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Re: For Your Information Submission:

The enclosed information is submitted on behalf of Dow Corning Corporation, Midland, Michigan, 48686-0994, on a For-Your-Information (FYI) basis as a follow-up to submissions made concerning octamethylcyclotetrasiloxane (OMCTS), which chemical substance was the subject of a health and safety data rule issued under Section 8(d) of the Toxic Substances Control Act (TSCA) and with an effective date of December 28, 1984 (sunset date December 28, 1994), as codified at 40 CFR 716 (Health and Safety Data Reporting). The information presented in this submission was generated as part of our Siloxane Research Program. This program was the subject of a memorandum of understanding, dated April 9, 1996, between Dow Corning and EPA.

**Listed Chemical Substance:**

556-67-2 Octamethylcyclotetrasiloxane (OMCTS, D<sub>4</sub>)

**Final Study Report:**

Non-Regulated Study: Development of an *in vitro* Assay to Measure the Binding of D<sub>4</sub> to the Estrogen Receptor

Dow Corning Corporation  
2001-I0000-50522  
January 10, 2003



**Manufacturer:**

Dow Corning Corporation  
PO Box 994  
2200 West Salzburg Road  
Midland, Michigan 48686-0994

For purposes of this TSCA For-Your-Information (FYI) submission, the general INTERNAL designation on the attached health and safety report is waived by Dow Corning.

If you require further information regarding this submission, please contact Michael Thelen, Manager of U.S. EPA Regulatory Affairs, at 989-496-4168 or at the address provided herein.

Sincerely,

A handwritten signature in cursive script that reads "Kathleen P. Plotzke". The signature is written in black ink and is positioned above the typed name.

Kathleen P. Plotzke  
Director, Health and Environmental Sciences  
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DOW CORNING CORPORATION  
TECHNICAL REPORT

Report No.: 2001-10000-50522  
Study No.: 9226  
Study Leader: Anne L. Quinn, Ph.D.  
Testing Facility: Dow Corning Corporation  
Health and Environmental Sciences  
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Study Completion date: 10 January, 2003

Title: Non-Regulated Study: Development of an *in vitro* Assay to Measure the Binding of D4 to the Estrogen Receptor.

**Distribution List**

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GLP Compliance Statement: The work described in this report was carried out using the best available scientific methodology, and procedures were followed to assure accurate, high quality results. However, this non-regulated study was *not* conducted to meet all of the requirements described in Good Laboratory Practices Regulations such as those documented in the Federal Register 40 CFR Part 792.

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#### **ABSTRACT**

The primary objective of this non-regulated method development study was to evaluate the potential binding of octamethylcyclotetrasiloxane (D4) to the estrogen receptors. In order to achieve this objective, several development steps had to be completed to establish this assay in house. Of particular concern was the method of recovery of the bound receptor from the assay mixture and the reduction of non-specific binding. Several positive control materials were evaluated using the hydroxylapatite (HAP) isolation method and the measured kinetic parameters were consistent with values found in the literature. An additional technical concern was the consistent delivery of D4 to an aqueous-based reaction mixture. It was determined that saturation of the headspace above the reaction mixture with a 900 ppm D4 vapor, resulted in molecular D4 partitioning into the aqueous reaction mixture in a reliable and predictable manner. GC/MS and <sup>14</sup>C-D4 analyses indicated that D4 was present in the reaction mixture at micromolar concentrations. A time-course experiment indicated that after ~30 minutes, the levels plateaued and remained constant over the three-hour incubation. This delivery system allowed for measurement of D4 binding to the estrogen receptors, which previously had not been reliably demonstrated.

Once developed, this method was used to measure the ability of D4 to bind to the estrogen receptors. Incubation of the 900 ppm D4 with the ER-alpha resulted in ~20% displacement of the <sup>3</sup>H-estradiol after a three hour incubation. The D4 did not significantly displace the <sup>3</sup>H-estradiol from the ER-beta under these conditions.

In summary, this method proved to be a way to measure D4 binding to the estrogen receptor. D4 weakly competes for the ER-alpha but does not appear to compete for the ER-beta.

DC Study Number: 9226  
Non-Regulated

Report Number: 2001-10000-50522  
INTERNAL

**STUDY INFORMATION**

Experimental Start Date:	26 April 1999
Experimental Termination Date:	20 December 2001
Study Completion Date:	10 January 2003
Study Leader:	Anne L. Quinn, Ph.D.
Sponsor:	Dow Corning Corporation
Study Personnel:	Anne L. Quinn, Ph. D. Debra McNett, B.S. Associate Research Specialist Renee L. Jezowski, B.S. Technician

**APPROVAL SIGNATURES**

This report, consisting of pages 1-17, including Tables 1-3, Figure 1-2 and Appendix A (A1-A21) is signed this 10 day of, Jan 2003.

Anne L. Quinn 10-January-2003  
Anne L. Quinn, Ph.D. Date  
Study Leader

Steven D. Crofoot 1/10/2003  
Steven D. Crofoot, M.S. Date  
Team Leader, Toxicology  
Health and Environmental Sciences

## OBJECTIVE

The purpose of this method development study was to optimize a method for the measurement of ligand binding to the estrogen receptor. Both isoforms of the estrogen receptor, alpha and beta were utilized in the presence of the positive control compound, diethylstilbestrol (DES), and the test material, D4.

## INTRODUCTION

Due to the importance of estrogens in reproductive systems, there is considerable interest in exogenous compounds that may mimic the action of estradiol by binding to the endogenous receptors. There are two known receptors that bind estradiol (ER-alpha and ER-beta). They share nearly perfect homology in the DNA binding and dimerization regions, showing differences in the ligand binding region. Thus ligands may show preferential binding for one of the two receptors. The two receptors show distinct but overlapping tissue distribution<sup>1</sup>. It is well established that binding of estradiol to either ER results in a conformational change in the hormone binding domain. Activation of the receptor by ligand binding initially results in an increase in overall phosphorylation<sup>2</sup>. This is followed by dimerization of the receptor, which results in an increase in transcription of ER target genes.

The mechanism by which compounds regulate gene transcription is particularly complex in the case of the estrogen receptors. Generally, site-specific transcription factors, such as steroid receptors stimulate gene expression by promoting the assembly of basal transcription factors into a stable pre-initiation complex that facilitates an increased rate of transcription by RNA polymerase II<sup>3</sup>. Additional proteins, termed coactivators, are usually involved that act as bridging factors between specific activators and general transcription factors. Within any one cell type there will be differences in the type of transcriptional factors and coactivators present as well as differences in the promoters preceding estrogen regulated genes. The literature abounds with examples where compounds have different effects within different cell systems or animal models. Additionally, activation of the receptors can be achieved *via* the hormone-dependent or the hormone-independent mechanism. The hormone-independent pathway involves activation of the receptor by growth factors *via* their membrane bound receptors and activating the MAP kinase cascades. This pathway of ligand-independent activation of the ER is not clearly understood, however a growing amount of literature in the last 5 years clearly support such a mechanism<sup>4</sup>.

Estradiol has been shown to both up- and down-regulate ER-alpha in rodent uterine tissues (stromal and epithelial cells). ER-alpha is felt to have a major role in the early events associated with estradiol induced cell proliferation in the uterus<sup>5</sup>. The role of ER-beta is less clearly defined. It is thought that the beta receptor has some repressor activity associated with its action. It is expressed in the uterus and ovary, however its transcriptional activation and expression patterns are not clearly understood<sup>6,7</sup>.

A variety of functionally diverse compounds have been shown to bind to the estrogen receptor with a range of affinities and have been able to activate transcription via the estrogen response element (ERE) to differing degrees<sup>8</sup>. In spite of all that is not understood about the ligand-receptor binding interactions, it is accepted that initial binding is a requirement to biological activity. Thus *in vitro* measurement of ligand binding is of value in determining if a compound may potentially affect estrogen regulated processes.

The evaluation of the siloxane, octamethylcyclotetrasiloxane (D4) in this aqueous *in vitro* system, presented some technical problems due to the very low water solubility and moderate volatility of the material. It has been reported that the addition of pure siloxanes to aqueous medium is not useful as the material would immediately separate and prevent any interaction between the test article and the receptor<sup>9</sup>. In spite of this, previous studies have found ways to use this material in an aqueous cell culture assay successfully. (Dow Corning Report Numbers 1999-10000-47034 and 2000-10000-48477)<sup>10, 11</sup>. Initial experiments here utilized a delivery system of D4 that resulted in variable data even though efforts were made to minimize headspace during the incubations, and to introduce test material in a manner that reduces loss. A superior method was then tried that involved saturating the headspace over the aqueous reaction mixture with a 900-ppm atmosphere of D4. Over time the D4 partitioned into the aqueous reaction mixture based on its partitioning coefficient between air and the aqueous protein mixture. It was anticipated that the D4 would partition into the reaction mixture in a manner that would allow molecular D4 to interact with the estrogen receptor. Under these conditions, binding would be dictated by the binding affinity rather than by D4 availability in the aqueous mixture.

