Appendix B1

Protocol for Androgen Receptor Competitive Binding Assay Using Rat Prostate Cytosol

(Provided by Dr. Vickie Wilson, U.S. EPA, NHEERL, Research Triangle Park, NC and Mr. Gary Timm, U.S. EPA, Washington, DC, USA)

[This page intentionally left blank]

ANDROGEN RECEPTOR COMPETITIVE BINDING PROTOCOL

RAT PROSTATE CYTOSOL*

Section 5.5 revised June 1, 2001

Final version of EPA Work Assignment 2-19 Appendix B. This slightly reformatted version incorporates editorial changes (typographic error corrections, pagination, etc.). It also contains several clarifications and standardization. For example, earlier versions contained permissive statements such as "Add 10 to 20 ml of scintillation cocktail". In this version, that step reads "Add 14 ml of scintillation cocktail". The prototype worksheet included as an example at the end of the protocol has been replaced with an example of the implemented worksheet.

This protocol was followed throughout Task 3

***** This protocol was provided in November, 2000 by the EPA as an attachment to the Statement of Work for Contract Number: 68-W-99-033 Work Assignment 2-19, " Development of Estrogen Receptor and Androgen Receptor Binding Data". The protocol has been reformatted, edited, and slightly revised as discussed and approved by the EPA.

Androgen Receptor Competitive Binding Protocol-Rat Prostate Cytosol

1. **Purpose and Applicability**-Determine ability of unknown compounds to compete with ^{3}H-ligand for binding to rat prostate homogenate.

2. **Safety and Operating Precautions**-All procedures with radioisotopes will follow the regulations and procedures as described in the Radiation Work Permit (RWP) and the Integrated Operations System (IOPS) hazard assessment summaries for the tracer laboratory. All staff working the tracer laboratory shall be DOE certified at the Radiation Worker II level.

3. **Animal Use**-The Battelle Animal Use Protocol for this assay is O-40. It and all appropriate Animal Resource Center protocols will be followed.

4. **Equipment and Materials**

4.1. Equipment

-Corning Stir/hot Plates

-Digital Pipettes

-Balance

-Polytron PT 35/10 Tissue Homogenizer

-Vacuum Concentrator

-Refrigerated General Laboratory Centrifuge

-High-Speed Refrigerated Centrifuge (up to 30,000 x *g*)

-pH Meter with Tris Compatible Electrode

-Scintillation Counter

4.2. Chemicals

-Negative Control (Corticosterone)

-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 (Optifluor)

-Ethyl Alcohol, anhydrous

-^{3}H-R1881 (NEN)

- Radioinert R1881 (NEN)

-Triamcinolone Acetonide

-Steroids (Steraloids - recrystallized)

4.3. 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 µl) pipette tips

5. Stock Preparations

5.1. Preparation of TEDG Stock Solutions

5.1.1. Add 7.444g disodium EDTA to 100 ml ddH_2O = 200mM. Store at 4ºC. Use 750 µl/100ml TEDG buffer = 1.5 mM.

5.1.2. Add 1.742 g PMSF to 100 ml ethanol = 100 mM. Store at 4ºC. Use 1.00 ml/100ml TEDG buffer = 1.0 mM.

5.1.3. Add 2.419 g sodium molybdate to 8.0 ml ddH_2O in a 10 ml volumetric flask; bring the total volume to 10 mls = 1.0 M. Store at 4ºC. Use 100µl/100ml TEDG buffer = 1.0 mM.

