

DRAFT REPORT

**VALIDATION OF AN ANDROGEN RECEPTOR (AR)
COMPETITIVE BINDING ASSAY: TASK 3
ESTABLISHING INTER-LABORATORY VARIABILITY USING A
STANDARD RAT VENTRAL PROSTATE CYTOSOL
BATTELLE STUDY NO. WA 4-11**

ABC LABORATORIES STUDY NO. 49655

Analytical Site:	ABC Laboratories, Inc. 7200 E. ABC Lane Columbia, Missouri 65202 (573) 474-8579
Study Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, Ohio 43201-2693
ABC Study Initiation Date:	TBA
ABC Study Completion Date:	<i>To be determined</i>

1.0 SIGNATURE PAGE

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David P. Houchens, Ph.D. Date

2.0 GLP COMPLIANCE STATEMENT

The ABC Laboratories, Inc. study number 49655 (portions conducted at ABC Laboratories, Inc.) was conducted in accordance with the principles of Good Laboratory Practice set forth in 21 CFR Part 58 by the Food and Drug Administration (FDA) with the following exception:

Sample identification generated by other co-operators is also included in this report and is presumed to be correct as supplied to ABC Laboratories. Characterization and stability of the test article, supplied by the sponsor is considered accurate. Characterization and stability of the internal standard as supplied by commercial vendor are considered accurate. ABC is responsible for accurate transcription of data from other sources creating this report and is only responsible for the validity and accuracy of work conducted by ABC Laboratories' personnel.

Camelia Gliser, B.S.
Study Director
Associate Scientist

Date

3.0 QUALITY ASSURANCE STATEMENT

Quality Assurance statement for ABC Laboratories, Inc. study number 49655, "Validation of an Androgen Receptor Competitive Binding Assay: Task 3, Establishing Inter-Laboratory Variability Using a Standard Rat Ventral Prostate Cytosol."

The report was reviewed by ABC's Quality Assurance Unit. The following inspections were conducted on study number 49655.

Date of Inspection	Phase Inspected	Date Reported to Analytical Investigator	Date Reported to Management	Date Reported to Study Director

The undersigned indicates that the report submitted is an accurate reflection of the raw data.

Michelle Haines, B.A.
Quality Assurance Associate II

Date

4.0 STUDY PERSONNEL

The following ABC Laboratories' personnel assisted with various portions of this study:

Name	Title
Amy Mize, Ph.D.	Director
Robert Howell, B.S.	Senior Scientist
Camelia Gliser, B.S.	Associate Scientist
Wes Kabler	Senior Technician

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6.0 LIST OF ABBREVIATIONS

% ACC	Percent Accuracy
AR	Androgen Receptor
Bmax	Density of the Receptor
°C	Degrees Celsius
%CV	Percent Coefficient of Variance
cpm	Counts per Minute
cps	Counts Per Second (Intensity)
dpm	Disintegrations per minute
FDA	Food and Drug Administration
kg	Kilogram
LSC	Liquid Scintillation Counter
HAP	Hydroxylapatite Slurry
hr	Hour
IC50	Half Life
Kd	Dissociation constant
mg	Milligram
mL	Milliliter
μCi	Micro Curie
μL	Microliter
μg	Microgram
min	Minute
n	Number
NC	No Comment
ng	Nanogram
QC	Quality Control
SD	Standard Deviation

7.0 LIST OF FIGURES

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9.0 INTRODUCTION

Analyte: Dexamethasone (see **Figure 1** for the amino acid sequence)
Reference: R1881 (Methyltrienolone)
Marker: Radiolabeled R1881 (³H-Methyltrienolone)
Test System: Rat prostate cytosol

Rat prostate cytosol samples collected in the study required quantitative analysis for the determination of Dexamethasone concentration. This report summarizes the results for Dexamethasone following Scintillation Counter analysis of rat prostate cytosol samples.

10.0 SAMPLE RECEIPT

A total of 10 study samples were received in acceptable condition; frozen, on dry ice (solid CO₂) on June 29, 2005. Samples were collected at Battelle Pacific Northwest Laboratory, then shipped by Federal Express to ABC Laboratories and stored at < -20°C.

Shipment Date	Receipt Date	# of Samples Received	# of Samples Analyzed	Storage Conditions
28-Jun-05	29-Jun-05	10	10	< -20°C

11.0 BRIEF DESCRIPTION OF THE PROTOCOL

Battelle Study Number WA 4-11, Task 3 is being conducted to determine the ability of Dexamethasone to compete with [³H] ligand for binding in rat ventral prostate tissue homogenate. Saturation binding assay experiments using R1881 were performed according to the protocol included in Appendix II.

12.0 METHODOLOGY

12.1 Preparation and Determination of Rat Prostate Cytosol

Samples were thawed on wet ice and pooled into 26 50-mL blue cap centrifuge tubes. Approximately 20 mL of TEDG buffer was added to each of the 26 tubes, with the exception of Tubes 8 and 15, where 15 mL of TEDG buffer was added. Contents were blended, centrifuged, decanted, and stored until analysis for protein determination.

Samples were prepared and analyzed for protein determination by microplating. The initial cytosol concentration was determined to be 7.39 mg/mL.

12.2 Saturation Assay Procedure

Day 1: The assay consisted of 48 tubes at 9 concentrations in triplicate each with and without 100X inert. Approximately 30 µL of Hot R1881 and

50 μL of triamcinolone acetonide were added to each glass tube (see Assay Tube Layout for details). Tubes 1-48 were dried and 300 μL of diluted cytosol (final concentration – 7.39 mg/mL) was added to each tube before vortexing and storing on ice at 4°C overnight.

Day 2: HAP slurry tubes were prepared and 100 μL of each assay tube was pipetted into individual HAP slurry tubes. After vortexing, centrifuging, aspirating the supernatant, and repeating a TRIS washing procedure multiple times, the supernatants were decanted and placed in scintillation vials for analysis.

12.3 List of Test Substances

Dexamethasone (CAS 50-28-2)					
Formula	Molecular Weight	Date Received	ABC Number	Lot Number	Chemical Purity
$\text{C}_{18}\text{H}_{24}\text{O}_2$	272.4				
Storage:			Supplied By: Battelle		
Reference Substance					
R-1881 (Methyltrienolone; CAS 965-93-5)					
Formula	Molecular Weight	Date Received	ABC Number	Lot Number	Chemical Purity
$\text{C}_{19}\text{H}_{24}\text{O}_2$	284.4				
Storage:			Supplied By: Battelle		
Marker					
Radiolabeled R-1881 (^3H -Methyltrienolone; CAS 68-23-5)					
Formula	Molecular Weight	Date Received	ABC Number	Lot Number	Chemical Purity
$\text{C}_{20}\text{H}_{26}\text{O}_2$	298.4				
Storage:			Supplied By: Battelle		

12.4 Equipment and Calibration

A Beckman Model LS 6000 or 6500 liquid scintillation counter (LSC; Beckman Instruments, Inc; Schaumburg, IL) was used to record the radioactivity levels of each sample after assay. The LSC is equipped with the H number method for cpm (counts per minute) to dpm (disintegrations per minute) conversion. Prior to analysis, the LSC calibration was checked daily to ensure proper functioning of the instrument.

12.5 Analytical Run Sequence

Each analytical batch was analyzed using three blank samples at the beginning of the run, followed by analysis of the assayed samples. The LSC data was corrected for background by subtracting the dpm value measured from the analysis of blank samples. All samples were analyzed in triplicate. Samples were counted for 5 minutes.

13.0 STUDY SAMPLE RESULTS

Graph pad prism was used by Nancy Holter of Battelle/PNL to calculate the dissociation constant (Kd) and the density of the receptor (Bmax) for dexamethasone as compared to R1881 from the saturation binding study portion.

The performance of the LSC system was qualified at ABC Laboratories. The data was, subsequently, imported electronically, using the maximum number of digits, into the Microsoft® Excel 2003 spreadsheets and is being reproduced for this report through Microsoft® Word 2003 software. After all calculation procedures, the final results were rounded to 3 significant figures or to the units place for numbers >1000.

Sample data was tabulated in Excel 2003 (Microsoft Corporation).

Density of Receptor (Bmax) and Dissociation Constant (Kd) of Saturation Experiments

Experiment No.	Date	Bmax	Kd
Saturation #1	11-Aug-05	0.00	0.00
Saturation #2	16-Aug-05	0.83	0.74

Bmax and Kd values are taken from Appendix II.