## MATERIALS AND METHODS

### Reagents

[2,4,6,7,16,17-<sup>3</sup>H]-estradiol (S.A. 118 Ci/mole, lot # 3363296) was purchased from DuPont NEN (Boston, MA.), Non-radioactive estradiol and diethylstilbestrol (DES) were purchased from Sigma Chemical Co, St. Louis, MO. Unlabeled D4 (octamethylcyclotetrasiloxane), lot # LL024510 (exp. 4 March, 2004) was provided by DCC. The <sup>14</sup>C-D4 was prepared by Wizard Laboratory. The 900-ppm D4 vapor was prepared and used within 1 hour of preparation. The positive control articles were prepared in ethanol and stored at ~4°C for up to 2 weeks. The final ethanol concentration for all incubations never exceeded 0.5% of the total volume.

### Test System and Procedure

Estrogen receptors alpha and beta were purchased from PanVera Corporation, (Madison WI). These were human purified proteins, recombinantly expressed from baculovirus-infected insect cells. They were provided by the manufacturer at varying concentrations and were used in assays following dilution in binding buffer. The reaction mixtures contained either one of the estrogen receptors, a known amount of radiolabeled estradiol and a range of competitor concentration. The reaction proceeded to equilibrium based on reported incubation times. The bound ligand-receptor complex was separated from the unbound radioligand using the HAP assay described below.

The binding buffer used throughout the study contained the following: 10 mM Tris, pH 7.5; 10% glycerol; 2 mM dithiothreitol and 1 mg/ml of BSA. Isolation of the bound component was carried out using the HAP protocol, recommended by the PanVera Corporation. The HAP isolation procedure is as follows: a 50% hydroxylapatite slurry was equilibrated with 50 mM Tris-Cl, pH 7.4; 1 mM EDTA. An equal volume of the HAP slurry was added to the equilibrated incubation mixture and placed on ice with intermittent mixing. Samples were washed using ER- $\alpha$  and  $\beta$  wash buffers which contained the following: ER- $\alpha$  wash: 40 mM Tris, PH 7.5; 100 mM KCl; 1 mM EDTA; 1 mM EGTA and ER- $\beta$  wash: 40 mM Tris, pH 7.5. A volume of 10 X wash buffer was added, the sample was vortexed, centrifuged for 5 minutes at 10,000 g and the supernatant discarded. The resultant pellet was washed 3 more times. The pellet was

resuspended in 2 volumes of ethanol and transferred to a liquid scintillation vial containing Ultima Gold Scintillation cocktail.

### GC/MS Analysis

The concentrations of octamethylcyclotetrasiloxane (D<sub>4</sub>) in solution in aliquots of the incubation samples were determined by extraction into toluene and subsequent gas chromatography-mass spectrometry (GC-MS) analysis. An aliquot (approximately 800µl) of each incubation sample was transferred to a vial containing approximately 1ml of toluene containing an internal standard solution consisting of 150 ng of tetrakis[trimethylsiloxy]silane (M<sub>4</sub>Q) / g of toluene. The samples were vortexed / mixed for at least 4 minutes using moderate vortexing, followed by separation of the organic layers from the aqueous layers via centrifugation at 3000g for at least 4 minutes (Beckman GS-6R). Emulsions formed after this process in 5 of the 6 samples. In order to facilitate separation of these emulsions, approximately 250 mg of sodium chloride (NaCl) was added to each of the samples with emulsions, followed by vortexing briefly and centrifuging for approximately 30 minutes at 3000g. The toluene extract layers were then removed to 2 mL round bottom glass vials containing approximately 250 mg of magnesium sulfate in order to dry the samples. Aliquots of each of the dried extracts were taken following a minimum of 1hr of drying time and transferred to limited volume inserts in GC autosampler vials for analysis by GC-MS.

Quantitation of the D<sub>4</sub> in the extracts was performed by comparison to toluene solvents standards containing M<sub>4</sub>Q added in the same volume as for the samples and D<sub>4</sub> in various amounts from approximately 10 to 18000 ng. Linear response plots were prepared from the results of the analyses of these toluene standards, and amounts of D<sub>4</sub> in the incubation samples were calculated based on comparison to these linear response plots. The ng amounts of D<sub>4</sub> calculated in this manner were converted to concentrations (ng/mL of incubation sample) based on the known dilution volumes of the samples. The GC-MS conditions utilized for the analyses can be found in Table A9.

## **EXPERIMENTAL DESIGN**

### Competition Experiments:

For competition experiments, a typical incubation mixture consisted of a constant <sup>3</sup>H-estradiol concentration (3.4 nM), with increasing amounts of unlabeled competitor, and a constant amount of estrogen receptor (0.2 nM). A separate non-specific binding tube containing a 1000-fold excess of cold estradiol was used as a competitor. The amount of specific binding was calculated as total dpm minus the dpm of the non-specific binding. This was then expressed as % estradiol binding with 100% binding being equal to the maximum <sup>3</sup>H-estradiol bound in the absence of any competitor. The incubations were carried out using crimped vials with very gentle inversion. The vials were incubated at 37°C for 3 hours. In these experiment, the DES utilized only 4 concentrations due to technical constraints, thus making IC<sub>50</sub> calculations impossible. For the D<sub>4</sub>, only the 900ppm concentration was tested. Therefore, the data is expressed only as the % of estradiol remaining and the trends observed for DES.

Delivery of D<sub>4</sub> as a Vapor: A previous method development study (Dow Corning Report # 2000-10000-49694) defined a method by which D<sub>4</sub> was delivered to a liver microsomal system via saturation of the headspace. D<sub>4</sub> was injected into a Tedlar bag containing 8L of N<sub>2</sub> so as to attain a 900ppm D<sub>4</sub> concentration. The volume of air was delivered through a calibrated dry test meter. To achieve the 900ppm concentration, 91 µl of D<sub>4</sub> was added to the bag using a

Hamilton syringe. After the test article was injected, the bag was gently heated using a heat gun to ensure complete vaporization of the test article. It was calculated that flushing the headspace above the reaction mixture with 10X volumes would replace the ambient air with approximately 99.9% test article as a 900ppm vapor. The reaction was incubated at 37°C with gentle inversion, for 3 hours.

## RESULTS

This study began with a significant method development component. The results for the method development part of the study are presented and discussed in Appendix A. The following are the results with the optimized method for competition binding experiments using D4 as a 900ppm vapor.

### Tracking of D4 in the Reaction Mixture:

D4 was measured in the reaction mixture using two separate methods; GC/MS analysis of the assay mixture and LSC analysis using labeled  $^{14}\text{C}$ -D4. GC/MS analysis of the samples taken at the end of the 3 hour incubation indicated that the concentration of D4 that had partitioned into the aqueous mixture was approximately 0.49  $\mu\text{M}$ . This is compared with the radiolabeled method, which calculated that approximately 0.42  $\mu\text{M}$  D4 was present in the aqueous mixture (Table 3). The addition of 0.2 mg/ml uterine lysate increased the amount of D4 partitioning from the headspace to the aqueous layer. By GC/MS, the approximate concentration of D4 was 1.6  $\mu\text{M}$ , reflecting a 3.3 fold increase in the amount of D4. By the radiolabeled tracking, it was calculated that 2.62  $\mu\text{M}$  D4 was present. One factor that may account for the difference in the values for the GC/MS and the  $^{14}\text{C}$ -D4 values is that the uterine lysate was from two separate animals. Thus the protein composition may be slightly different and affect the binding of D4. Both methods of quantitation of D4 in the reaction mixture indicate that delivery of test article via a vapor route is effective in delivering a consistent level of molecular D4. Additionally, as D4 loss was not a concern the reaction was carried out at 37°C; reducing equilibration times and decreasing stability and aggregation issues associated with the receptor.

### Addition of Uterine lysate to the Assay:

The addition of uterine lysate into the reaction mixture resulted in several notable effects. Overall, the additional protein enhanced the ability of DES to effectively displace  $^3\text{H}$ -estradiol in the reaction medium for both the alpha and beta receptor. At 500 $\mu\text{M}$ , DES was able to displace ~100% of the bound estradiol compared to ~80% displaced when the uterine lysate was not added. The additional protein in the assay mixture also resulted in a higher concentration of D4 in the aqueous reaction mixture. However, this was not associated with an increase in the receptor binding measured in this assay.