5.1.4. Add 15.4 mg DTT directly to 100 ml TEDG buffer the morning of the receptor isolation = 1.0 mM.

5.1.5. Add 147.24 g Tris-HCL + 8.0 g Tris base to 800mls ddH_2O in a volumetric flask; bring the final volume to 1.0 liter. Refrigerate to 4ºC and pH (using 4ºC pH standardizing solutions) the cooled solution to 7.4. Store at 4ºC. Use 1.0 ml/100 ml TEDG buffer = 10mM. (50 mM Tris = 50 ml 1 M Tris/1 L H2O)

5.1.6. Add 298.2 g KCL to 600 ml ddH_2O 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.

5.2. 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 ddH_2O

-1.0 ml 1M TRIS

-10.0 ml glycerol

-100 µl 1M sodium molybdate

-750 µl 200mM EDTA

-1.0 ml 100mM PMSF

-15.4 mg DTT

5.2.1. Check pH of the final solution to make sure it is 7.4 at 4ºC.

(Preparation of high salt buffer has been omitted by EPA)

5.3. Preparation of 50 mM TRIS Buffer

5.3.1. Add 50.0 ml 1.0 M TRIS to 950 ml ddH_2O. Store at 4ºC. Check pH of the final solution to make sure it is 7.4 at 4ºC.

5.4. Preparation of 60% Hydroxylapatite (HAP) Slurry

5.4.1. Shake BIO-RAD HT-GEL until all the HAP is in suspension (i.e., looks like milk).

5.4.2. 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 2h.

5.4.3. 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.

5.4.4. The next morning, repeat the washing steps x 2 with fresh 50 mM TRIS buffer.

5.4.5. 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).

5.4.6. Store at 4ºC until ready for use in the extraction.

5.5. Preparation of [^{3}H-17á-Methyl]-R1881 Stock Solutions

5.5.1. Steps 5.5.2 through 5.5.4 describe the **general** preparation, section 5.5 describes the preparation at Battelle..

5.5.2. Dilute the original 1.0 mCi/ml stock of [^{3}H-17á-methyl]-R1881 to 0.1 µM (i.e., 1 x 10^{-7} M). This is most easily accomplished by pipetting 1 µl 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 pipette 86.0 µl into an amber colored vial (i.e., R1881 is photosensitive) and add 10.0 mls ethanol to the vial; this solution is 1 x 10^{-7}M.

5.5.3. Calculation Check

5.5.3.1. 86 µl x 1.0 mCi/1000µl = 86 x 10^{-3} mCi R1881 = 86 x 10^{-6} Ci R1881

5.5.3.2. 86 x 10^{-6} Ci; 86.0 Ci/mmol = 1 x 10^{-6} mmol R1881 = 1 x 10^{-9} moles R1881

5.5.3.3. 1 x 10^{-9} moles R1881; 0.010 liters = 1 x 10^{-7} moles/liter = 0.1 µM

5.5.4. To prepare the 1 x 10^{-8}M stock simply make a 10-fold dilution of the 1 x 10^{-7} M stock (i.e., pipette 1.0 ml of the 1 x 10^{-7} M stock into a clean amber colored vial and add 9 mls ethanol = 0.01 µM).

5.5.5. **Specific:** The R1881 acquired by Battelle in January, 2001 had a specific activity of 75.2 Ci/mmol (rather than the 86 used in the example in section 5.5.1) at 1 mCi/ml. The stock solution was prepared by adding 75.2 µl of R1881 to 10 ml EtOH. This solution is 1 x 10^{-7}M. To prepare the 10^{-8} M stock, a ten-fold dilution of the 1 x 10^{-7}M stock was made by adding 1 ml of the 1 x 10^{-7}M stock to 9 ml of EtOH.

5.6. Preparation of 100X Radioinert R1881 Solutions

5.6.1. 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 µl and dilute to 20 ml in an amber vial with ethanol = 1 x 10^{-5} M R1881. This is the 10 µM Radioinert (cold) R1881 stock.

5.6.2. To make the 1.0 µM cold R1881 stock, pipette 2 ml of the 10 µM stock into an amber vial and dilute to 20 ml with ethanol = 1 x 10^{-6}M = 1.0 µM cold R1881 stock.

5.7. Compound Stock Preparations

5.7.1. Battelle-Sequim will supply test chemicals diluted in ethanol (200 proof) at a concentration of 3.0 x 10^{-2} M (i.e., 30 mM).