In order to verify the low protein cytosol recoveries are consistent with the cytosol preparation, Battelle did a separate analysis of the cytosol. Results of the comparison are included in the raw data in Appendix I.

14.0 CONCLUSIONS

Saturation experiments were conducted on August 11 and 16, 2005. Results of the saturation experiments indicate that the saturation experiment was not successful.

15.0 SAMPLES, STUDY DATA AND REPORT ARCHIVAL

At the sponsor's discretion, study samples will be destroyed upon finalization of the report.

The original project plan and raw data will be kept at ABC Laboratories' archives for at least 20 years after finalization of the report.

The original final report and an electronic copy will be sent to the sponsor. A copy of the report will be kept in ABC Laboratories' archives for at least 20 years.

All facility records (for example, equipment, logbooks, and temperature records) will be kept in ABC Laboratories indefinitely.

16.0 REFERENCES

ABC SOP PH-BA 2.1.8, "Routine Drug Analysis and Re-Analysis"
Effective date: 10-Nov-04 and subsequent revision.

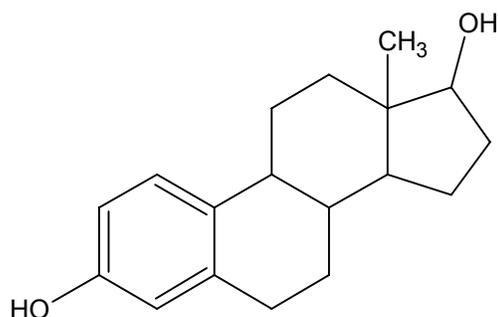
U.S. Food and Drug Administration. Good Laboratory Practice Standards for Nonclinical Laboratory Studies, 21 CFR Part 58 (1998).

U.S. Food and Drug Administration. Center for Drug Evaluation and Research. Guidance for Industry. Bioanalytical Method Validation.
Issue date: May 2001.

FIGURES

Figure 1. Structure of Dexamethasone, R1881, and Radiolabeled R1881

Test Compound:	Dexamethasone
Supplier:	Battelle
CAS No. :	CAS 50-28-2
Chemical Name:	dexamethasone
Formula:	$C_{18}H_{24}O_2$
Molecular Weight:	272.4
Storage Conditions:	
Structure:	



Reference Compound:	R1881 (Methyltrienolone)
Supplier:	
Formula:	$C_{19}H_{24}O_2$
Molecular Weight:	284.4
Storage conditions:	
Structure:	

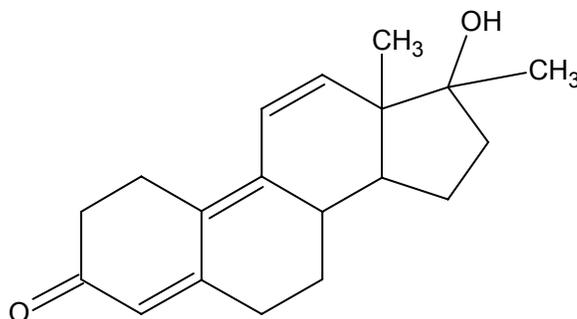
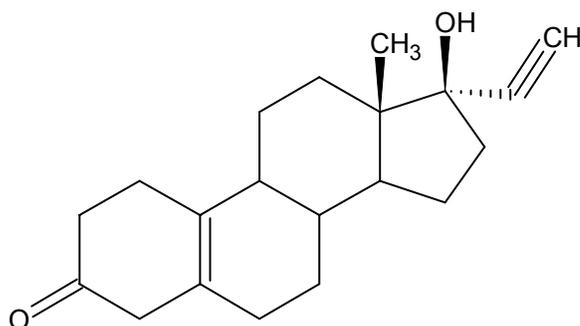


Figure 1. Structure of Dexamethasone, R1881, and Radiolabeled R1881 (continued)

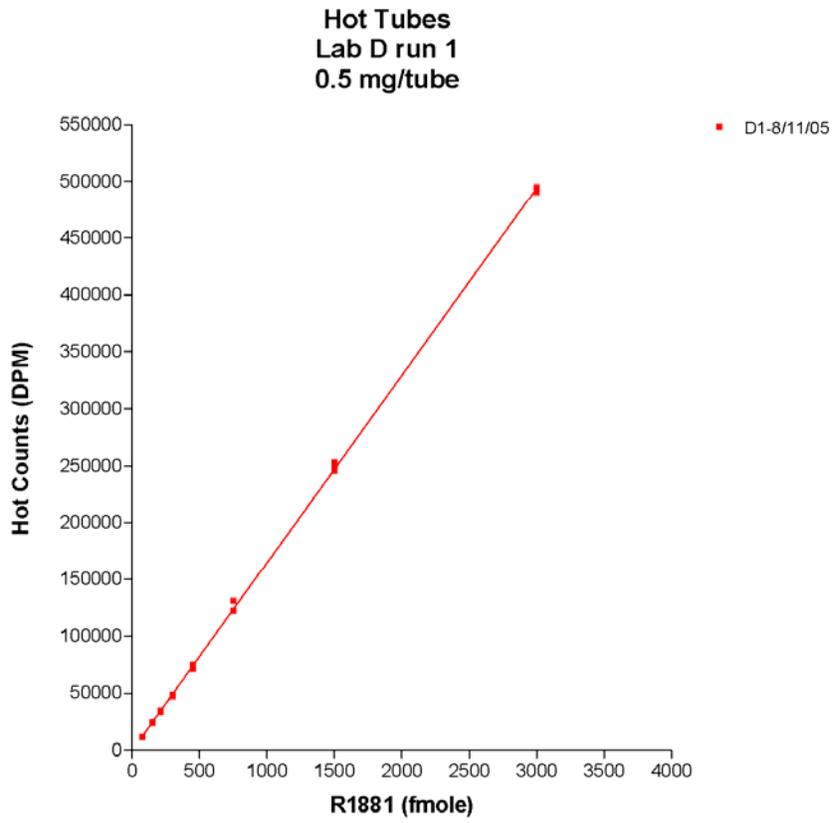
Marker Compound:	Radiolabeled R1881 (³H-Methyltrienolone)
Supplier:	Battelle
CAS No. :	CAS 68-23-5
Chemical Name:	methyltrienolone
Formula:	C ₂₀ H ₂₆ O ₂
Molecular Weight:	298.4
Storage Conditions:	
Structure:	