### Estrogen Receptor Binding:

DES: Four concentrations of DES were used as the positive control samples. The DES was tested at 1nM, 10nM, 100nM and 500nM. In the presence of increasing DES, there was the expected concentration dependent displacement of the  $^3\text{H}$ -estradiol. At the highest dose used, 500 nM, the displacement was approaching 100% (Table 1 and 2 and Figure 1 and 2).

D4: When 900 ppm D4 vapor was incubated with the ER-alpha, the percent of  $^3\text{H}$ -estradiol displaced was 17.1%. There was not a significant displacement of  $^3\text{H}$ -estradiol from the beta receptor in the presence of D4 (Table 1 and 2 and Figure 1 and 2).

## DISCUSSION

The data generated from this study indicate that D4 has the potential to bind to the estrogen receptor alpha. Using the 900 ppm vapor as the source of test material, D4 partitions into the reaction mixture in the molecular form and can weakly compete for the receptor. Under these test conditions, approximately 20% of the bound <sup>3</sup>H-estradiol was displaced by the D4. The D4 did not significantly compete with <sup>3</sup>H-estradiol for the ER beta in this system. The addition of the uterine lysate resulted in some noteworthy observations. Overall, the ability of DES to compete for both the ER-alpha and ER-beta was improved without altering the non-specific binding component for the reaction. The uterine lysate itself had no appreciable binding in the absence of the human purified receptor. The likely affect of adding the uterine lysate was to provide proteins that may be present in an *in vivo* system. The natural state of the receptors involves a variety of ancillary proteins, providing structural stability as well as function. It is not clear specifically, what the uterine lysate is contributing to the overall binding scenario, however the positive control compound, DES showed more favorable binding kinetics in the presence of the uterine protein. This suggests that a more complex binding environment may be required to fully characterize receptor-ligand interactions. The uterine lysate did not alter the affect of the D4 binding to either receptor at the concentration of D4 used in this study. The GC/MS analysis and radiolabeled tracking data indicate that D4 was partitioning into the aqueous reaction mixture, and the presence of uterine lysate enhanced this by 3-6 fold depending upon the method of quantitation. However, there was no increased displacement of estradiol in the presence of more D4. It is likely that although more D4 partitions into the aqueous mixture and is retained over the course of the reaction incubation, the D4 is tightly bound to the extra proteins and is not bioavailable. The concentration of D4 that resulted in the displacement of the <sup>3</sup>H-estradiol cannot be determined by these experiments and may need to be theoretically calculated using the partitioning coefficients of the test material. This type of analysis highlights the importance of the technical aspects of study design, when working with difficult materials, such as D4.

## CONCLUSIONS

This study was conducted to determine if D4 had the potential to bind to the estrogen receptors, alpha and beta. Under the conditions used in this *in vitro* assay system, D4 was able to weakly compete with estradiol for the ER-alpha only.

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Table 1  
Summary of Receptor Binding: ER-alpha

Compound	% of <sup>3</sup> H-Estradiol Bound				SD
	Run 1	Run 2	Run 3	Mean	
DES-0 nM	100	100	100	100	
DES-1 nM	31.1	28.2	60.7	40	18.0
DES-10 nM	18.9	21.7	54.3	31.6	19.7
DES-100 nM	6.1	7.6	12	8.6	3.1
DES-500 nM	0.8	-6.7	2.4	-1.2	4.9
D4-900 ppm	76.5	77.3	94.9	82.9	10.4

Table 2  
Summary of Receptor Binding: ER-beta

Compound	% of <sup>3</sup> H-Estradiol Bound			Mean	SD
	Run 1	Run 2	Run 3		
DES-0nM	100	100	100	100	
DES-1 nM	49.8	57.3	69.9	59.0	10.2
DES-10 nM	32.6	38.6	50.6	40.6	9.2
DES-100 nM	5.8	21.2	3.2	10.1	9.7
DES-500 nM	-3.4	3.8	1.4	0.6	3.7
D4-900 ppm	101.4	112.8	93.5	102.6	9.7

Table 3

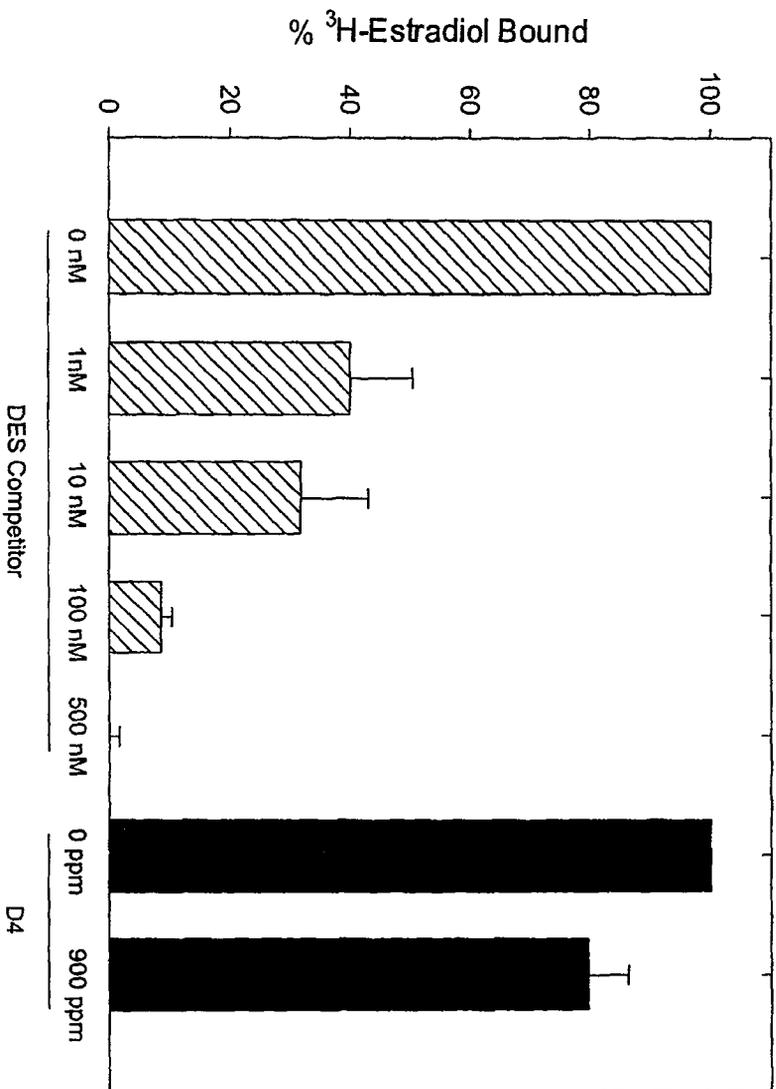
GC/MS Analysis of D4 in the Reaction Mixture at 3 Hours				
Sample	ng D4/ml	nmoles/L	µM	Mean
900 ppm D4-1	115.76	526.21	0.53	
900 ppm D4-2	11.79	39.83	0.04	
900 ppm D4-3	135.95	459.28	0.46	0.49 <sup>A</sup>
900 ppm D4 w/lysate-1	465.28	1571.9	1.57	
900 ppm D4 w/lysate-2	574.32	1940.27	1.94	
900 ppm D4 w/lysate-3	382.23	1291.32	1.29	1.60
Radiolabeled <sup>14</sup> C-D4 in the Reaction Mixture				
Sample	Mean dpm	dpm/ml	µM	µM at 3 Hours
0 Minute	1403.5	1754.4	0.04	
15 Minute	23006.5	28758.1	0.66	
30 Minute	19035.0	23793.8	0.55	
1 Hour	13129.3	16411.6	0.38	
2 Hour	16269.0	20336.3	0.47	
3 Hour	14534.7	18168.4	0.42	0.42
0 Minute w/lysate	5123.0	6403.8	0.15	
15 Minute w/lysate	68508.0	85635.0	1.97	
30 Minute w/lysate	66857.0	83571.3	1.92	
1 Hour w/lysate	83859.3	104824.1	2.41	
2 Hour w/lysate	73121.3	91401.6	2.10	
3 Hour w/lysate	91207.0	114008.8	2.62	2.62

<sup>A</sup> For the GC/MS data without lysate: The middle value was not included in the calculation of the mean.

