Appendix B3

Protocol for Measuring Androgen-Binding Sites on Androgen Receptors or Binding Proteins

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1. Source of androgen receptors:

Androgen receptors are present in tissues of the male reproductive tract, for example, the prostate, epididymis, and seminal vesicle. In intact adult males, the binding site on the androgen receptors is occupied by endogenous androgen, therefore, it is necessary that they be castrated to eliminate endogenous androgens, freeing the androgen binding-site for occupation by exogenous radiolabeled ligand used in the assay. While the epididymis contains androgen receptor, it also contains androgen-binding protein (ABP), which is present at a concentration at least 10-times greater than that of the receptor. Special precautions are necessary when assaying androgen receptor in the presence of ABP, therefore, it is suggested that the epididymis not be used as a routine source of androgen receptor. A good source of androgen receptor is the prostate of adult rats that have been castrated for 24-48 hr. In sexually immature male animals, the binding site on the androgen receptors is not occupied by endogenous hormone, therefore, reproductive tract tissues of immature males provide a good source of androgen receptor. We successfully have used frozen prostates from 21 day-old rats, purchased from Harlan Bioproducts for Science, Indianapolis, IN, as a source of androgen receptor. Androgen receptors are also present in female reproductive tract tissues, for example, the chicken oviduct, and the uterus and vagina of most mammals. Thus, these tissues are also potential sources of androgen receptor for assay purposes.

2. Preparation of Cytosol:

a. Weigh the androgen receptor-containing tissues, mince thoroughly, on ice, using razor blades or scissors [1]. If using frozen tissues, pulverize in a mortar and pestle. Transfer the minced (or pulverized) tissue to an appropriately sized cold test tube and add ice cold TE buffer(10mM Tris-HCl, pH 7.5, 1.0 mM EDTA) at a ratio of 1:10 or 1:4 weigh/volume to the minced tissue. Keep samples on ice. It is recommended that a ratio of 1:4, or even 1:2, be tried initially. You will be diluting the cytosol later in the assay and you want to make sure that you have enough receptor present to detect. Once you become familiar with the amount of receptor in the cytosol from a given source, you can standardize your tissue to buffer ratio. As a precaution against proteolysis of the androgen receptor, you may wish to add a cocktail of protease inhibitors to the buffer, for example, 2 mM PMSF, 10 ug/ml antipain, 10 mM molybdate, 5 mM leupeptin [2, 3]. If you are planning to freeze the cytosol for later use, homogenize in TE buffer containing 10% v/v glycerol.

b. Homogenize the tissues in the above buffer using a Polytron (Brinkman Instruments, Westbury , NY) in a cold room (cool Polytron before use) or use cold glass-glass or glass-teflon homoginizers.

c. Centrifuge the homogenate at about 200,000 x g for 1 hr in a refrigerated centrifuge—the supernatant is the cytosol. Pour off and save cytosol for the assay—keep on ice.

3. The "binding check":

The purpose of the binding check is to determine how much cytosol you will need to use in the assay to obtain an androgen receptor measurement that is clearly above background.

a. Decide on the volumes of cytosol that you wish to test, for example, 10 ul, 50 ul, 200 ul.

b. We want the final volume of the assay to be 500 ul, so set up as follows in an ice bath, add components to the tubes in the order shown:

Tube No.*	Vol. of TE	Vol oflabel**	Vol. ofcold***	Vol. of cytosol
1-2	440 ul	50 ul	-------	10 ul
3-4	340 ul	50 ul	100 ul	10 ul
5-6	400 ul	50 ul	--------	50 ul
7-8	300 ul	50 ul	100 ul	50 ul
9-10	250 ul	50 ul	------	200 ul
11-12	150 ul	50 ul	100 ul	200 ul
13-14	450 ul	50 ul	------	------

*We typically use 12 x 75 mm borosilicate tubes.

**To standardize the assay, we dilute the label ([^{3}H]5α-DHT, 130 Ci/mmole, Dupont/NEN, Boston, MA) with TE so that when 50 ul of it are added to the assay tubes, the appropriate concentration of radiolabeled steroid (approx. 7 nM) will be present (working solution).

***This is the volume of unlabeled 5α-DHT at 1 ng/ in TE, will completely inhibit binding of label to the receptor.

c. Incubate the samples on ice for 2 hr; binding equilibrium may not have occurred during this time, but you will probably have enough binding to see what volume of cytosol to use. If not, incubate longer, for example 4 hr or overnight.

d. At the conclusion of the incubation add 0.5 of ice-cold dextran-coated charcoal to each tube, vortex each tube for 10 sec, then incubate all tubes on ice for 10 min [1]. When the incubation time is up, centrifuge the tubes for 10 min at 1500 x g in a swinging- bucket- type rotor at 4 C. When centrifugation is completed, carefully, but swiftly decant the supernatant from each tube into a separate scintillation vial, add scintillation fluid that is designed for aqueous samples, and count. The decanting process soon becomes almost innate—shortly, you will have less than a