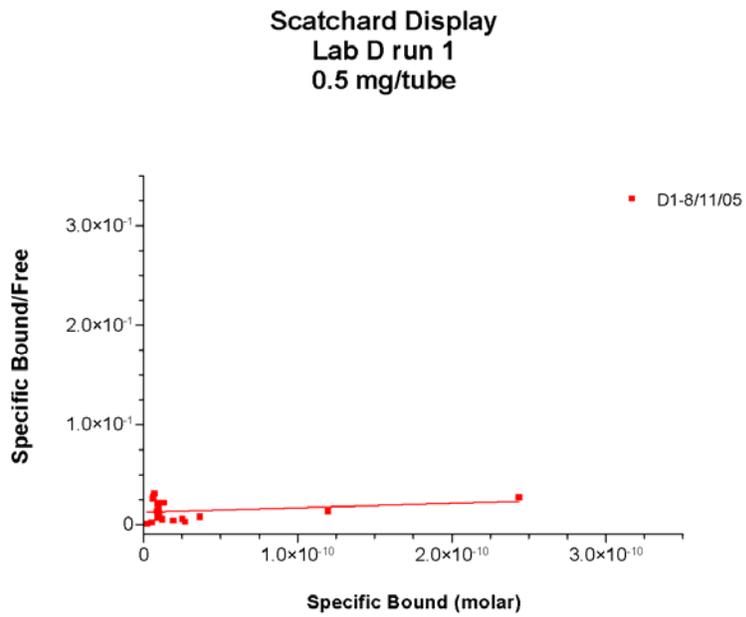


APPENDIX I
RAW DATA

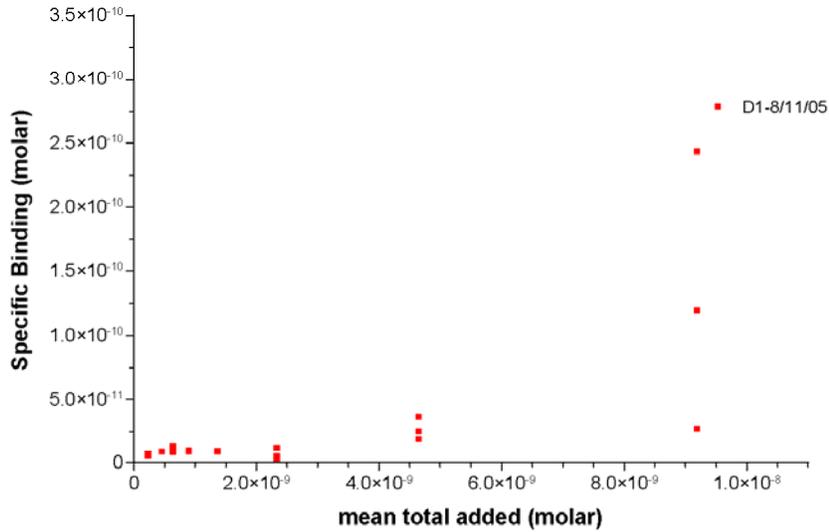
SATURATION ASSAYS

4-11-3-Saturation_D.pzf.D1 Hot - Mon Aug 22 11:39:46 2005



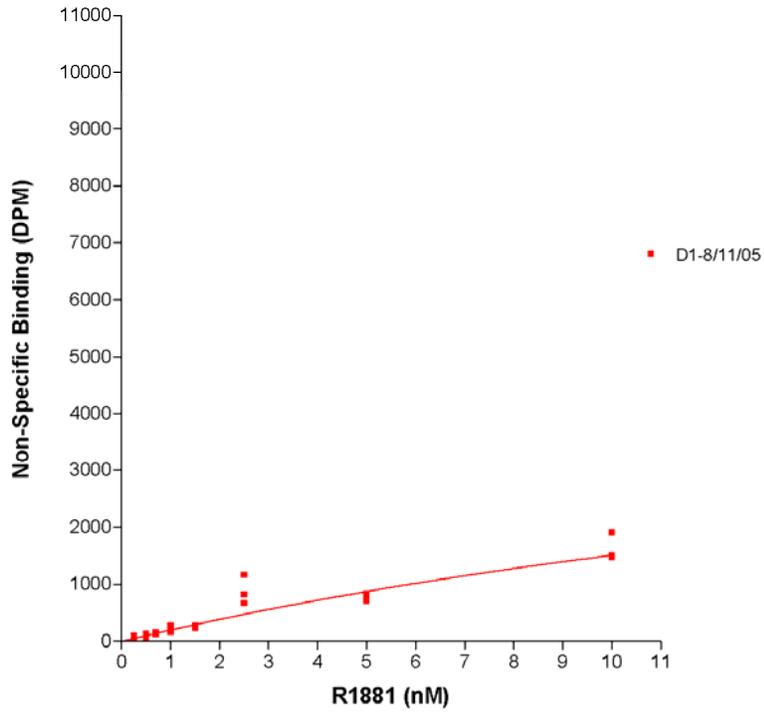


**Lab D run 1
0.5 mg/tube**



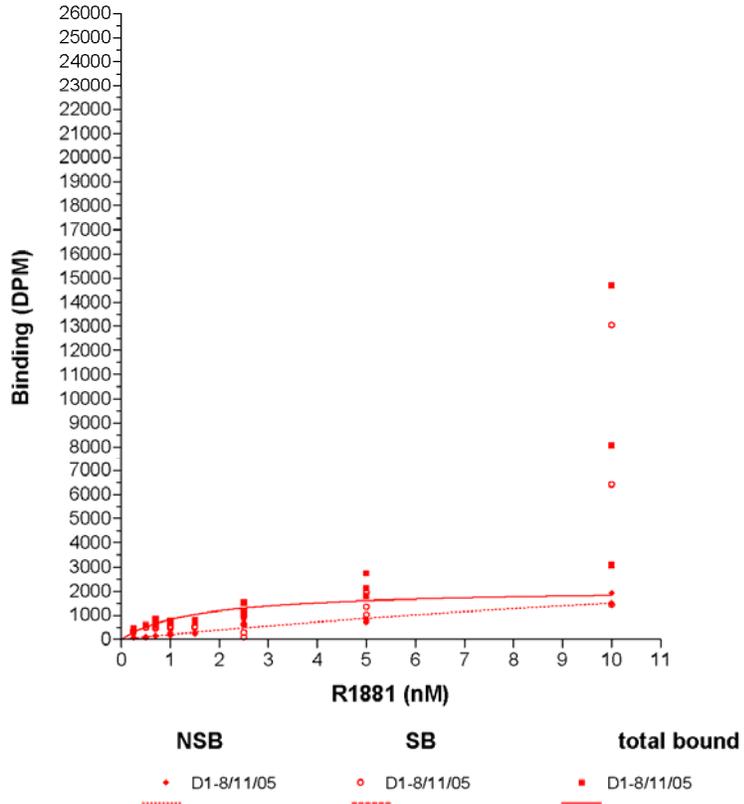
Specific bound	D1-8/11/05
One site binding (hyperbola)	Does not converge.
Best-fit values	
BMAX	
KD	
Std. Error	
BMAX	
KD	
95% Confidence Intervals	
BMAX	
KD	
Goodness of Fit	
Degrees of Freedom	
R ² (unweighted)	
Weighted Sum of Squares (1/Y ²)	
Absolute Sum of Squares	
Sy.x	
Data	
Number of X values	
Number of Y replicates	
Total number of values	
Number of missing values	

