# Appendix B4

## Standard Operating Procedure for the Rat Estrogen Receptor Equilibrium Exchange Assay

**(Provided by Dr. Weida Tong, Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Jefferson, AR, USA)**

***[This page intentionally left blank]***

# *Rat Estrogen Receptor Equilibrium Exchange Assay*

# *Standard Operating Procedure*

1.0 Purpose & Applicability

The purpose of this SOP is to outline a procedure for the quantitation of estrogen receptor number and binding affinity in ovariectomized adult female rat reproductive tissue (i.e., uterus). 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 radiolabeled synthetic ligand (i.e., [$^3$H]-Estradiol) by cytosolic and/or nuclear receptor extracts. Total [$^3$H]-Estradiol binding is determined by incubating the extracts with increasing concentrations of [$^3$H]-Estradiol during which time the labeled 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 100-300 fold molar excess of radioinert estradiol is included in each incubation together with the increasing concentrations of [$^3$H]-Estradiol (i.e., binding of [$^3$H]-Estradiol in the presence of a 100-300 fold molar excess of radioinert estradiol represents unsaturable binding). Specific binding is calculated as total - nonspecific binding and is analyzed via Scatchard analysis.

2.0 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.

3.0 Equipment and Materials

3.1 Equipment

* Corning Stir/Hot Plates
* Digital Pipets
* Balance
* Polytron PT 35/10 Tissue Homogenizer
* Beckman HPLC with on-line Radiochromatograph
* Vacuum Concentrator
* Hamilton Syringes (50 µl)
* Refrigerated General Laboratory Centrifuge
* Beckman LX Ultracentrifuge with 90 TI Rotor
* pH Meter with Tris-compatible electrode
* Scintillation counter

3.0 Equipment and Materials (cont.)

3.2 Chemicals

* Tris HCL
* Tris Base
* Glycerol (99% +)
* Ethylenediaminetetraacetic Acid (EDTA); Disodium Salt
* Dithiothreitol (DTT)
* Hydroxylapatite (Bio-Rad)
* Scintillation Cocktail
* Ethyl Alcohol, anhydrous
* [$^3$H]-Estradiol
* Radioinert Estradiol (Steraloids; recrystallized)
* Steroids (Steraloids; recrystallized)

3.3 Supplies

* 20 ml Polypropylene Scintillation vials
* 12x75 mm Borosilicate glass test tubes
* 1000 ml graduated cylinders
* 500 ml erlenmeyer flasks
* yellow (0-200 µl) pipet tips

4.0 Methods

4.1 Preparation of TEDG Stock Solutions

(A) 200 mM EDTA

Add 7.444 g EDTA (disodium salt) to 100 ml ddH$_2$O. Store at 4$^o$C

Use 750 µl/100 ml TEDG buffer = 1.5 mM final concentration of EDTA

(B) 1.0 M Tris

Add in a volumetric Flask:
147.24 g Tris HCL
8.0 g Tris Base
800 ml ddH2O
Stir until dissolved. QS to 1.0 Liter. Refrigerate to 4$^o$C and then pH the cooled solution to 7.4. pH using standardizing solutions which are also at 4$^o$C. Store at 4$^o$C. Use 1.0 ml of 1.0 M Tris/100 ml TEDG buffer = 10 mM final Tris concentration.

4.2 Preparation of TEDG Buffer (pH 7.4)

Add the following in this order:

| Ingredient | To make 100 ml | To make 500 ml | To make 1.0 L | To make 2.0 L |
|---|---|---|---|---|
| dd H2O | 87.15 ml | 435.75 ml | 871.5 ml | 1743.0 ml |
| 1.0 M Tris | 1.00 ml | 5.00 ml | 10.0 ml | 20.0 ml |
| Glycerol | 10.00 ml | 50.00 ml | 100.0 ml | 200.0 ml |
| 200 mM EDTA | 750 µl | 3.75 ml | 7.5 ml | 15.0 ml |

Immediately prior to use in the assay, add:

15.4 mg Dithiothreitol/100 ml TEDG buffer. pH final solution to make sure it is 7.4 at 4 $^{o}$C