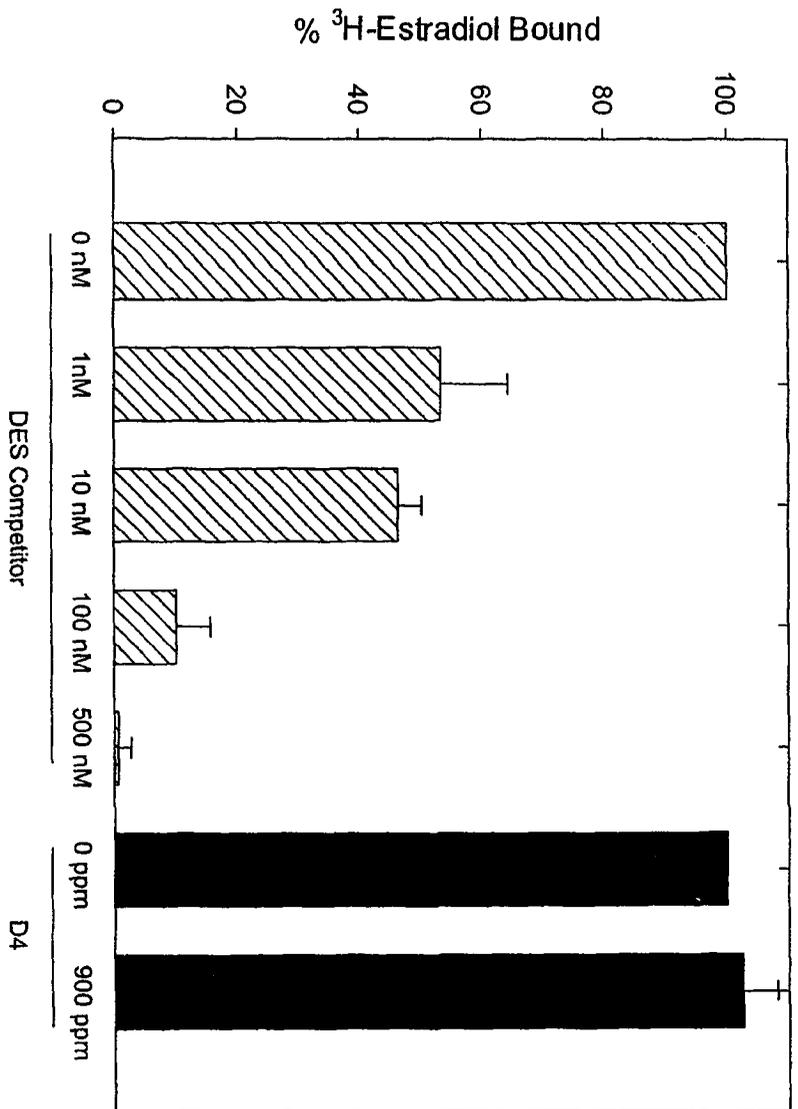
Figure 1

Receptor Binding Data : ER-Alpha

Competition Experiment in the presence of 0.2 mg uterine lysate/ml



**Figure 2**  
**Receptor Binding Data : ER-Beta**  
Competition Experiment in the presence of 0.2 mg uterine lysate/ml



## Appendix A METHOD DEVELOPMENT

### METHODS

In order to evaluate D4 binding to the estrogen receptor, multiple parameters were evaluated: type of vial used, temperature of the incubation, concentration of receptor and ligand, the presence of BSA and most critical the method of delivering D4 to the test system. All of the parameters that were evaluated are discussed below

Vial type: It was evident from the very first experiments that although the positive control compounds could be incubated in small test tubes, the D4 material would experience extensive loss. This was initially considered because the cell culture system did not restrict head space and was successful as an *in vitro* method. The use of small test tubes was more convenient in the post incubation work up but was unfortunately not suitable for this type of material. Initially, the 2ml crimped vials were filled completely so as to restrict headspace for the D4. As the method of D4 delivery was changed from delivery as a neat liquid to delivery as a vapor, the reaction volume was reduced to 1ml so as to have a 1ml headspace. In either case, regardless of the vial/tube used, the data for the positive control materials was not affected.

Isolation of Bound Component: The majority of the data collected for the positive control compounds was carried out using the Brandel Cell Harvester. As the experiments progressed and the difficulty with using neat D4 as a liquid became apparent, the method was switched back to the hydroxylapatite procedure, as this was the procedure recommended by the manufactures of the receptor. Although more labor intensive, the HAP assay was used so that we could rule out the Brandel Harvester as an additional source of variability. The Brandel Harvester is a very convenient method to isolate the bound component, however for the series of experiments involving D4, it was determined that the more conventional HAP was the preferred method of isolation. Briefly, the method is as follows. Isolation of the bound component was carried out using the Brandel Cell Harvester system. A strip of Whatman paper was placed over the tubing outlets and the tubing apparatus was locked into position. A vacuum was applied and the reaction mixtures were aspirated through the tubing onto the filter paper. All excess fluid passes through the filter paper and is collected in the side-arm flask. Radiolabeled ligand that is bound to the receptor is retained on the filter paper. The reaction tubes and filter paper are then washed 3 times using an equal volume of wash buffer. Following washing, the vacuum was removed and individual filter blots were placed in 7 ml LSC vials containing 6 ml of Ultima Gold scintillation cocktail. The data obtained for the positive control material was similar using either method of isolation.

Incubation temperature: Initial experiments were run at room temperature. Reports had indicated that D4 was successful in binding to the receptor when the incubation was carried out at 37°C<sup>12</sup>. In order to determine an optimal temperature, a comparison of room temperature versus 37°C was carried out. The positive control, DES resulted in equal displacement at both temperatures. The advantage to using 37°C was that equilibrium times were reduced at higher temperatures. However, for D4 higher temperatures would result in increased material loss from the reaction assay into the headspace. The optimal temperature for D4 delivered as a liquid was subsequently determined to be 18°C throughout the initial experiments. The rationale for this was to minimize loss of material and to slow down the off-rate of the compound from the receptor. The overall kinetic parameter Kd is a combination of the on and off rate of the

material. Generally, incubations would be carried out at 4°C in order to slow down the off-rate so as to maximize binding. Due to the high freezing point of D4 (17.5°C) the incubations were carried out at 18°C, minimizing loss and slowing down the off-rate as much as possible without freezing the test article. The choice of the incubation temperature was made to be consistent with the majority of the methods used in the Additionally, it was decided that the samples would be incubated with constant rotation. Samples were placed on a rotating apparatus at 18°C overnight. Constant motion should enhance mixing of the test article and possibly promote binding. Once the decision was made to use D4 as a vapor, the temperature was increased to 37°C, as this would reduce the time to equilibrate, reduce concerns with stability and aggregation of the receptor and enhance partitioning of the material into the aqueous reaction mixture.

Concentration of Radioligand and Receptor: Several experiments were carried out to determine the optimal ligand to receptor ratio. In determining this parameter, the specific activity of the radioligand needs to be considered. For relative binding experiments, it is typical to choose a radioligand concentration that is around the  $K_d$  of the compound. Preliminary experiments indicated that the  $K_d$  for estradiol is around 1-2nM. Based on the counts that were being obtained for the positive controls, a value of 3.4nM estradiol was used. This would allow for measurement of binding even at the very high levels of displacement. The concentration of the receptor is ideally set out to be 10 times lower than the ligand concentration. However, at these low receptor concentration, the amount of radiolabeled ligand bound is too low to measure accurately. Experiments evaluated the effect of lowering the ER concentration to 10 times the E2 concentration and lowering the [E2] to 1 nM. Under these conditions, the counts were barely detectable over the non-specific binding counts. The experiments presented in the main body of the report used ER concentrations that were 3-4 times lower than the ligand concentration. It was determined experimentally that it is not useful to decrease this ratio under these conditions. Due to this fact, the  $IC_{50}$  values calculated may be slightly higher than those found in the literature, however the ability of a compound to compete can be unequivocally determined.

Bovine Serum Albumin (BSA): The role of BSA was examined in the incubation mixture. Based on the hydrophobic composition of BSA, it is likely D4 binds albumin, probably with significant affinity. The presence of this protein in the binding buffer may limit the amount of free molecular D4 available to bind to the ER. Experiments were run that omitted BSA from the reaction mixture. Under these experimental conditions, there was no apparent binding of any of the positive control compounds for both ER-alpha and beta. According to the manufacturer, the BSA is required to prevent aggregation of the receptor when it is diluted prior to use. Therefore, it is not surprising that under these conditions the DES and estrone showed no binding. Overall the counts were very low compared to comparable experiments, so that even in the absence of competitor, the E2 binding was at a reduced level. This would be consistent with the receptor aggregation. All further experiments were carried out in the presence of 1mg/ml BSA.

Pre-incubation of the receptor: An experiment was setup to evaluate the order of adding compounds to the incubation mixture. This experiment had two parts. In the first part estradiol was preincubated with the receptor to achieve equilibrium prior to the addition of D4. In the second part both E2 and D4 were added simultaneously. The D4 range, ER and ligand concentrations were the same as previously optimized. There was no apparent difference in the magnitude of competition when the material was added directly with the estradiol or whether the estradiol was pre-incubated. The binding of any compound is measured after the system has

reached a steady state equilibrium. Estradiol alone reaches equilibrium by 30 minutes of incubation at 37°C. As the estradiol binding is reversible, it is expected that the presence of the D4 would still be able to compete even if the estradiol component had reached equilibrium. As the overall incubation was allowed to interact for at least 12-15 hours, it is assumed that the entire system had reached equilibrium. All data reported in the main body were with the estradiol and D4 added at the beginning of the incubation.

