body weight at weaning, is experimentally controlled. In addition, body weight at weaning is also statistically controlled, by assigning the thirty males to two treatment groups in a manner that provides each group with similar means and variances in weaning weight. This source of variance is included in the data analysis as a blocking factor or by using weaning weight as a covariate. In addition, it is imperative that treatments should be initiated no later than 33 days of age, as waiting just a few days longer can result in failure of the study as control/untreated male rats will begin to display "puberty" (i.e. preputial separation) within five to seven days.

C. Experimental Design

The design is a randomized complete block (bodyweight at weaning is the blocking factor) design with fifteen weanling male rats in each of two treatment groups. The treatment conditions are 1) Vehicle-treated and 2) Xenobiotic-treated. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should contain all treatment conditions and be balanced with respect to numbers of animals in each block (i.e. two blocks with two treatment conditions, with eight males/treatment/block).

D. Treatment

Treatments are administered daily by oral gavage from 33 days of age for 20 days. This duration of treatment is unnecessary to detect androgenic chemicals, but is required for the detection of pubertal delays and antithyroid effects. Using a #18 gavage needle and a 1 cc glass tuberculin syringe for each treatment. Xenobiotics are administered in corn oil at 2.5 ml/kg body weight at 0700-1000 daily. The treatments should be administered on a mg/kg body weight basis, adjusted daily for weight changes and body weight and volume of the dose administered should be recorded each day.

E. Preputial Separation (PPS)

Males are examined daily for PPS. The appearance of partial and complete PPS, or a persistent thread of tissue between the glans and prepuce should all be noted if and when they occur. In addition, the weight at complete PPS should be noted.

F. Necropsy

On the last day of treatment, males are anesthetized in CO₂ and body weight is recorded. The rat is subsequently euthanized by decapitation, and serum collected for optional hormonal analysis. At necropsy, the paired testicular, paired epididymal, liver, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani plus bulbocavernosus muscles, to the nearest mg and body weights (nearest 0.1 g) should be recorded. During necropsy care must be taken to remove mesenteric fat with small surgical iris scissors from these tissues such that the fluid in the sex accessory glands is retained. Once free from the fat and adnexa the weight with fluid is recorded and these tissues, and the thyroid, also can be examined histologically. Tissues should be placed in Bouins for 24 hours, after which they are rinsed and stored in 70 % alcohol, until being embedded in paraffin, stained with H and E, and examined for histological alterations. In addition, serum thyroxine (T4) and TSH should be measured. Serum LH and androgen levels are optional.

G. Statistical Analysis

All data (age at PPS, weight at PPS, body and organ weights at necropsy, and serum hormones) are analyzed as one-way ANOVAs (Control versus Treatment), using PROC GLM the SAS version 6.08 on the USEPA IBM mainframe. The regression model should include bodyweight at weaning as a covariate. If the study was conducted in blocks, then the analysis is a two-way ANOVA with Block and Treatment as main effects, again, bodyweight at weaning is used as a covariate. Statistically significant effects ($p < 0.05$, F/t statistic) should be examined using the LSMEANS procedure on SAS (two-tailed t-test) to compare group 1) vehicle to the 2) xenobiotic treated group. For organ weight data, bodyweight at necropsy could be used as a covariate in the model, although this is rarely useful for endocrine-related endpoints. If treatment reduces growth and delays PPS, the mechanism responsible for the delay is always in question. In this regard, body weight change from day 22 until the average age of PPS in the control group could be used as a covariate in the regression model, however, this is not the best use of ANOCOVA. If serum hormone levels, or any other data, display heterogeneity of variance, then appropriate data transformations should be employed. Often log transformation of serum hormone data is required because the variance is proportional to the mean.

H. Data Summarization

Data should be summarized in tabular form containing the mean, standard error of the mean and sample size for each group in the table. Individual data tables should also be included. The mean, SE and CV values for the control data should be examined to determine if they meet acceptable QA criteria for consistency with normal values. Data presented should include at least, age and weight at PPS, testicular, epididymal, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani, liver and body weights at necropsy, body weight change from day 21 to necropsy and serum T4 and TSH. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data.