1% variance among replicates. Alternatively, you may use a pipette to aspirate a specific volume of each supernatant.

e. Determining your results:
1) Average the duplicates 2) Subtract the duplicates containing unlabeled hormone from those that contained labeled hormone only. This will give you the amount of specific binding in cpm; appropriate calculations will yield fmol or pmol of bound label. This specifically bound 5α-DHT is presumed to be bound by the androgen receptor. One or more of the volumes of cytosol used should give you the amount to be used for measuring the androgen receptor. 3) The tubes containing only label and buffer give you the true background, that is, the amount of label not adsorbed by the charcoal. This number should be low--500-1500 cpm. If the background is higher than this, reduce the amount of label added per tube. This background number also gives an indication of the quality of the labeled preparation; as the solution of labeled hormone ages, the background increases. To reduce the occurrence of degradation of the labeled compound, store the stock solution (in ethanol as it comes from Dupont or diluted with additional ethanol) at –20 C and only make up enough working solution (stock solution diluted in TE) to last for a few days. Store the working solution at 4 C and keep on ice while in use. The stock solution stored under these condition should last at least a year without significant signs of degradation.

f. The "binding check" seems to be a tedious procedure, and it is, but you should do it to get an estimate of how much receptor is in a given tissue. Once you know this, you can use cytosol from that tissue source in the future without having to do a binding check.

4. Competitive Binding Assay:

a. Set up the assay essentially as shown for the "binding check", however, for the assay we always use triplicate determinations and incubate on ice or at 4 C for 4 hr or overnight. For the assay a standard curve is set up in which a fixed concentration of labeled hormone and a fixed amount of cytosol (receptor) is used together with varying concentrations of unlabeled hormone—add cytosol last! For example:

Tube No.	Vol. of TE	Vol of label*	Vol. of cold**	Vol. of cytosol
1-3	250 ul	50 ul	-----	200 ul
4-6	240 ul	50 ul	0.1 ng (10ul, 0.1ng.ul)	200 ul
7-9	200 ul	"	0.5 ng(50 ul ")	"
10-12	240 ul	"	1.0 ng(10ul, 0.1 ng/ul)	"
13-15	230 ul	"	2.0 ng(20ul ")	"
16-18	200 ul	"	5.0 ng(50ul ")	"
19-21	150 ul	"	10ng(100ul ")	"
22-24	200 ul	"	50 ng (50ul, 1 ng/ul)	"
25-27	150 ul	"	100 ng (100ul, ")	"
28-30	450 ul	"	-------	-----

*Using labeled 5α-DHT at 7 nM
** Using unlabeled 5α-DHT in TE buffer.

b. As with the binding check: average triplicates, subtract the value of the average of tubes 25-27 from the average of the other samples to yield specific binding.

c. Plotting: Calculate the percentage of control of each concentration of inhibitor, using the value of the samples having no competitor added as 100%, and plot against the log of the unlabeled hormone concentration—this will yield a sigmoid curve.

d. When using competitors other than unlabeled 5α-DHT, for example environmental toxicants, set up assays as for the standard curve and plot the resulting data in the same manner. Remember that steroid hormone receptors have affinities for toxicants that are orders of magnitude lower than for the physiological hormones and plan to use concentrations of competitors to reflect this. We make up stock solutions of toxicants DMSO. The toxicant stocks are diluted with TE to make the working solutions. Higher concentrations of toxicants may come out of solution at 4 C. To minimize this, add cytosol to assay tubes that are at room temperature, vortex, and then add to the ice bath.

e. Calculating the RBA (relative binding affinity): Use the standard curve, determine the concentration of unlabeled 5α-DHT that causes a 50% inhibition of binding of radiolabeled 5α-DHT (IC_{50}). Use the competition curve, calculate the concentration of unlabeled toxicant, etc. that causes a 50% inhibition of radiolabeled 5α-DHT. Divide the IC_{50} of the physiological ligand by the IC_{50} of the toxicant and multiply by 100 to obtain the RBA [4].

5. Costs

a. The [^{3}H]5α-DHT is the most expensive item with 250 uCi costing approximately $500.00. This is enough radioactivity to set up about 4000 tubes.
b. Frozen prostates from 21 day-old rats are about $4.00 each. To do a standard curve and a competition curve could take 25-30 prostates. A less expensive source of androgen receptor is the 24-hr castrated adult rats, approximate cost $30.00.
c. The costs of unlabeled 5α-DHT, charcoal, dextran, and reagents for buffers is negligible.
d. Technical costs: It would take about 1 hr to prepare the solutions and reagents necessary for the assay. A technician can set up the 57 tubes required for the two curves in less than 30 min. Another 30 min is necessary for adding of charcoal to the assay tubes, centrifuging them, decanting the supernatants, adding the scintillation fluid to the assay vials, and placing the vials in the scintillation counter. Calculations and plotting takes about 1 hr. Total technician time—approximately 3 hr.
e. The total cost for the two curves as shown above would be about $175 (high end) or about $85 (low end).

References

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