Appendix B2

Protocol for the Fluorescence Polarization Assay of the Competitive Binding of Ligands to Estrogen-Receptor Complexes

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Fluorescence Polarization Assay of the Competitive Binding of Ligands

to Estrogen-Receptor Complexes

Purified expressed human estrogen receptor alpha (ER) and human estrogen receptor beta (ER), fluorescent-labeled 17 -estradiol (ES2), and estrogen screening buffer were purchased from Pan Vera Corporation.

A solution of ER-ES2 complex in estrogen screening buffer containing 20 nM ES2, 26 nM human-ER and 10 mM dithiothreitol (DTT) was prepared, and 50 µl of this solution was added to borosilicate test tubes containing 50 µl of serially diluted compounds to be tested as potential estrogenic ligands. Samples tubes containing 50 µl of the estrogen screening buffer and 50 µl of the ER-ES2 complex solution were included as negative controls to determine the initial polarization value (P_o, polarization value with no estrogen competitor present). Sample tubes containing 100 µl of the estrogen screening buffer with 10 nM ES2 and 5 mM DTT were also included to determine the polarization value of nonbound ES2 (P_{100}). The samples were incubated at room temperature for 1 hour and the fluorescence polarization (FP) then measured using a Beacon 2000 Fluorescence Polarization Instrument (Pan Vera) equipped with a 530 nm emission filter and a 490 nm excitation filter.

FP values were converted to percentage inhibition using the equation

$$I_\% = (P_o - P)/(P_o - P_{100}) \times 100$$

where P_0 is the FP value at 0%, P_{100} is the FP value when 100% of the ES2 has been competitively displaced from the ER complex, and P is the experimental FP value at each concentration of the competing ligand being tested. The percentage inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting and the concentration of competing ligand required to displace half of the bound ES2 fluorescent ligand determined (IC_{50}). The IC_{50} values were converted to relative binding affinities (RBA) using 17 -estradiol (E_2) as a standard. The RBA for E_2 was set equal to 100 and the RBA value for each competing ligand calculated using the following formula:

$$RBA = (IC_{50}E_2 / IC_{50} \text{ competitor} \quad) \times 100 \; .$$

Measuring Estrogen Receptor-Estrogen Response Element Binding by
Fluorescence Polarization

Preparation of fluorescence labeled estrogen response elements.
Sense and antisense oligonucleotide strands 35 bases long containing either estrogen response elements (EREs) from the Xenopus vit A2 gene or the human pS2 gene, or the consensus glucocorticoid response element (negative control) were obtained from Oligos Etc. (Wilsonville, OR). The sense DNA strands were labeled with fluorescein attached via a six-carbon spacer at the 5' terminus. Double stranded oligonucleotides were then prepared by annealing equimolar concentrations of the separate sense and antisense strands in 10 mM Tris-HCl, pH 7.8, and 1.50

mM NaCl as follows: 1 nmole of the sense and 1 nmole of the antisense DNA strands in 500 µL buffer were heated in water bath to 95°C for 10 min and slowly cooled (30 min) to room temperature. To remove any hairpin formations the double stranded DNA was purified by electrophoresis on 12% polyacrylamide (1:19 bisacrylamide:acrylamide) gels containing 89 mM Tris-borate, 2.5 mM EDTA, pH 8.3, and 10% ammonium persulphate.

ER-ERE binding studies.

The abilities of ligand bound ER and ER to associate with Xenopus vit A2 ERE or human pS2 ERE were measured using fluorescence polarization (FP). Purified, expressed human ER and ER obtained from Pan Vera Corporation were serially diluted from 450 nM to 0.8 nM in DNA binding buffer (10 mM potassium phosphate, pH7.8; 0.1 mM EDTA; 50 µM magnesium chloride; 10% glycerol). The concentrations of the ligands required to saturate ER or ER were determined by FP competitive binding experiments. Each ER was then incubated with saturating levels of the individual estrogenic ligands for 30 min, and then for 10 min with poly (dI-dC) (1 µg/5 µg of protein) at room temperature. The binding, initiated by adding fluorescein-labeled synthetic oligonucleotide EREs (final concentration 0.5 nM), was allowed to proceed at room temperature for 60 min in dark. The same experiment was performed with the ERs bound to 17 -estradiol (E_2) as positive controls. The samples were prepared and measured in borosilicate test tubes with final reaction volume of 100 µl. The FP at each ER concentration was measured on Beacon 2000 Fluorescence Polarization Instrument (Pan Vera Corporation) equipped with 490 nm excitation and 530 nm emission filters. Binding isotherms were constructed by plotting percent saturation versus ER concentration using the formula:

$$S_\% = (P-P_0)/(P_{100}-P_0) \times 100$$

where P_0 is the polarization value at 0% saturation, P_{100} is the polarization value at 100% saturation, and P is the observed fluorescence polarization (FP) at each concentration point. The equilibrium dissociation constant (K_d) was calculated from the binding curves using a nonlinear least-squares curve fitting program. To compare the binding affinities of ER -ligand and ER -ligand complexes for the various EREs the K_d values were converted to relative binding affinities (RBA) using the following formula with the K_d for the E_2-ER complex as the standard.

$$RBA = (K_d E_2 / K_d \text{ competitor}) \times 100.$$