4.3 Preparation of 50 mM Tris Buffer

| Ingredient | To make 1.0 L | To make 2.0 L | To make 5.0 L |
|---|---|---|---|
| 1.0 M Tris | 50 ml | 100 ml | 250 ml |
| $ddH_2O$ | 950 ml | 1900 ml | 4750 ml |

Store at 4 $^{o}$C. pH final solution to make sure it is 7.4 at 4$^{o}$C

4.4 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 ml of the suspension 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 up to 100 ml with 50 mM Tris buffer. Resuspend the HAP by sealing the top of the graduated cylinder with parafilm and inverting the cylinder several times. Place in the refrigerator overnight. The next morning, repeat the washing steps twice more with fresh 50 mM Tris buffer. After the last wash, add enough 50 mM Tris buffer to make the final solution a 60% slurry (i.e., if the volume of settled HAP is 60 ml, bring the final volume of the slurry up to 100 ml). Store at 4$^{o}$C until ready for use in the extraction.

4.5 Preparation of [$^3$H]-Estradiol Stock Solutions

Dilute the original 1.0 mCi/ml stock of [$^3$H]-Estradiol to 0.1 µM (i.e., 1 x $10^{-7}$ M). This is most easily accomplished by pipeting 1 µl of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 10.0 ml with ethanol. Thus, if the specific activity of the stock vial = 86 Ci/mmol, then pipet 86.0 µl into an amber colored vial and add 10.0 ml ethanol to the vial; this solution is 1 x $10^{-7}$ M.

**Calculation Check**:
86 µl x 1.0 mCi/1000 µl = 86 x $10^{-3}$ mCi Estradiol = 86 x $10^{-6}$ Ci Estradiol
86 x $10^{-6}$ Ci ÷ 86.0 Ci/mmol = 1 x $10^{-6}$ mmol Estradiol = 1 x $10^{-9}$ moles Estradiol
1 x $10^{-9}$ moles Estradiol ÷ .010 liters = 1 x $10^{-7}$ moles/liter = 0.1 µM

To prepare the 1 x $10^{-8}$ M stock, simply make a 10-fold dilution of the 1 x $10^{-7}$ M stock. To do this, pipet 1.0 ml of the 1 x $10^{-7}$ stock into a clean amber colored vial and add 9 ml ethanol. Final concentrations = 0.01 µM.

4.6 Preparation of 100X Radioinert Estradiol Solutions

Add 27.24 mg Estradiol to a 100 ml volumetric flask, QS to 100 ml with 95% ethanol (1 x $10^{-3}$ M or 1 mM stock).

Take 1.0 ml of the 1 mM stock estradiol and place in another 100 ml volumetric flask, QS new flask to 100 ml with 95% ethanol (1 x $10^{-5}$ or 10 µM). This is the 10 µM radioinert estradiol stock and should be stored in the freezer when not in use (storage in 20 ml aliquots works well).

To make the **1.0 µM radioinert estradiol stock**: Pipet 2.0 ml of the 10 µM estradiol stock into a vial and dilute to 20 ml with 95% ethanol (1 x $10^{-6}$).

To make the **0.1 µM radioinert estradiol stock**: Pipet 2.0 ml of the 1.0 µM estradiol stock into a vial and dilute to 20 ml with 95% ethanol (1 x $10^{-7}$).

4.7 Standard Curve Construction for Saturation and Scatchard Analysis

The first step is to pipet the radioactive ligand (i.e., [$^3$H]-Estradiol) with and without a 100-fold excess of radioinert estradiol into each tube so that the final concentrations of [$^3$H]-Estradiol are 7.0, 3.5, 1.17, 0.7, 0.35, 0.117, 0.035, and 0.0117 nM in a 300 µl total volume. To accomplish this, label tubes and pipet the following into duplicate 12x75 mm borosilicate glass test tubes:

| **Tube** | **Volume[$^3$H]-$E_2$ (µl)** | **Final Conc. [$^3$H]-$E_2$ (nM)** | **Volume Radioinert $E_2$ (µl)** | **Final Conc. Radioinert $E_2$ (nM)** | **Volume of50 mM Tris (µl)** | **Cytosolic Extract (µl)** |
|---|---|---|---|---|---|---|
| 1 | **21 of 1x$10^{-7}$** | 7.00 | ***** | ***** | **229** | **50** |
| 2 | **10.5 of 1x$10^{-7}$** | 3.50 | ***** | ***** | **239.5** | **50** |
| 3 | **3.5 of 1x$10^{-7}$** | 1.17 | ***** | ***** | **246.5** | **50** |
| 4 | **21 of 1x$10^{-8}$** | 0.70 | ***** | ***** | **229** | **50** |
| 5 | **10.5 of 1x$10^{-8}$** | 0.35 | ***** | ***** | **239.5** | **50** |
| 6 | **3.5 of 1x$10^{-8}$** | 0.117 | ***** | ***** | **246.5** | **50** |
| 7 | **10.5 of 1x$10^{-9}$** | 0.035 | ***** | ***** | **239.5** | **50** |
| 8 | **3.5 of 1x$10^{-9}$** | 0.0117 | ***** | ***** | **246.5** | **50** |
| 9 | **21 of 1x$10^{-7}$** | 7.00 | **21 of 1x$10^{-5}$** | 700 | **208** | **50** |
| 10 | **10.5 of 1x$10^{-7}$** | 3.50 | **10.5 of 1x$10^{-5}$** | 350 | **229** | **50** |
| 11 | **3.5 of 1x$10^{-7}$** | 1.17 | **3.5 of 1x$10^{-5}$** | 117 | **243** | **50** |
| 12 | **21 of 1x$10^{-8}$** | 0.70 | **21 of 1x$10^{-6}$** | 70 | **208** | **50** |
| 13 | **10.5 of 1x$10^{-8}$** | 0.35 | **10.5 of 1x$10^{-6}$** | 35 | **229** | **50** |
| 14 | **3.5 of 1x$10^{-8}$** | 0.117 | **3.5 of 1x$10^{-6}$** | 11.7 | **243** | **50** |
| 15 | **10.5 of 1x$10^{-9}$** | 0.035 | **10.5 of 1x$10^{-7}$** | 3.5 | **229** | **50** |
| 16 | **3.5 of 1x$10^{-9}$** | 0.0117 | **3.5 of 1x$10^{-7}$** | 1.17 | **243** | **50** |

After all ingredients have been pipeted, gently vortex the incubation tubes, place them in the tube rotator at 4°C and incubate for approximately 20 hours. Set rotator speed at approximately 40%. Proceed to **Day 2** of assay instructions.

**Note: tubes #1-8 are Total Binding Tubes and tubes #9-16 are Non-specific Binding Tubes**

4.8 Estrogen Receptor Assay Procedure (Keep everything at 4°C!!!)

1. Estrogen Receptor Preparation:
   a) Make TEDG buffer (add the DTT and check pH) and place in ice.
   b) Ovariectomize 10-12 Sprague-Dawley rats at least 10 days prior to receptor preparation.
   c) Sacrifice the rats and remove the uterus from each animal. Trim fat from the uteri.
   d) Weigh each uteri and record the data.
   e) Place all the uteri into a homogenization tube containing TEDG buffer at 4°C.
   f) Decant storage buffer from uteri and add 1.0 ml TEDG buffer per 0.1 gm of tissue.
   g) Homogenize the tissue at 4°C with a Polytron homogenizer using 5-sec bursts. Note: Polytron should be cooled prior to use by placing the probe in TEDG buffer in an ice water bath.
   h) Transfer the homogenate to pre-cooled ultracentrifuge tubes, balance and centrifuge at 105,000 x *g* (approximately 33,000 rpm with TI-90 Beckman ultracentrifuge rotor) for 60 min. at 4°C.
   i) The supernatant contains the cytosolic estrogen receptors. Decant supernatant and assay directly or freeze (-70°C) until ready for use.