NSB Tubes
Lab D run 1
0.5 mg/tube

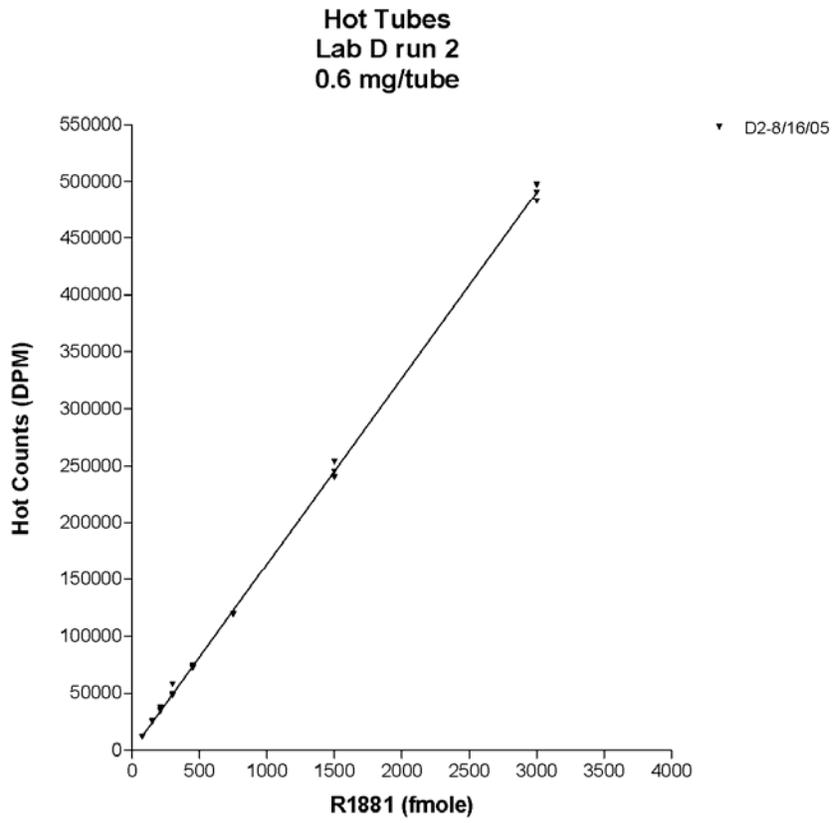


4-11-3-Saturation_D.pzf:D1 data (DPM) - Mon Aug 22 11:39:46 2005

**bound counts
Lab D run 1
0.5 mg/tube**

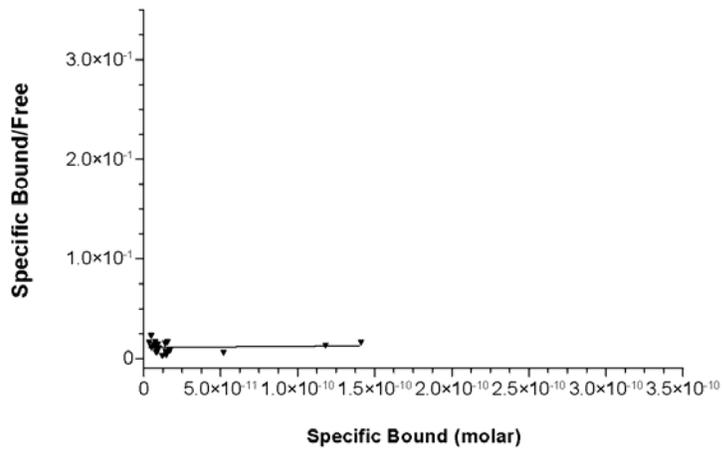


4-11-3-Saturation_D.pzf.D2 Hot - Mon Aug 22 11:39:46 2005



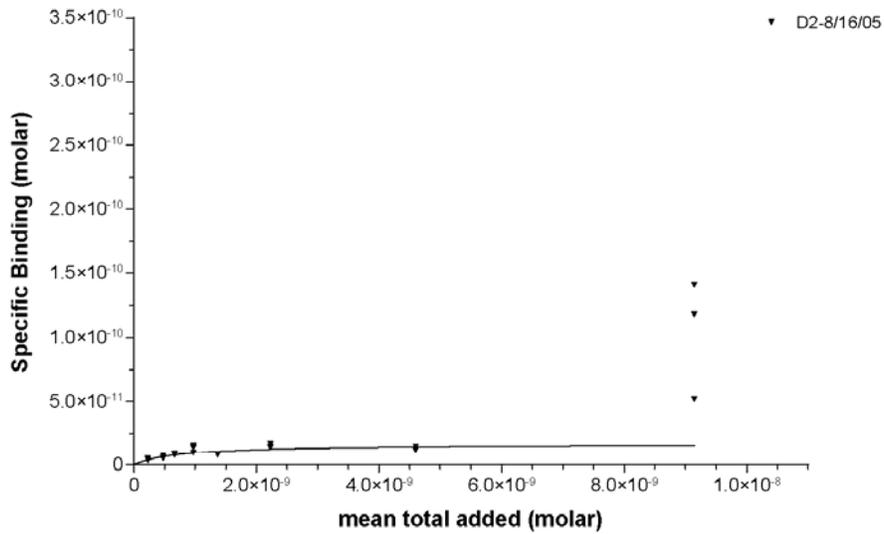
Scatchard Display
Lab D run 2
0.6 mg/tube

▼ D2-8/16/05



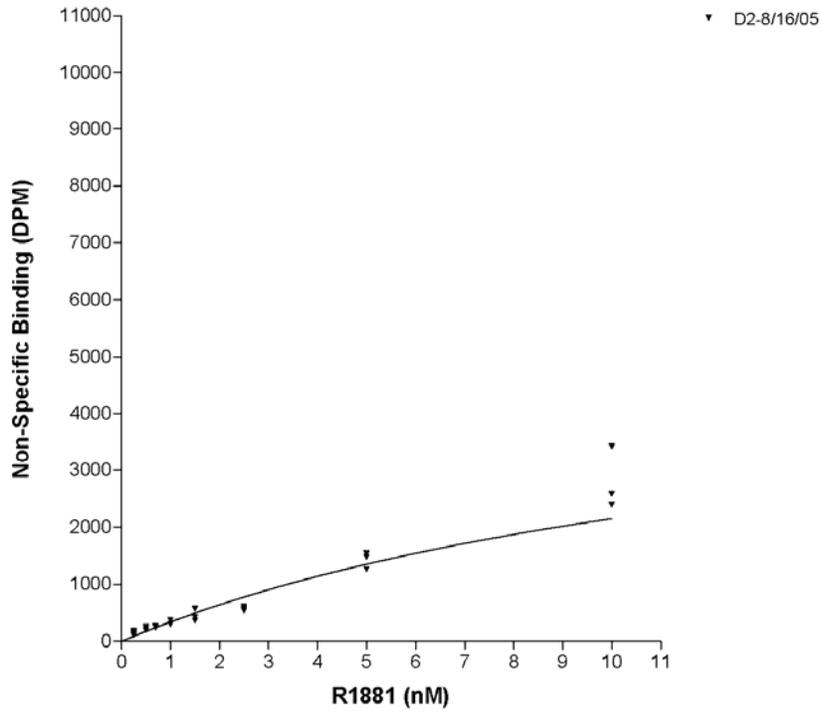
4-11-3-Saturation_D.pzf:D2 Curve (molar) - Mon Aug 22 11:39:46 2005

Lab D run 2
0.6 mg/tube



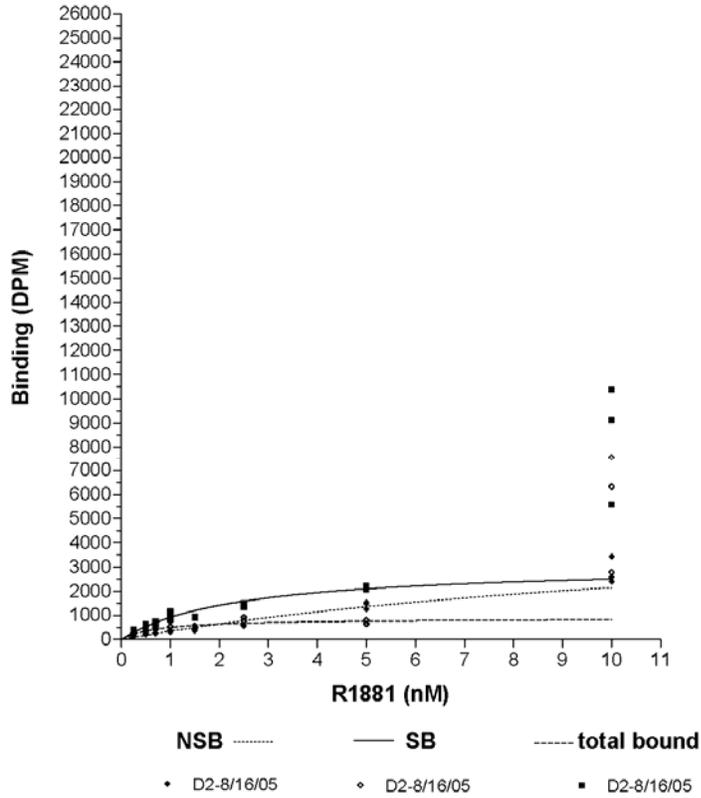
D2-8/16/05	
BMAX	1.662e-011
KD	7.389e-010
Std. Error	
BMAX	3.107e-012
KD	2.959e-010
95% Confidence Intervals	
BMAX	1.018e-011 to 2.306e-011
KD	1.252e-010 to 1.353e-009
Goodness of Fit	
Degrees of Freedom	22
R ² (unweighted)	-0.003867
Weighted Sum of Squares (1/Y ²)	2.952
Absolute Sum of Squares	2.779e-020
Sy.x	3.554e-011
Data	
Number of X values	24
Number of Y replicates	1
Total number of values	24
Number of missing values	0