5.7.2. Note: Battelle-Sequim may determine that some chemicals are not soluble at this concentration, so adjustments will need to be made in the protocol depending upon the specific chemical. Likewise, some chemicals (e.g., CdCl) may not be soluble in ethanol at all, so appropriate modifications in this assay should be made to accommodate any change in solvent. Such changes must be documented.

5.7.3. Prepare serial dilutions of R1881 for standard curve and test chemical in ethanol to yield the Initial Concentrations as indicated in Table 1.

Table 1 Standard Curve

Standards	***Initial R1881 Concentration (Molar)***	****Final R1881 Concentration (Molar) in AR assay tube***
Negative Control	0 (Corticosterone)	1×10^{-4}
0	0 (EtOH)	0
NSB	3×10^{-5}	1×10^{-6}

S1	3×10^{-6}	1×10^{-7}
S2	3×10^{-7}	1×10^{-8}
S3	3×10^{-8}	1×10^{-9}
S4	3×10^{-9}	1×10^{-10}
S5	3×10^{-10}	1×10^{-11}

**When 10 µl of each standard is added to the AR assay tube, the final concentration will be as indicated when the total volume in the AR assay tube is 310 µl.*

5.7.4. Prepare serial dilutions of the test chemicals as indicated in Table 2.

Table 2 – Test Chemical Concentrations		
Serial Dilutions of Test Chemical	***Initial Concentration (30 X) (Molar)***	****Final Concentration (Molar) in AR assay tube***
Concentration 1	3 x 10^{-4}	1 x 10^{-5}
Concentration 2	3 x 10^{-5}	1 x 10^{-6}
Concentration 3	3 x 10^{-6}	1 x 10^{-7}
Concentration 4	3 x 10^{-7}	1 x 10^{-8}
Concentration 5	3 x 10^{-8}	1 x 10^{-9}
Concentration 6	3 x 10^{-9}	1 x 10^{-10}
Tube 7	0 (vehicle only)	0
**Final Concentration of test chemical in assay tube when 10 µl of Initial Concentration is used in a total volume of 310 µl.*		

Example for use at Battelle-Sequim:

Make stocks 30X above desired final (this accounts for the use of 10µl stock in 300µl cytosol)

4 (t) octyl phenol FW 206.33
1M = 206.33g/L
1mM= .20633mg/ml
final conc *x30mM*
1) 1mM = 6.1899mg x 2 = 12.37 mg/2 ml ethanol (100%)
2) 316µM =316µl of 1 +684µl ethanol (100%)
3) 100µM =100µl of 1 + 900µl ethanol (100%)
4) 31.6µM =100µl of 2 + 900µl ethanol (100%)
5) 10µM =100µl of 3 + 900µl ethanol (100%)
6) 3.16µM =100µl of 4 + 900µl ethanol (100%)
7) 1µM =100µl of 5 + 900µl ethanol (100%)

6. Tissue Homogenate Collection

6.1. Castrate 60-90 day old rats as per laboratory animal protocols.

6.2. 24 hours after castration, make low-salt TEDG buffers and place in an ice-water bucket.

6.3. Kill rat and excise ventral prostate. Tissue should be trimmed of fat and pooled. The weight of the pooled prostate tissue will be recorded.

6.4. Add low-salt TEDG buffer at 10ml/g tissue

6.5. Mince tissues with Metzenbaum scissors until all pieces are small 1-2mmcubes. Then homogenize the tissues at 4ºC with a Polytron homogenizer using 5-sec 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]

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

6.7. The supernatant is the low-salt cytosolic receptor. Pool the supernatant from all rats. Aliquot into 5ml and store -80ºC until needed for assay.

6.8. Determine the protein content for each batch of cytosol using the BioRad Protein Assay Kit (BioRad Chemical Division, Richmond, CA).