Delivery of D4 as a Liquid: The problems associated with using D4 in an aqueous based in vitro system necessitated special attention for the preparation of stock solutions. The optimized procedure was as follows: The neat D4 was injected into a 2ml crimped vial already containing buffer so that the final volume was 2ml. The vial was vortexed vigorously for 2 minutes in order to form a homogenous microsuspension of the D4 material. For each sample dosed, the appropriate volume of material was removed using a Hamilton syringe and added to the crimped 2ml vial containing the incubation mixture. Under these circumstances, headspace was minimized in an effort to reduce loss. As material was removed from the stock vial, it is likely that increasing amounts of D4 would be lost in the headspace with each subsequent removal. In order to minimize this loss the dosing was carried out as quickly as possible.

The experiments that utilized D4 delivered as a liquid were inconsistent due to the nature of the test material. The delivery of D4 as a liquid was found not to be a viable means of determining receptor binding under these conditions, even after special care was taken to reduce loss.

Tracking of D4 in the Aqueous Reaction Mixture: In order to determine the amount of D4 that partitioned from the 900ppm headspace into the aqueous reaction mixture, a time course was setup. Reactions were setup that mimicked the binding assays, however the D4 used was <sup>14</sup>C-D4 labeled. Separate reactions were setup for the following time points, 0 hour, 15 minutes, 30 minutes, 1 hour, 2 hour and 3 hour. Samples containing 0.2 mg/ml uterine lysate were also prepared to evaluate the benefit of adding extra protein to the mixture. At the appropriate time an 800µl aliquot was removed from each of the vials and counted by liquid scintillation.

Uterine Lysate: The addition of uterine protein to the reaction mixture was undertaken after it appeared that additional protein enhanced the partitioning of D4 into the aqueous mixture. Uterine lysates previously prepared for another method development study (Study Number 9511) were provided frozen for use in this study. Briefly, uteri were crushed in liquid N<sub>2</sub>, and resuspended in 5X volume of lysis buffer. The lysis buffer is prepared as follows: 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM sodium vanadate, 0.2 mM PMSF, 1mM Hepes, pH 7.6, 2 µg/ml leupeptin and 2 µg/ml aprotinin. Lysates were stored at -20°C until used. BCA protein analysis was carried out to determine protein concentration.

Saturation Binding Experiments: In order to determine K<sub>d</sub> values for the endogenous ligand, saturation binding experiments were performed. A typical reaction would contain ER alpha or beta, binding buffer and an increasing amount of radiolabeled estradiol. A separate non-specific binding tube containing a 1000 fold excess of cold estradiol was used for each concentration of estradiol. Incubations were carried out for 3 hours at room temperature. The specific binding was calculated as the total dpm minus the non-specific binding component. Non-linear regression analysis (Sigma Plot version 5.0, hyperbola, single rectangular) was used to generate plots and determine K<sub>d</sub> values.

## RESULTS AND DISCUSSIONS

### Saturation Binding:

In order to ensure that the test system was responding appropriately, saturation binding experiments were carried out for both the alpha and beta receptor. Using a range of radiolabeled estradiol (5 pM-10 nM), specific binding curves were generated (Figure A1 and A2). The K<sub>d</sub> values (the concentration of estradiol that equaled ½ maximum binding) were calculated using non-linear regression analysis. The HAP isolation procedure resulted in a K<sub>d</sub> value for ER beta of 1.7 nM. The ER alpha was not carried out using the HAP procedure. The Cell Harvester resulted in a K<sub>d</sub> value of 2.7 nM for ER beta and 1.6 nM for ER alpha. These two methods of isolation resulted in comparable K<sub>d</sub> values and these values were consistent with the range (0.1-5 nM) generally reported for estradiol binding to ER's in various systems.

### Relative Binding Assays:

Diethylstilbestrol (DES), estradiol (E<sub>2</sub>) and estrone (E<sub>1</sub>) were utilized as positive controls for these experiments.

*Diethylstilbestrol:* DES exhibited a typical competition curve using ER alpha with a dose range of 5 pM-100 nM. Initial experiments utilized ER alpha at a 1:100 fold dilution and an <sup>3</sup>H-estradiol concentration of 3.5 nM. Under these conditions, DES resulted in an IC<sub>50</sub> of 9.65 nM. (Figure A3 and Table A7). DES effectively displaced estradiol with ER beta however an IC<sub>50</sub> was not calculated due to the limited number of doses used. The relative binding affinity for ER alpha was 89%. However, it appears that the addition of uterine lysate resulted in more favorable kinetics for the DES and it is likely that had this experiment been repeated using uterine lysate, the IC<sub>50</sub> would be lower and closer to literature reported values.

*Estrone:* E<sub>1</sub> exhibited typical competition curves using ER alpha and beta with a dose range of 50 pM-2.0 μM. The beta receptor exhibited the typical sigmoidal curve with the alpha receptor exhibiting a shallower slope. (Figure A4 and A5). The IC<sub>50</sub> calculated for ER alpha was 12.2 nM and 30.1 nM for ER beta (Table A7). The RBA for estrone was calculated for ER alpha to be 69.7%.

*Estradiol:* E<sub>2</sub> was used as a positive control for relative binding affinity calculations. ER alpha was used and the expected displacement of the radioligand was observed. Estradiol has equal affinity for the two receptor subtypes, so the IC<sub>50</sub> value obtained for ER alpha was used for the beta receptor calculations. The IC<sub>50</sub> calculated for ER alpha was 8.5 nM (Figure A6). All of the RBA values are summarized in Table A7.

**Table A1**

Saturation Binding Assay: [ER-alpha] = 1:100 dilution			
[Estradiol]	Total dpm (n = 2)	Non-Specific Binding	Specific Binding (corrected for background)
5.0pM	216.0	2.0	214.0
10.0pM	234.5	4.0	230.5
50.0pM	950.0	28.0	922.0
100.0pM	1653.5	46.0	1607.5
500.0pM	8253.0	277.0	7976.0
1.0nM	17086.5	667.0	16419.5
5.0nM	38774.5	2768.0	36006.5
10.0nM	43230.0	5662.0	37568.0

Table A2

Saturation Binding Assay: [ER-beta] = 1:100 dilution			
[Estradiol]	Total dpm (n = 2)	Non-Specific Binding	Specific Binding (corrected for background)
5.0pM	54.0	9.0	45.0
10.0pM	113.0	64.0	49.0
50.0pM	552.5	47.0	505.5
100.0pM	1061.0	68.0	993.0
500.0pM	4801.0	349.0	4452.0
1.0nM	6860.5	471.0	6389.5
5.0nM	16882.5	2567.0	14315.5
10.0nM	19999.0	4387.0	15612.0

**Table A3**

Relative Binding Assay (RBA) Diethylstilbestrol binding to ER-alpha				
[DES]	Mean dpm n = 2	Non-Specific Binding	Specific Binding	% E2 Binding
0.0 pM	30139.5	3735.0	26404.5	100.0
5.0 pM	29480.5	2534.0	26946.5	102.1
10.0 pM	32217.5	2412.0	29805.5	112.9
50.0 pM	33131.5	2594.0	30537.5	115.7
100 pM	31499.0	2944.0	28555.0	108.1
500 pM	30197.5	2478.0	27719.5	105.0
1 nM	27702.0	3041.0	24661.0	93.4
5 nM	20283.0	2602.0	17681.0	67.0
10 nM	15167.0	2607.0	12560.0	47.6
50 nM	8870.0	2956.0	5914.0	22.4
100 nM	4845.5	3168.0	1677.5	6.4

**Table A4**

Relative Binding Assay (RBA) Estrone binding to ER-alpha			
[estrone]	Experiment 1	Experiment 2	Average % Estradiol Binding
0.0 pM	100.00	100.00	100.00
50 pM	78.34	72.88	75.61
100 pM	97.37	68.57	82.97
500 pM	87.06	65.90	76.48
1.0 nM	83.85	51.39	67.62
5.0 nM	51.93	45.26	48.60
10 nM	46.59	69.42	58.00
50 nM	40.01	45.43	42.72
100 nM	26.82	31.26	29.04
500 nM	8.07	8.38	8.23
1.0 µM	4.45	4.78	4.61
2.0 µM	2.92	2.77	2.85