NSB Tubes
Lab D run 2
0.6 mg/tube

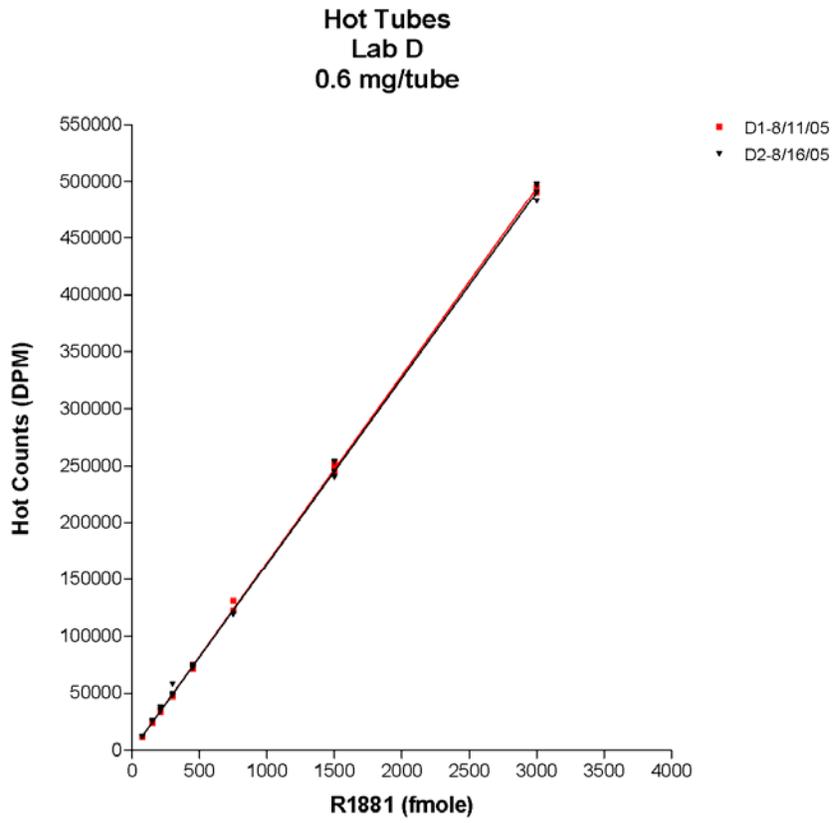


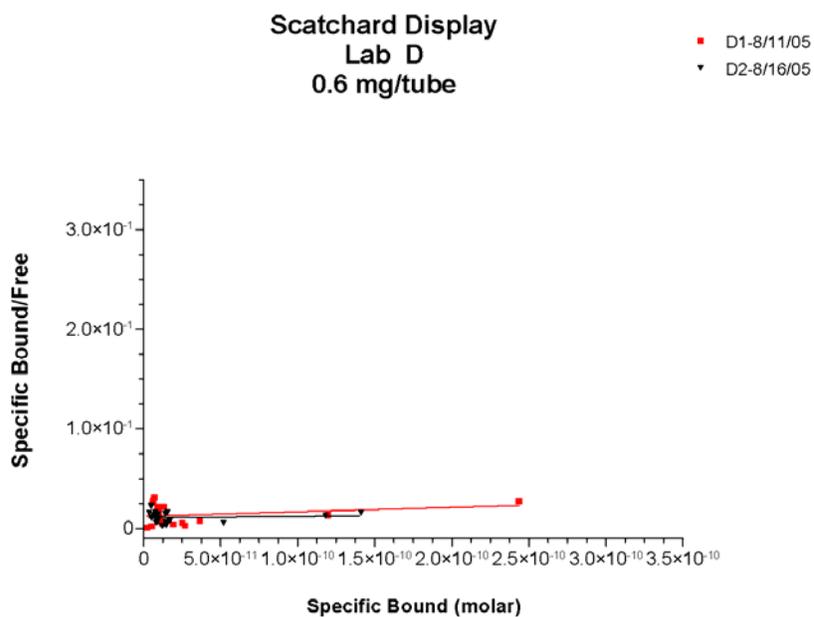
4-11-3-Saturation_D.pzf.D2 data (DPM) - Mon Aug 22 11:39:47 2005

bound counts
Lab D run 2
0.6 mg/tube



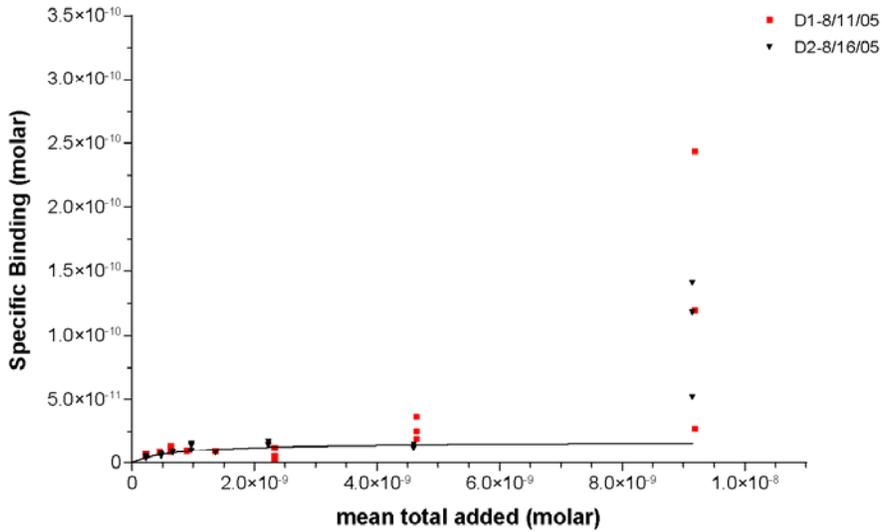
4-11-3-Saturation_D.pzf:D Hot - Mon Aug 22 11:39:47 2005