7. Assay Procedure, Day 1

7.1. Set up tubes:

7.1.1. Label 12 x 75mm glass tubes 1-90 (or if using pre-labeled tubes, note starting number). Place tubes in centrifuge tube holders following numbering scheme. See worksheet for assignment of tube numbers. 12x75 mm glass tubes

7.1.2. Add 30µl of 0.01µM [^{3}H] R1881 + 50µl Triamcinolone Acetonide (60µM stock) to ALL tubes

7.1.3. For 2 tubes, also add 100x inert R1881 (30µl of 10µM)

7.1.4. Place tubes in speed-vac and dry the tubes according to instructions. Remove when dry.

7.2. Add 10µl of compound stocks (see Table 2 for concentrations 1-7 in duplicate)

7.3. Remove aliquot of prostate cytosol and thaw on ice.

7.4. Add 300µl of cytosol to every tube ON ICE. Gently vortex and place tubes in refrigerator overnight in rotor (20hr).

7.5. Before leaving for the day, prepare the first wash of the HAP slurry as described in section 4.5 above.

7.6. Also, if necessary, label the HAP tubes and the scintillation vials to be used the following day.

8. Assay Procedure, Day 2

8.1. 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.

8.2. While the HAP slurry is constantly being stirred, pipette 500 µl of the HAP slurry into the assay tubes. 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.

8.3. One tube should be prepared for each incubation tube (duplicate omitted by EPA).

8.4. Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipette 100 µl (duplicate omitted by EPA) from each of the incubation tubes into the appropriate pre-labeled 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 5 minutes for 20 minutes.

8.5. Centrifuge the HAP tubes for 2-3 minutes at 4ºC and 600 x *g* (i.e., 1780 rpm in a Beckman GLC refrigerated centrifuge). Place the tubes back into the rack and into the ice-water bath.

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

8.7. 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.

8.8. Repeat the TRIS washing procedure 3 or 4 times (to be determined empirically) keeping the tubes at 4ºC at all times.

8.9. Following the last wash and decanting, add 1.5 ml of ethanol to each tube, vortex 3 times at 5 minute intervals and centrifuge the tubes at 600 x *g* for 10 minutes. Decant the supernatants into pre-labeled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

9. Data Processing

9.1. <u>Concentration of Free [^{3}H]-R1881</u>

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

9.1.1.0.1. <u>Calculation Check</u>

X DPM = 4.5045 x 10^{-13} Ci = 5.4141 x 10^{-15} mmole = 5.4141 x 10^{-18} moles

2.22 x 10^{12} dpm/Ci   83.2 Ci/mmole   1000 mmole/mole0.0003 liters

= 1.8047 x 10^{-14} moles/liter = X (1.8047 x 10^{-5}) nM   1 x 10^{-9} moles/nmole

9.2. <u>Calculation of Total, Nonspecific and Specific [^{3}H]-R1881 Binding</u>

Total binding is calculated by multiplying the DPM from the tubes that contained only radiolabelled R1881 x (1.6242 x 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 cold R1881 x (1.6242 x 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.

9.3. Calculation Check

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

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

9.4. Graphical Presentation of the Data

9.4.1. Standard Curve and Test Chemical Competitive Binding Curves: Data for the standard curve and each test chemical will be plotted as the percent ^{3}H-R1881 bound versus the molar concentration. Estimates of the IC_{50}s will be determined using appropriate non linear curve fitting software such as GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A Scatchard analysis may also be preformed for the standard curve using R1881 to demonstrate that the assay meets acceptable QA standards.

9.4.2. Relative Binding Affinity: The RBA for each competitor should be calculated by dividing the IC_{50} for R1881 by the IC_{50} of the competitor and expressing as a percent (e.g., RBA for R1881 =100 %).

10. References

file: `chemreceptor.sop (8/24/99)` supplied by EPA with Statement of Work for Contract Number: 68-W-99-033 Work Assignment 2-19, “Development of Estrogen Receptor and Androgen Receptor Binding Data”.

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

11. Example Worksheet

The first sixteen positions of the assay run are used to establish background and standards for the run. Positions 1 and 2 are the replicate “zero” vials, designated “0". Positions 3 and 4 are non specific binding vials containing cold receptor, designated “NSB”. Positions 5 through 14 are the standard curve, designated “S1" through “S6". Positions 15 and 16 are the negative control, designated “Neg.”.