**Table A5**

Relative Binding Assay (RBA) Estrone binding to ER-beta			
[estrone]	Experiment 1	Experiment 2	Average % Estradiol Binding
0.0 pM	100.00	100.00	100.00
50 pM	95.68	Sample lost	95.68
100 pM	93.39	102.98	98.18
500 pM	99.74	92.81	96.27
1.0 nM	99.43	110.90	105.17
5.0 nM	68.76	102.77	85.76
10 nM	32.75	87.21	59.98
50 nM	24.08	60.92	42.50
100 nM	24.97	36.56	30.77
500 nM	5.22	20.78	13.00
1.0 µM	8.95	9.75	9.35
2.0 µM	1.21	6.62	3.92

**Table A6**

Relative Binding Assay (RBA)				
Estradiol binding to ER-alpha				
[Estradiol]	dpm	Non-Specific Binding	Specific Binding	% E2 Binding
0 pM	38042.0	2560	35481.6	100.0
1 nM	35768.0	2560	33207.6	93.6
5 nM	29919.0	2560	27358.6	77.1
10 nM	17398.0	2560	14837.6	41.8
100 nM	7318.0	2560	4757.6	13.4
200 nM	5632.0	2560	3071.6	8.7

**Table A7**

Kinetic Parameters of Estrogen Receptor Binding			
Measurement of Kd <sup>1</sup>	HAP Method	Cell Collector Method	
ER-alpha	N/T	1.6 nM	
ER-Beta	1.7 nM	2.7 nM	
<b>Measurement of RBA<sup>3</sup></b>			
	ER-alpha	ER-beta	
	IC <sub>50</sub> <sup>2</sup>		
Estradiol	8.5 nM	100	100
DES	9.6 nM	89	N/T
Estrone-alpha	12.2 nM	69.7	N/A
Estrone-beta	30.1 nM	N/A	28.1

<sup>1</sup> Kd was calculated as the concentration of ligand required to provide 1/2 maximal binding using non-linear regression (hyperbolic; singular rectangular; Sigma plot version 5.0)

<sup>2</sup> IC<sub>50</sub> was calculated as the concentration of competitor required to displace 50% of the radiolabelled estradiol using non-linear regression (sigmoidal Hill plot; 4th parameter; Sigma Plot version 5.0)

<sup>3</sup> RBA was calculated as the ratio of IC<sub>50</sub> of E<sub>2</sub>/IC<sub>50</sub> of test compound

N/A = non applicable; N/T = samples were not tested under these conditions

Table A8

GC-MS Instrument Parameters	
Sample Introduction and Separation:	
Instrument:	HP 7673 Automatic Liquid Sampler and HP 6890 Gas Chromatograph (Agilent Technologies; Palo Alto, CA)
Inlet:	Splitsless, 250°C
Column Head Pressure:	8.8 psi initially, constant flow
Inlet Purge Time:	0.15 min
Injection:	1 µL
Column:	HP-5MS; 30 m x 0.25 mm I.d., 0.25 µm film (Agilent Technologies; Palo Alto, CA)
Column Oven:	70°C for 2.0 min 20°C/min to 190°C, no hold 50°C/min to 230°C, no hold
Run Time:	8.8 min
<b>Detection:</b>	
Instrument:	HP 5973 Mass Selective Detector (Agilent Technologies; Palo Alto, CA)
Acquisition:	Selected Ion Monitoring (SIM)
Quantitation Ions:	D <sub>1</sub> : m/z 281, dwell 100 M <sub>1</sub> Q: m/z 281, dwell 100
Retention Times:	D <sub>1</sub> : ~4.3 min M <sub>1</sub> Q: ~5.8 min
Heated Transfer Line:	280°C
Solvent Delay:	3.8 min
Tune:	Autotune
Electron Multiplier Voltage	Value obtained from autotune (~1700 V)

Figure 1A  
Saturation Binding Analysis  
ER-Alpha

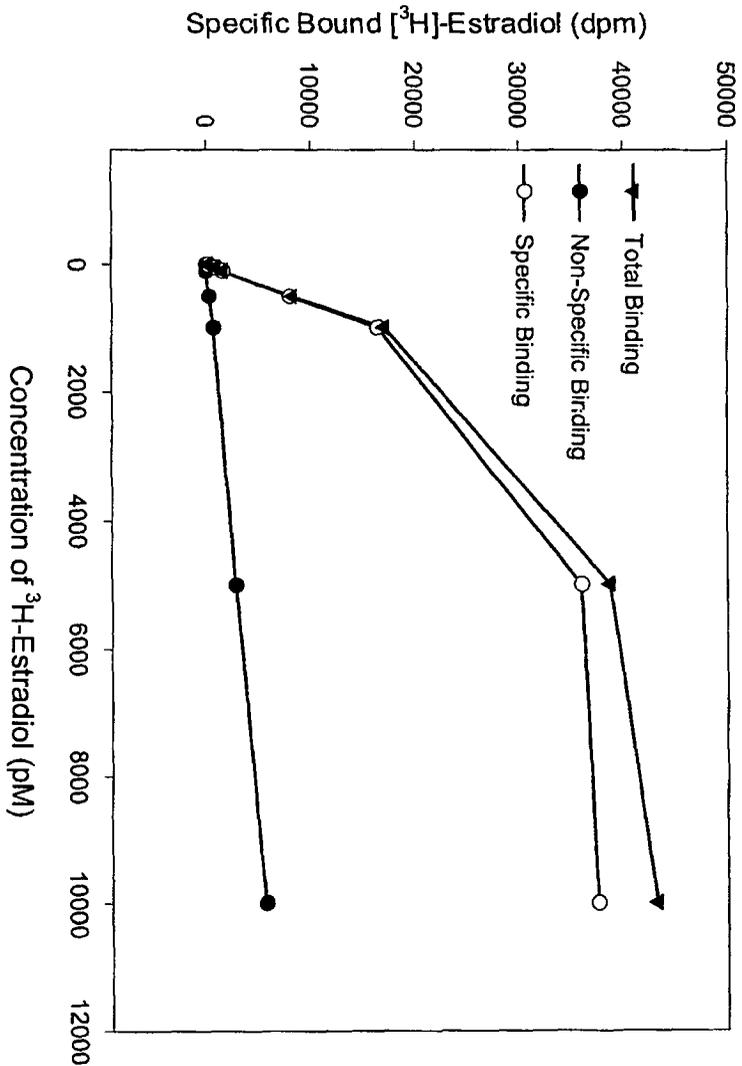
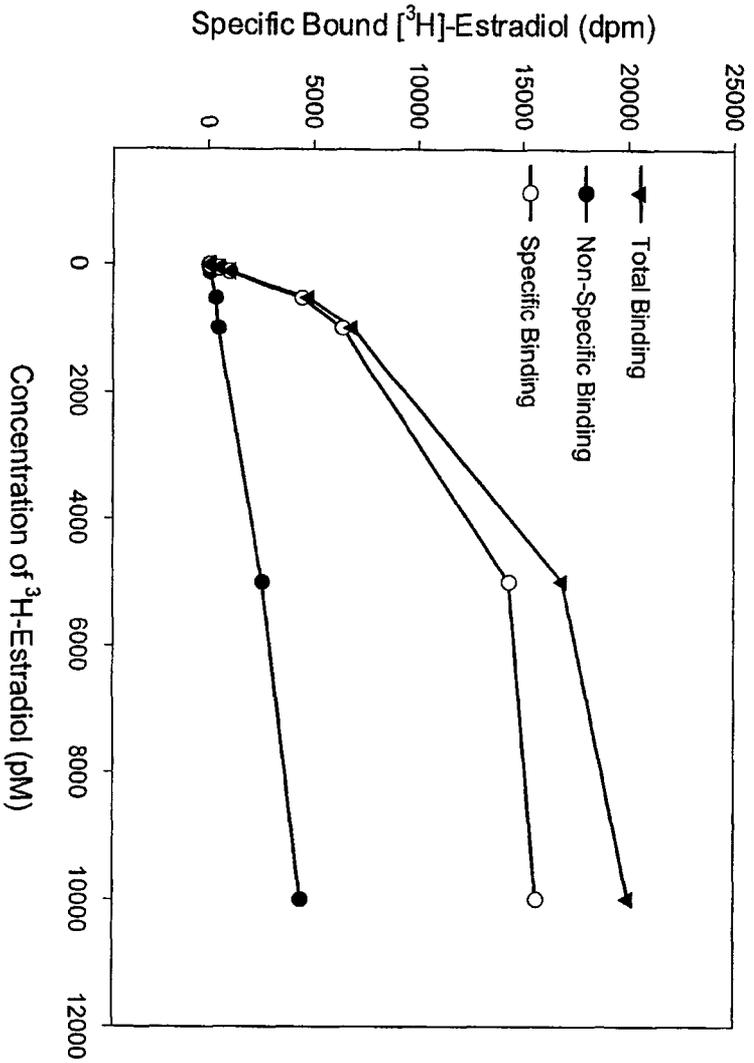
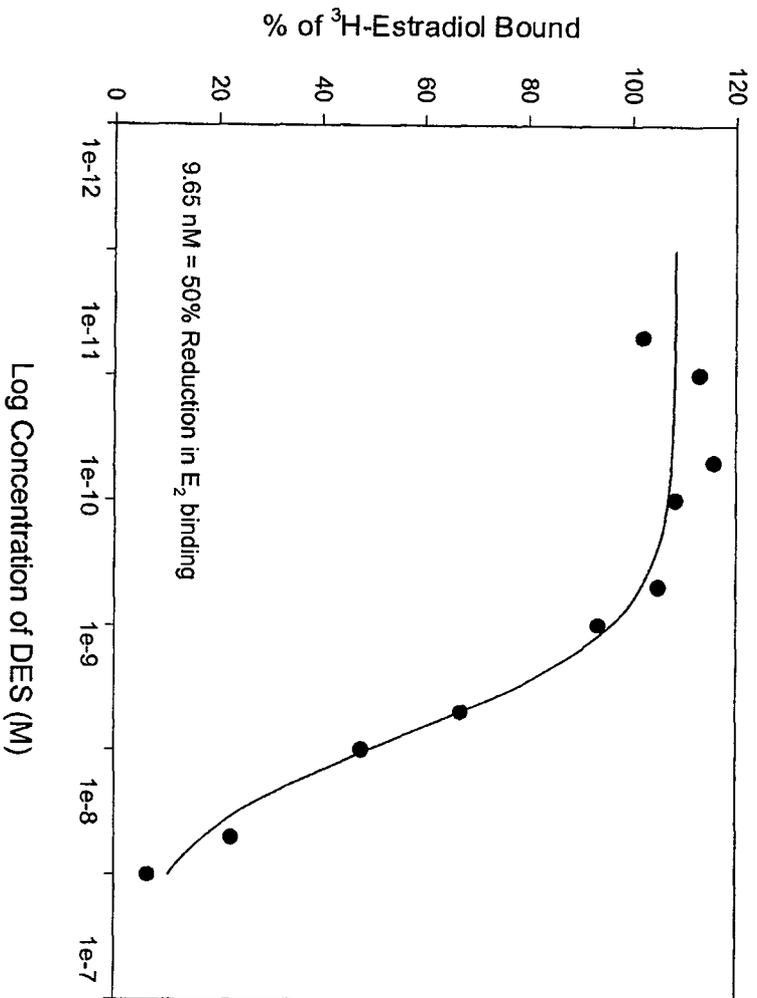