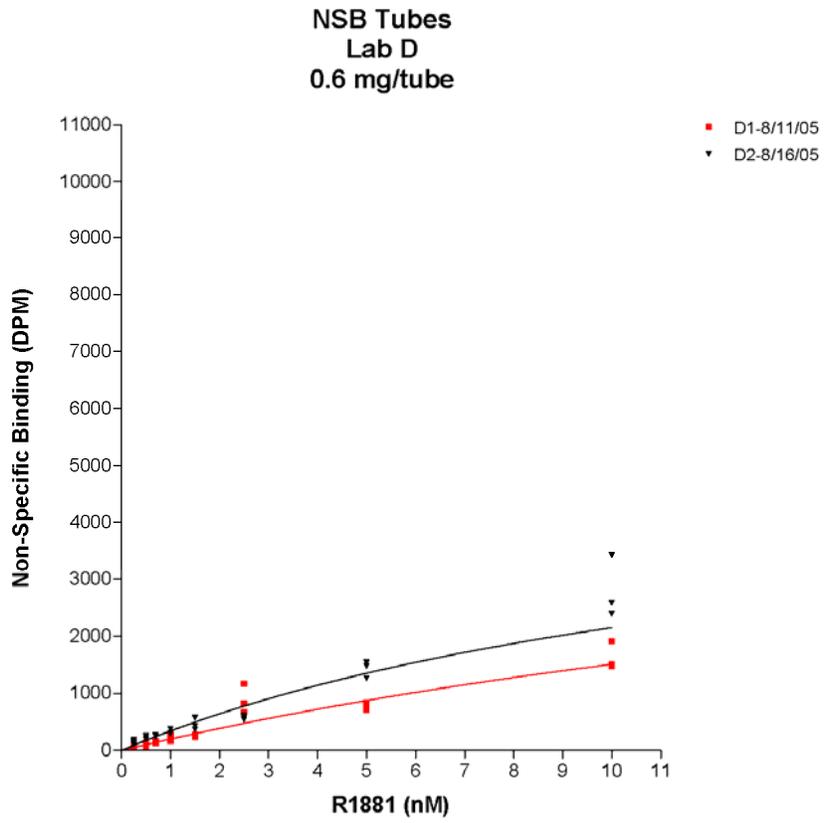


4-11-3-Saturation_D.pzf:D Curve (molar) - Mon Aug 22 11:39:47 2005

Lab D
0.6 mg/tube

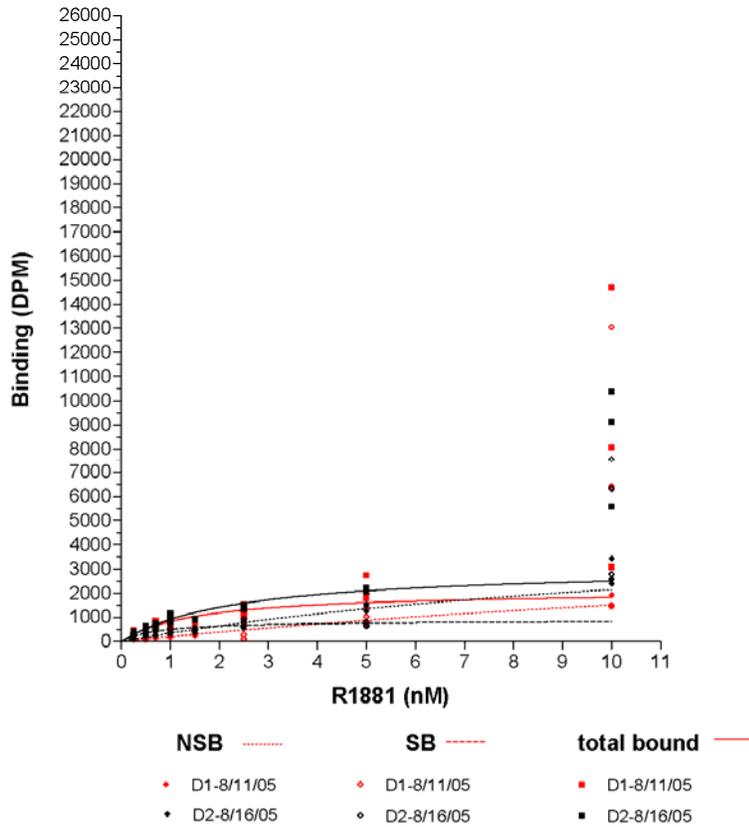


Specific bound	D1-8/11/05	D2-8/16/05
One site binding (hyperbola)	Does not converge.	
Best-fit values		
BMAX		1.662e-011
KD		7.389e-010
Std. Error		
BMAX		3.107e-012
KD		2.959e-010
95% Confidence Intervals		
BMAX		1.018e-011 to 2.306e-011
KD		1.252e-010 to 1.353e-009
Goodness of Fit		
Degrees of Freedom		22
R ² (unweighted)		-0.003867
Weighted Sum of Squares (1/Y ²)		2.952
Absolute Sum of Squares		2.779e-020
Sy,x		3.554e-011
Data		
Number of X values		24
Number of Y replicates		1
Total number of values		24
Number of missing values		0



4-11-3-Saturation_D.pzf:D data (DPM) - Mon Aug 22 11:39:47 2005

**bound counts
Lab D
0.6 mg/tube**



Saturation Data for Experiment #1

Laboratory D

AR Saturation Assay (cold R1881 and WP supplied by Sequim)
72 assay tubes

Please return by eMail to n.a.Holter@pnl.gov

Provide information in all blue cells in columns O and DK

If the DPM value for a tube was judged unreliable,

Include the DPM value in column O

Provide a reason in column R

The value in column Q will automatically change to FALSE

For your convenience, data reduction is performed in columns

U through BZ, and the values needed for analysis are presented

in columns CF through CN

Cells in column S are presented with a grey background

if the total binding exceeds 10% of the hot added at that concentration,
the cytosol concentration is probably too high for good competitive assays

Laboratory Code: D

Run identification: 8112005

Assay start date: 8/11/2005

Tracer lot number: 3559-507

Specific activity on day of assay: 80.56 Ci/mmoles

Cytosol lot or vial number:

protein (cytosol) per tube: 600 ug

protein (cytosol) per tube: 0.6 mg

KD 0.00E+00 nM

Bmax 0.00 fmole/100 ug

total volume in tubes 300 uL

volume of ethanol counted: 2 mL

multiply DPM in sample by : 3

Receptor_Notes
diluted to 2 mg/ml for use (0.6 mg/300 ul)

protocol calls for counting decanted EtOH since
reflects 100ul of reaction mixture processed

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (uM)	Cold R1881 Volume (uL)	Cold Final Concentration (nM)	Triamcetenone Acetate (uL)	Cytosol (ul)	Significant portion of label on Vial	dpm as counted	corrected DPM for 2mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
1	1	H	10.0	7.5	0.25	—	—	—	50	300	—	133.07	399.21	TRUE	
2	2	H	10.0	7.5	0.25	—	—	—	50	300	—	152.34	457.02	TRUE	
3	3	H	10.0	7.5	0.25	—	—	—	50	300	—	139.59	418.77	TRUE	
4	1	H	10.0	15	0.50	—	—	—	50	300	—	198.61	595.83	TRUE	
5	2	H	10.0	15	0.50	—	—	—	50	300	—	193.98	581.94	TRUE	
6	3	H	10.0	15	0.50	—	—	—	50	300	—	200.02	600.06	TRUE	
7	1	H	10.0	21	0.70	—	—	—	50	300	—	199.66	598.98	TRUE	
8	2	H	10.0	21	0.70	—	—	—	50	300	—	224.72	674.16	TRUE	
9	3	H	10.0	21	0.70	—	—	—	50	300	—	289.24	867.72	TRUE	
10	1	H	10.0	30	1.00	—	—	—	50	300	—	256.99	770.97	TRUE	
11	2	H	10.0	30	1.00	—	—	—	50	300	—	244.14	732.42	TRUE	
12	3	H	10.0	30	1.00	—	—	—	50	300	—	230.51	691.53	TRUE	
13	1	H	10.0	45	1.50	—	—	—	50	300	—	256.93	770.79	TRUE	
14	2	H	10.0	45	1.50	—	—	—	50	300	—	251.55	754.65	TRUE	
15	3	H	10.0	45	1.50	—	—	—	50	300	—	265.99	797.97	TRUE	
16	1	H	100.0	7.5	2.50	—	—	—	50	300	—	332.84	998.52	TRUE	
17	2	H	100.0	7.5	2.50	—	—	—	50	300	—	508.42	1525.26	TRUE	
18	3	H	100.0	7.5	2.50	—	—	—	50	300	—	389.45	1168.35	TRUE	
19	1	H	100.0	15	5.00	—	—	—	50	300	—	708.30	2124.9	TRUE	
20	2	H	100.0	15	5.00	—	—	—	50	300	—	911.59	2734.77	TRUE	
21	3	H	100.0	15	5.00	—	—	—	50	300	—	601.43	1804.29	TRUE	
22	1	H	100.0	30	10.00	—	—	—	50	300	—	1028.02	3084.06	TRUE	
23	2	H	100.0	30	10.00	—	—	—	50	300	—	4899.37	14698.11	TRUE	
24	3	H	100.0	30	10.00	—	—	—	50	300	—	2683.64	8050.92	TRUE	
25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	32.91	98.73	TRUE	

CONFIDENTIAL

Saturation Data for Experiment #1 (continued)

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (uM)	Cold R1881 Volume (uL)	Cold Final Concentration (nM)	Triacelenone Acetate (uL)	Cytosol (ul)	Significant portion of label on Vial	dpm as counted	corrected DPM for 2mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	33.14	99.42	TRUE	
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	21.91	65.73	TRUE	
28	1	HC	10.0	15	0.5	1.00	15	50	50	300	C7	25.10	75.3	TRUE	
29	2	HC	10.0	15	0.5	1.00	15	50	50	300	C7	44.89	134.67	TRUE	
30	3	HC	10.0	15	0.5	1.00	15	50	50	300	C7	24.90	74.7	TRUE	
31	1	HC	10.0	21	0.7	1.00	21	70	50	300	C6	45.89	137.67	TRUE	
32	2	HC	10.0	21	0.7	1.00	21	70	50	300	C6	56.10	168.3	TRUE	
33	3	HC	10.0	21	0.7	1.00	21	70	50	300	C6	47.91	143.73	TRUE	
34	1	HC	10.0	30	1	1.00	30	100	50	300	C5	72.00	216	TRUE	
35	2	HC	10.0	30	1	1.00	30	100	50	300	C5	56.63	169.89	TRUE	
36	3	HC	10.0	30	1	1.00	30	100	50	300	C5	92.88	278.64	TRUE	
37	1	HC	10.0	45	1.5	1.00	45	150	50	300	C4	88.16	264.48	TRUE	
38	2	HC	10.0	45	1.5	1.00	45	150	50	300	C4	95.30	285.9	TRUE	
39	3	HC	10.0	45	1.5	1.00	45	150	50	300	C4	80.37	241.11	TRUE	
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	224.10	672.3	TRUE	
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	274.90	824.7	TRUE	
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	389.34	1168.02	TRUE	
43	1	HC	100.0	15	5	10.00	15	500	50	300	C2	258.28	774.84	TRUE	
44	2	HC	100.0	15	5	10.00	15	500	50	300	C2	277.17	831.51	TRUE	
45	3	HC	100.0	15	5	10.00	15	500	50	300	C2	237.58	712.74	TRUE	
46	1	HC	100.0	30	10	10.00	30	1000	50	300	C1	493.22	1479.66	TRUE	
47	2	HC	100.0	30	10	10.00	30	1000	50	300	C1	639.03	1917.09	TRUE	
48	3	HC	100.0	30	10	10.00	30	1000	50	300	C1	504.95	1514.85	TRUE	
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12160.86	12160.86	TRUE	
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12398.40	12398.4	TRUE	
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12496.30	12496.3	TRUE	
52	1	Hot	10.0	15	0.06	—	—	—	—	—	—	24039.13	24039.13	TRUE	
53	2	Hot	10.0	15	0.06	—	—	—	—	—	—	24534.80	24534.8	TRUE	
54	3	Hot	10.0	15	0.06	—	—	—	—	—	—	24449.59	24449.59	TRUE	
55	1	Hot	10.0	21	0.08	—	—	—	—	—	—	33620.61	33620.61	TRUE	
56	2	Hot	10.0	21	0.08	—	—	—	—	—	—	33973.64	33973.64	TRUE	
57	3	Hot	10.0	21	0.08	—	—	—	—	—	—	34602.49	34602.49	TRUE	
58	1	Hot	10.0	30	0.10	—	—	—	—	—	—	47529.20	47529.2	TRUE	
59	2	Hot	10.0	30	0.10	—	—	—	—	—	—	49128.96	49128.96	TRUE	
60	3	Hot	10.0	30	0.10	—	—	—	—	—	—	47765.37	47765.37	TRUE	
61	1	Hot	10.0	45	0.30	—	—	—	—	—	—	71666.65	71666.65	TRUE	
62	2	Hot	10.0	45	0.30	—	—	—	—	—	—	74826.73	74826.73	TRUE	
63	3	Hot	10.0	45	0.30	—	—	—	—	—	—	72743.20	72743.2	TRUE	
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—	—	122110.20	122110.2	TRUE	
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—	—	122262.30	122262.3	TRUE	
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—	—	130955.40	130955.4	TRUE	
67	1	Hot	100.0	15	1.00	—	—	—	—	—	—	245627.30	245627.3	TRUE	
68	2	Hot	100.0	15	1.00	—	—	—	—	—	—	248112.20	248112.2	TRUE	
69	3	Hot	100.0	15	1.00	—	—	—	—	—	—	253281.80	253281.8	TRUE	
70	1	Hot	100.0	30	3.00	—	—	—	—	—	—	490699.60	490699.6	TRUE	
71	2	Hot	100.0	30	3.00	—	—	—	—	—	—	495123.70	495123.7	TRUE	
72	3	Hot	100.0	30	3.00	—	—	—	—	—	—	492819.20	492819.2	TRUE	