There are twelve positions for each unknown, designated "U1", "U2", etc. After the last unknown, there are four positions for additional NSB and Neg., and four calibration positions for vials containing only the tracer and scintillation cocktail, these are designated as "Hot"

Androgen Receptor Competitive Binding Protocol-Rat Prostate Cytosol

Example Assay Worksheet Rat Androgen Receptor 5/2/2001

Person's Name here ***Num_Pts_Std_Curve:*** 6 ***Num_Test_Chem:*** 1 ***Num_Dilutions_Per_Chem:*** 6

Receptor: Rat Prostate, Lot 021401 ***Tracer:*** H-3 R1881, Lot 3363714

Position			**Competitor**	**Initial Concentration (M)**	Tracer (ul)	**Inert Tracer (ul)**	Tri. Acetate (ul)	**Speed Vac**	Competitor (ul)	**Receptor (ul)**	HAP (ul)	**Final Concentration (M)**
1	1	0	EtOH		30	-	50	<>	10	300	500	
2	2	0	EtOH		30	-	50	<>	10	300	500	
3	1	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
4	2	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
5	1	S1	Inert R1881	3E-06	30	-	50	<>	10	300	500	1E-07
6	2	S1	Inert R1881	3E-06	30	-	50	<>	10	300	500	1E-07
7	1	S2	Inert R1881	3E-07	30	-	50	<>	10	300	500	1E-08
8	2	S2	Inert R1881	3E-07	30	-	50	<>	10	300	500	1E-08
9	1	S3	Inert R1881	3E-08	30	-	50	<>	10	300	500	1E-09
10	2	S3	Inert R1881	3E-08	30	-	50	<>	10	300	500	1E-09
11	1	S4	Inert R1881	3E-09	30	-	50	<>	10	300	500	1E-10
12	2	S4	Inert R1881	3E-09	30	-	50	<>	10	300	500	1E-10
13	1	S5	Inert R1881	3E-10	30	-	50	<>	10	300	500	1E-11
14	2	S5	Inert R1881	3E-10	30	-	50	<>	10	300	500	1E-11
15	1	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
16	2	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
17	1	U1	Sample ID	3E-04	30	-	50	<>	10	300	500	1E-05
18	2	U1	Sample ID	3E-04	30	-	50	<>	10	300	500	1E-05
19	1	U1	Sample ID	3E-05	30	-	50	<>	10	300	500	1E-06
20	2	U1	Sample ID	3E-05	30	-	50	<>	10	300	500	1E-06
21	1	U1	Sample ID	3E-06	30	-	50	<>	10	300	500	1E-07
22	2	U1	Sample ID	3E-06	30	-	50	<>	10	300	500	1E-07
23	1	U1	Sample ID	3E-07	30	-	50	<>	10	300	500	1E-08
24	2	U1	Sample ID	3E-07	30	-	50	<>	10	300	500	1E-08
25	1	U1	Sample ID	3E-08	30	-	50	<>	10	300	500	1E-09
26	2	U1	Sample ID	3E-08	30	-	50	<>	10	300	500	1E-09
27	1	U1	Sample ID	3E-09	30	-	50	<>	10	300	500	1E-10
28	2	U1	Sample ID	3E-09	30	-	50	<>	10	300	500	1E-10
29	1	0	EtOH		30	-	50	<>	10	300	500	
30	2	0	EtOH		30	-	50	<>	10	300	500	
31	1	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
32	2	NSB	Inert R1881	1E-05	30	30	50	<>	-	300	500	1E-06
33	1	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
34	2	Neg.	Corticosterone	3E-03	30	-	50	<>	10	300	500	1E-04
35	1	Hot	Scint. Cocktail		30	-	-	<>	-	-	-	
36	2	Hot	Scint.Cocktail		30	-	-	<>	-	-	-	
37	1	Hot	Scint.Cocktail		30	-	-	<>	-	-	-	
38	2	Hot	Scint. Cokctail		30	-	-	<>	-	-	-	