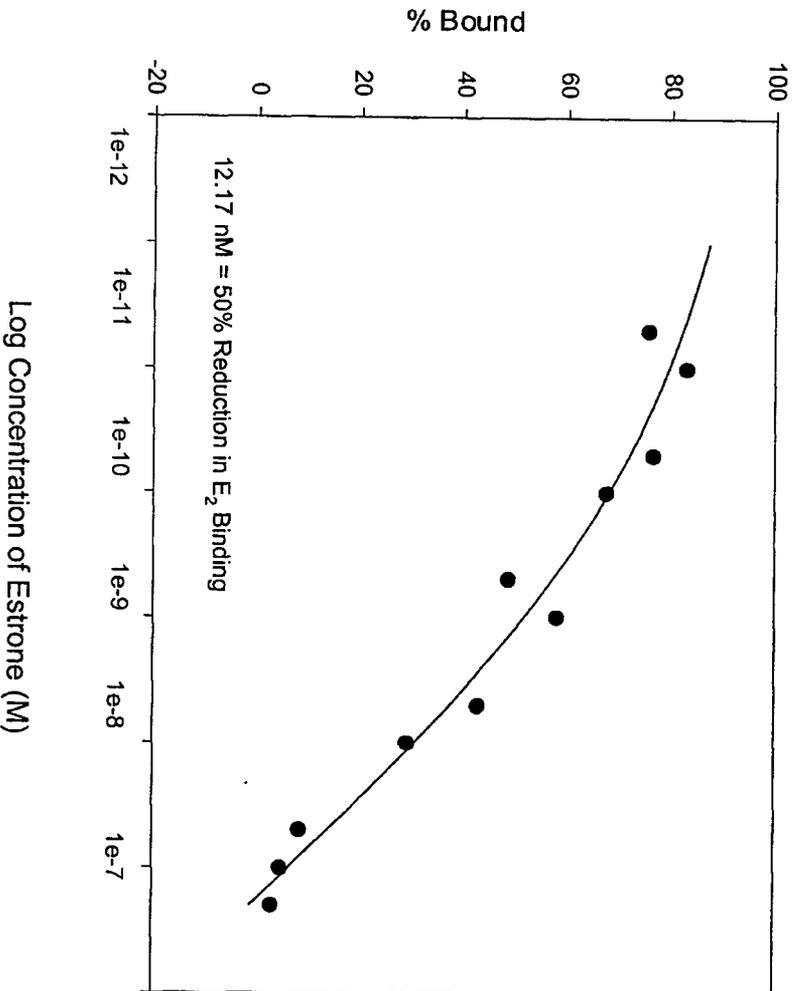
Figure A2  
Saturation Binding Analysis  
ER-Beta



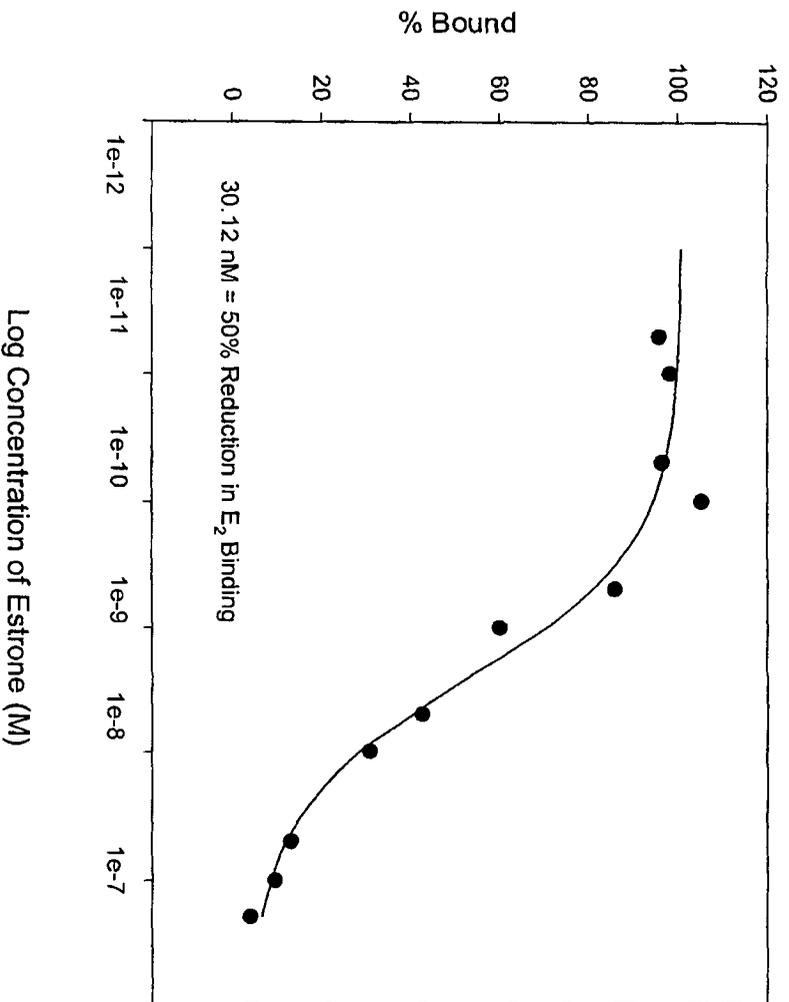
**Figure A3**  
**Diethylstilbestrol Binding to ER Alpha**  
**(RBA)**