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Saturation Data for Experiment #1 (continued)

Ten Percent Rule	Saturation X values, Bound y values, NSB y values			Total Binding -- Positions 1-24 radiolabeled R1881 plus cytosol (Panel A)													
				Tube Identification		Assay tube contents											Total Volume
				Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. Initial	Hot R1881 Volume	Cold R1881 Conc. Initial	Cold R1881 volume	Triamcetenone Acetate	Cytosol	Hot Conc. Final	Cold Conc. Final	
3.2%	0.25	399.2	88.0	8E+06	1	1	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
3.7%	0.25	457.0	88.0	8E+06	2	2	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
3.4%	0.25	418.8	88.0	8E+06	3	3	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
2.4%	0.5	595.8	94.9	8E+06	4	1	H	c2	10.0	15	—	—	—	300	0.50	—	300
2.4%	0.5	581.9	94.9	8E+06	5	2	H	c2	10.0	15	—	—	—	300	0.50	—	300
2.5%	0.5	600.1	94.9	8E+06	6	3	H	c2	10.0	15	—	—	—	300	0.50	—	300
1.8%	0.7	599.0	149.9	8E+06	7	1	H	c3	10.0	21	—	—	—	300	0.70	—	300
2.0%	0.7	674.2	149.9	8E+06	8	2	H	c3	10.0	21	—	—	—	300	0.70	—	300
2.5%	0.7	867.7	149.9	8E+06	9	3	H	c3	10.0	21	—	—	—	300	0.70	—	300
1.6%	1	771.0	221.5	8E+06	10	1	H	c4	10.0	30	—	—	—	300	1.00	—	300
1.5%	1	732.4	221.5	8E+06	11	2	H	c4	10.0	30	—	—	—	300	1.00	—	300
1.4%	1	691.5	221.5	8E+06	12	3	H	c4	10.0	30	—	—	—	300	1.00	—	300
1.1%	1.5	770.8	263.8	8E+06	13	1	H	c5	10.0	45	—	—	—	300	1.50	—	300
1.0%	1.5	754.7	263.8	8E+06	14	2	H	c5	10.0	45	—	—	—	300	1.50	—	300
1.1%	1.5	798.0	263.8	8E+06	15	3	H	c5	10.0	45	—	—	—	300	1.50	—	300
0.8%	2.5	998.5	888.3	8E+06	16	1	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
1.2%	2.5	1525.3	888.3	8E+06	17	2	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
0.9%	2.5	1168.4	888.3	8E+06	18	3	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
0.9%	5	2124.9	773.0	8E+06	19	1	H	c7	100.0	15	—	—	—	300	5.00	—	300
1.1%	5	2734.8	773.0	8E+06	20	2	H	c7	100.0	15	—	—	—	300	5.00	—	300
0.7%	5	1804.3	773.0	8E+06	21	3	H	c7	100.0	15	—	—	—	300	5.00	—	300
0.6%	10	3084.1	1637.2	8E+06	22	1	H	c8	100.0	30	—	—	—	300	10.00	—	300
3.0%	10	14698.1	1637.2	8E+06	23	2	H	c8	100.0	30	—	—	—	300	10.00	—	300
1.6%	10	8050.9	1637.2	8E+06	24	3	H	c8	100.0	30	—	—	—	300	10.00	—	300

Saturation Data for Experiment #1 (continued)

Run	Position							Number of molecules					Ratio	
		Total Counts (dpm)	Non Specific Binding (Mean of reps in pos. 25-48) (dpm)	Specific Binding (Total - Non Specific) (dpm)	Ratio of NSB/ total binding	Ratio Total binding/ Hot	Total Added (Mean of reps in pos. 49-72) (dpm)	Free (total added - bound) (dpm)	Total Binding molecules (fmole)	Non Specific Binding molecules (fmole)	Specific Binding molecules (fmole)	Total Added (Mean of reps in pos. 49-72) (fmole)	Free (total added - bound) (fmole)	Specific Bound /Free
8E+06	1	399.2	88.0	311.3	22.0%	3.2%	12351.9	11952.6	2	1	2	75	73	0.03
8E+06	2	457.0	88.0	369.1	19.2%	3.7%	12351.9	11894.8	3	1	2	75	72	0.03
8E+06	3	418.8	88.0	330.8	21.0%	3.4%	12351.9	11933.1	3	1	2	75	72	0.03
8E+06	4	595.8	94.9	500.9	15.9%	2.4%	24341.2	23745.3	4	1	3	148	144	0.02
8E+06	5	581.9	94.9	487.1	16.3%	2.4%	24341.2	23759.2	4	1	3	148	144	0.02
8E+06	6	600.1	94.9	505.2	15.8%	2.5%	24341.2	23741.1	4	1	3	148	144	0.02
8E+06	7	599.0	149.9	449.1	25.0%	1.8%	34065.6	33466.6	4	1	3	207	203	0.01
8E+06	8	674.2	149.9	524.3	22.2%	2.0%	34065.6	33391.4	4	1	3	207	203	0.02
8E+06	9	867.7	149.9	717.8	17.3%	2.5%	34065.6	33197.9	5	1	4	207	202	0.02
8E+06	10	771.0	221.5	549.5	28.7%	1.6%	48141.2	47370.2	5	1	3	292	288	0.01
8E+06	11	732.4	221.5	510.9	30.2%	1.5%	48141.2	47408.8	4	1	3	292	288	0.01
8E+06	12	691.5	221.5	470.0	32.0%	1.4%	48141.2	47449.6	4	1	3	292	288	0.01
8E+06	13	770.8	263.8	507.0	34.2%	1.1%	73078.9	72308.1	5	2	3	444	439	0.01
8E+06	14	754.7	263.8	490.8	35.0%	1.0%	73078.9	72324.2	5	2	3	444	439	0.01
8E+06	15	798.0	263.8	534.1	33.1%	1.1%	73078.9	72280.9	5	2	3	444	439	0.01
8E+06	16	998.5	888.3	110.2	89.0%	0.8%	125109.3	124110.8	6	5	1	760	754	0.00
8E+06	17	1525.3	888.3	636.9	58.2%	1.2%	125109.3	123584.0	9	5	4	760	751	0.01
8E+06	18	1168.4	888.3	280.0	76.0%	0.9%	125109.3	123941.0	7	5	2	760	753	0.00
8E+06	19	2124.9	773.0	1351.9	36.4%	0.9%	249007.1	246882.2	13	5	8	1512	1499	0.01
8E+06	20	2734.8	773.0	1961.7	28.3%	1.1%	249007.1	246272.3	17	5	12	1512	1496	0.01
8E+06	21	1804.3	773.0	1031.3	42.8%	0.7%	249007.1	247202.8	11	5	6	1512	1501	0.00
8E+06	22	3084.1	1637.2	1446.9	53.1%	0.6%	492880.8	489796.8	19	10	9	2993	2975	0.00
8E+06	23	14698.1	1637.2	13060.9	11.1%	3.0%	492880.8	478182.7	89	10	79	2993	2904	0.03
8E+06	24	8050.9	1637.2	6413.7	20.3%	1.6%	492880.8	484829.9	49	10	39	2993	2945	0.01