**Day 1**

Note: If constructing a standard curve for saturation and Scatchard analysis, label tubes and pipet reagents as described in section 4.7. If running a competitive binding assay, start at step 3 and proceed from there.

2. Label **duplicate** 12x75 glass tubes.
   a) *Standard Curve*: label tubes 0, NSB, S1, S2, S3, S4, S5

| Standard Label | Initial Conc. (M) | Final Conc. (M) |
|---|---|---|
| 0 | 0 | 0 |
| NSB | $3 \times 10^{-6}$ | $1 \times 10^{-7}$ |
| S1 | $3 \times 10^{-7}$ | $1 \times 10^{-8}$ |
| S2 | $3 \times 10^{-8}$ | $1 \times 10^{-9}$ |
| S3 | $1 \times 10^{-8}$ | $3.33 \times 10^{-10}$ |
| S4 | $3 \times 10^{-9}$ | $1 \times 10^{-10}$ |
| S5 | $1 \times 10^{-9}$ | $3.33 \times 10^{-11}$ |

b) *Test Chemicals*: Label tubes 1, 2, 3, 4, ..... The concentrations tested can vary, but a potential standard range of concentrations is outlined below. More than one chemical can be run in an assay if desired.

| Sample # | Initial Conc (M) | Final Conc. (M) |
|---|---|---|
| 1 | $3 \times 10^{-2}$ | $1 \times 10^{-3}$ |
| 2 | $3 \times 10^{-3}$ | $1 \times 10^{-4}$ |
| 3 | $3 \times 10^{-4}$ | $1 \times 10^{-5}$ |
| 4 | $3 \times 10^{-5}$ | $1 \times 10^{-6}$ |
| 5 | $3 \times 10^{-6}$ | $1 \times 10^{-7}$ |
| 6 | $3 \times 10^{-7}$ | $1 \times 10^{-8}$ |

3. Pipet 10 μl of [$^3$H]-estradiol (initial conc. = $3 \times 10^{-8}$ M; final conc. = $1 \times 10^{-9}$ M) into all tubes.
4. Pipet 10 μl of estradiol standard to appropriate standard tubes. The 0 tube receives 10 μl of ethanol.
5. Pipet 10 μl of radioinert test chemical to appropriate sample tubes.
6. Pipet 230 μl of 50 mM Tris buffer into each tube.
7. Pipet 50 μl of cytosolic estrogen receptor supernatant to all tubes.
8. Place reaction mixture tubes in rotator at 4°C for 20 hours.
9. Before leaving for the day, prepare the first wash of the HAP slurry as described in Section 4.4.

**Day 2**

10. Finish washing the HAP as described in Section 4.4. 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 of ice water. Stir the HAP slurry by placing the beaker on a stir plate.
11. Label duplicate 12x75 glass tubes with standard & sample numbers and place on ice. These are the HAP tubes.
12. While the slurry is constantly being stirred, pipet 500 μl of the HAP slurry into the cold, pre-labeled 12x75 tubes.
13. Remove the reaction mixture tubes from the rotator and place them in the ice water bath with the HAP tubes.
14. After mixing the contents of the reaction mixture tubes, pipet 200 μl of each reaction mixture into the appropriately-labeled, duplicate HAP tubes. *Discard remainder of reaction mixture, unless doing the Saturation & Scatchard Analysis.*
15. Vortex the HAP tubes at 5 minute intervals for a total of 20 minutes. *During this incubation, if you are running the Saturation & Scatchard Analysis, pipet 30 μl of the remaining reaction mixture into duplicate, appropriately-labeled, scintillation vials (these are called the TotalCount Tubes and will be used to estimate the concentration of total [$^3$H]-estradiol).*
16. Centrifuge the HAP tubes at 4°C for 3-4 minutes at 600 x *g* (~1700 rpm).
17. Place the tubes back into the ice water bath and aspirate and discard the supernatant. A vacuum aspiration apparatus is helpful with this step.
18. Add 2.0 ml of 50 mM Tris buffer to each HAP tube. Vortex to resuspend HAP pellet and then centrifuge at 4°C for 3-4 minutes at 1700 rpm.