**Figure A4**  
**Estrone Binding to ER-Alpha**



**Figure A5**  
**Estrone Binding to ER-Beta**



**Figure A6**  
**Estradiol Binding to ER-Alpha**

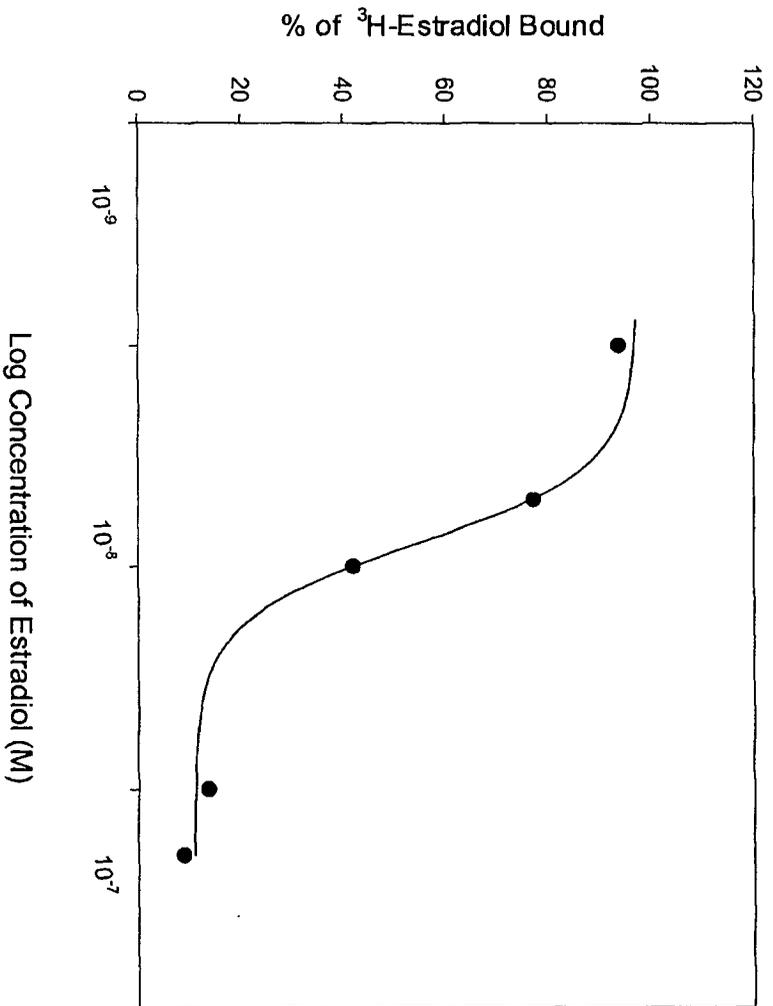
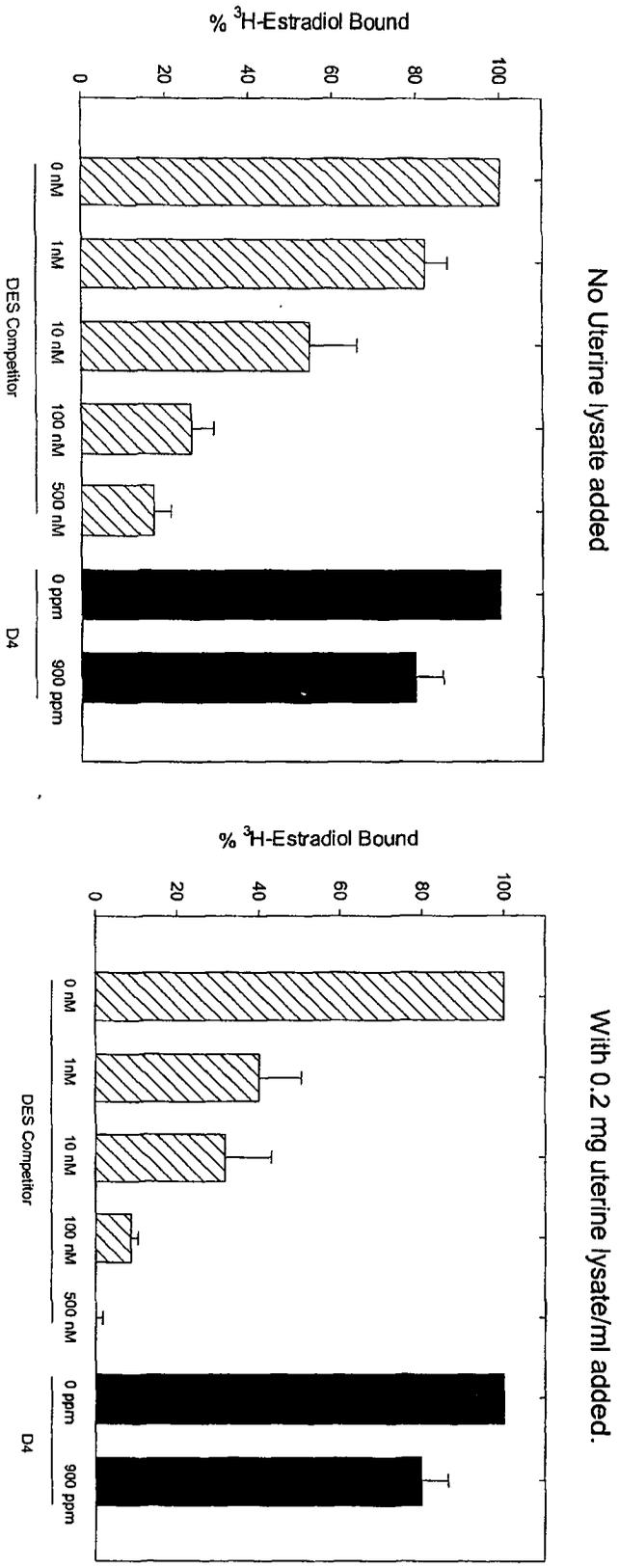


Figure A7

Receptor Binding ER-Alpha  
DES and D4 with and without Uterine Lysate



**Figure A8**  
**Receptor Binding ER-Beta**  
**DES and D4 with and without Uterine Lysate**

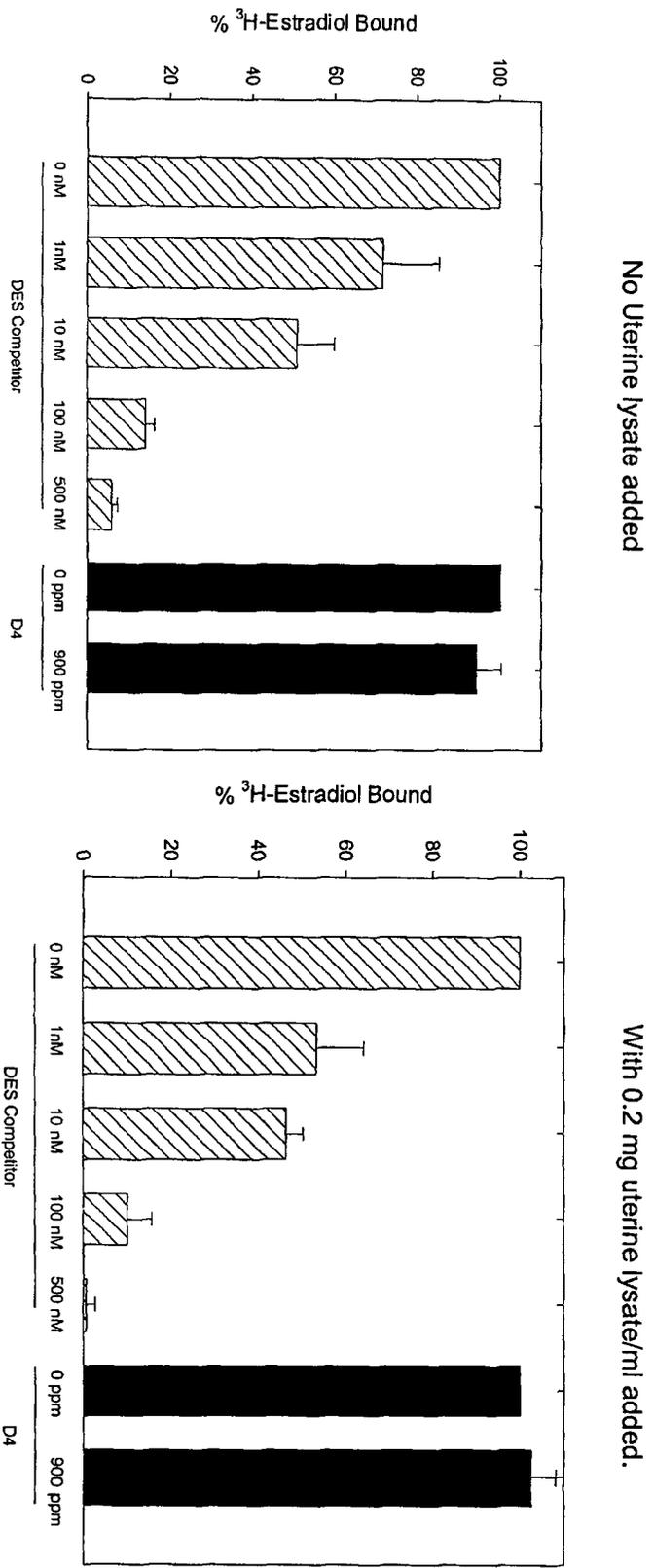


Figure A9  
Partitioning of 900 ppm D4 Vapor into the Aqueous Reaction Mixture