19. Aspirate and discard the supernatant. Repeat Step 18 twice more.
20. After the third wash, aspirate the supernatant. Add 2.0 ml of cold ($4^oC$) 100% ethanol to each HAP tube. Vortex and place in ice for 15 minutes, vortexing at 5 minute intervals.
21. Centrifuge the HAP tubes at $4^oC$ for 10 minutes at 1700 rpm.
22. Decant the supernatant into appropriately-labeled scintillation vials.
23. Add 10 ml of scintillation cocktail to each vial, cover and shake.
24. Place into scintillation counter and count DPMs.

**Evaluation of Data**

25. The counts of tube >0' (no radioinert compound added) serve as the comparator for the counts from tubes into which radioinert test compound was added.
26. Determine the percentage of binding: Number of counts from the HAP sample tubes divided by the number of counts from the >0' tube.
27. Plot the percentage of binding as a function of the concentration of the radioinert compound.
28. Determine the $IC_{50}$ by using the biostatistics program KELL or by noting where the binding curve intersects the 50% value of the ordinate.
29. The relative binding affinity (RBA) of each chemical is determined by:

$$RBA = IC_{50} \text{ Estradiol}/IC_{50} \text{ Test Compound}$$

5.0 Data Processing

5.1 Free Concentration of $[^3H]$-estradiol

Multiply the DPM in the total count tubes by $1.8047x10^{-5}$. **NOTE:** This number will change as the specific activity of new batches of isotope change. Use the equation below to calculate the new number for each batch of isotope.

This value will yield the free concentration (i.e., nM) of $[^3H]$-estradiol initially present in each incubation tube.

Calculation Check

(**X** DPM $\div$ $2.22x10^{12}$ dpm/Ci) = ($4.5045x10^{-13}$ Ci $\div$ Specific Activity of $[^3H]$-estradiol Ci/mmole)

= ($5.4141x10^{-15}$ mmole $\div$ 1000 mmole/mole) = ($5.4141x10^{-18}$ moles $\div$ Volume of reaction mixture)

= ($1.8047x10^{-14}$ moles/liter $\div$ $1x10^{-9}$ moles/nmole) = **X** = $1.8047x10^{-5}$ nM

**Notes:** 1. $2.22x10^{12}$ = a nuclear constant
2. Specific Activity will vary between batches of $[^3H]$-estradiol
3. 1000 mmole/mole is used to convert mmoles to moles

4. Volume of reaction mixture should be in Liters
5. $1x10^{-9}$ moles/nmole is used to convert moles to nmoles

5.2 Calculation of Total, Nonspecific and Specific [$^3$H]-Estradiol Binding

* Total binding = (**X** DPM from the tubes that contained only [$^3$H]-estradiol x $1.6242x10^{-2}$). This value will be total binding in fmoles.

* Nonspecific binding = (**X** DPM from the tubes containing both [$^3$H]-estradiol + 100-fold molar excess radioinert estradiol x $1.6242x10^{-2}$). This value will be nonspecific binding in fmoles.

* Specific binding = (fmoles total binding - fmoles nonspecific binding)

Calculation Check
To get fmoles, multiply the DPM values by $1.6242x10^{-2}$. This is simply nM x 300.

[$1.0847x10^{-5}$ nM x (Volume counted ÷ $1x10^{-6}$ nmoles/fmole)] = $1.6242x10^{-2}$ fmoles

Note: The value $1.6242x10^{-2}$ will change with specific activity of the [$^3$H]-estradiol batch and the volume of the reaction mixture counted.

5.3 Graphical Presentation of the Data

Maximal binding capacity (Bmax) and association/dissociation constants (Ka/Kd) 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 PJ, Rodbard D. Anal. Biochem. 1980; 107:220-239).

6.0 References

Nonneman DJ, Ganjam VK, Welshons WV, Vom Saal FS. Biol. Reprod. 1992; 47:723-729

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

Korach KS. Endocrinology 1979; 140:1324-1332

Korach KS et al. Molecular Pharmacology 1988; 33:120-126

Kun Chae et al. Molecular Pharmacology 1991; 40:806-811