Saturation Data for Experiment #1 (continued)

		Non Specific Binding -- Positions 25-48 radiolabeled R1881 plus 100 X inert R1881 plus cytosol													
Run	Position	Tube Identification			Assay tube contents								Scintillation Results		
		Rep	Tube Type Code	Conc. Code	Hot Conc. R1881 Initial		Cold R1881 Conc. Initial		Triamcelenone Acetate	Cytosol	Hot Conc. Final		Cold Conc. Final	Counts per Scintillation Vial (Total Binding)	Non Specific Binding (Mean of reps in pos. 25-48)
					(nM)	(ul)	(mM)	(ul)			(nM)	(nM)			
8E+06	25	1	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	98.7	88.0	
8E+06	26	2	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	99.4	88.0	
8E+06	27	3	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	65.7	88.0	
8E+06	28	1	HC	c2	10.0	15	1.00	15	50	300	0.5	50	75.3	94.9	
8E+06	29	2	HC	c2	10.0	15	1.00	15	50	300	0.5	50	134.7	94.9	
8E+06	30	3	HC	c2	10.0	15	1.00	15	50	300	0.5	50	74.7	94.9	
8E+06	31	1	HC	c3	10.0	21	1.00	21	50	300	0.7	70	137.7	149.9	
8E+06	32	2	HC	c3	10.0	21	1.00	21	50	300	0.7	70	168.3	149.9	
8E+06	33	3	HC	c3	10.0	21	1.00	21	50	300	0.7	70	143.7	149.9	
8E+06	34	1	HC	c4	10.0	30	1.00	30	50	300	1	100	216.0	221.5	
8E+06	35	2	HC	c4	10.0	30	1.00	30	50	300	1	100	169.9	221.5	
8E+06	36	3	HC	c4	10.0	30	1.00	30	50	300	1	100	278.6	221.5	
8E+06	37	1	HC	c5	10.0	45	1.00	45	50	300	1.5	150	264.5	263.8	
8E+06	38	2	HC	c5	10.0	45	1.00	45	50	300	1.5	150	285.9	263.8	
8E+06	39	3	HC	c5	10.0	45	1.00	45	50	300	1.5	150	241.1	263.8	
8E+06	40	1	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	672.3	888.3	
8E+06	41	2	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	824.7	888.3	
8E+06	42	3	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1168.0	888.3	
8E+06	43	1	HC	c7	100.0	15	10.00	15	50	300	5	500	774.8	773.0	
8E+06	44	2	HC	c7	100.0	15	10.00	15	50	300	5	500	831.5	773.0	
8E+06	45	3	HC	c7	100.0	15	10.00	15	50	300	5	500	712.7	773.0	
8E+06	46	1	HC	c8	100.0	30	10.00	30	50	300	10	1000	1479.7	1637.2	
8E+06	47	2	HC	c8	100.0	30	10.00	30	50	300	10	1000	1917.1	1637.2	
8E+06	48	3	HC	c8	100.0	30	10.00	30	50	300	10	1000	1514.9	1637.2	

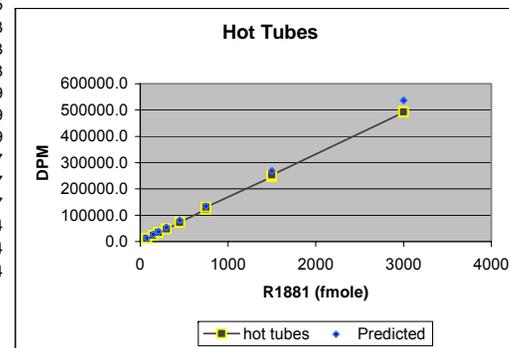
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Saturation Data for Experiment #1 (continued)

Free -- Positions 49-72, radiolabeled R1881 without cytosol										
Run	Position	Rep	Tube Type Code	Conc. Code	Hot R1881 Conc. Initial	Hot R1881 Volume	Molecules of R1881	Counts per Scintillation Vial	Experimental number of molecules	Total Added (Mean of reps in pos. 49-72)
					(nM)	(ul)	(fmole)	(dpm)	(fmole)	(dpm)
8E+06	49	1	Hot	c1	10	7.5	75	12160.9	74	12351.9
8E+06	50	2	Hot	c1	10	7.5	75	12398.4	75	12351.9
8E+06	51	3	Hot	c1	10	7.5	75	12496.3	76	12351.9
8E+06	52	1	Hot	c2	10	15	150	24039.1	146	24341.2
8E+06	53	2	Hot	c2	10	15	150	24534.8	149	24341.2
8E+06	54	3	Hot	c2	10	15	150	24449.6	148	24341.2
8E+06	55	1	Hot	c3	10	21	210	33620.6	204	34065.6
8E+06	56	2	Hot	c3	10	21	210	33973.6	206	34065.6
8E+06	57	3	Hot	c3	10	21	210	34602.5	210	34065.6
8E+06	58	1	Hot	c4	10	30	300	47529.2	289	48141.2
8E+06	59	2	Hot	c4	10	30	300	49129.0	298	48141.2
8E+06	60	3	Hot	c4	10	30	300	47765.4	290	48141.2
8E+06	61	1	Hot	c5	10	45	450	71666.7	435	73078.9
8E+06	62	2	Hot	c5	10	45	450	74826.7	454	73078.9
8E+06	63	3	Hot	c5	10	45	450	72743.2	442	73078.9
8E+06	64	1	Hot	c6	100	7.5	750	122110.2	742	125109.3
8E+06	65	2	Hot	c6	100	7.5	750	122262.3	743	125109.3
8E+06	66	3	Hot	c6	100	7.5	750	130955.4	795	125109.3
8E+06	67	1	Hot	c7	100	15	1500	245627.3	1492	249007.1
8E+06	68	2	Hot	c7	100	15	1500	248112.2	1507	249007.1
8E+06	69	3	Hot	c7	100	15	1500	253281.8	1538	249007.1
8E+06	70	1	Hot	c8	100	30	3000	490699.6	2980	492880.8
8E+06	71	2	Hot	c8	100	30	3000	495123.7	3007	492880.8
8E+06	72	3	Hot	c8	100	30	3000	492819.2	2993	492880.8

predicted dpm

Computation Check	
13414	
13414	
13414	8/11/05 specific activity date
26828	80.56 Ci/mMole 3H R1881
26828	2.22E+12 DPM/Ci (definition)
26828	
37559	1.7885E+14 DPM/mMole
37559	1.7885E+11 DPM/nMole
37559	178.9 DPM/fmole
53655	0.005591 fmole/DPM
53655	



Linear regression results (LINEST function)	
(Regression line forced through 0,0)	
Slope	164.6552726 dpm/fmole
1/slope	0.006073295 fmole/dpm
x	y
origin	0 0
end point	3007.0 495123.7
SLOPE function, used if missing HOT tubes	
Slope	164.7 dpm/fmole
1/slope	0.006071 fmole/dpm
x	y
origin	0 0
end point	3005.8 495123.7

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Saturation Data for Experiment #1 (continued)

Prism input for bound/free		Prism input for specific bound							
specific bound/molar	bound/free	average total added molar	specific bound/molar	specific bound/dpm	total added dpm	total bound dpm	NSB dpm	Hot Final Concentration (nM)	
5.80091E-12	0.02604	2.30207E-10	5.80091E-12	311.3	12160.9	399.2	98.7	0.25	
6.87834E-12	0.03103	2.30207E-10	6.87834E-12	369.1	12398.4	457.0	99.4	0.25	
6.16545E-12	0.02772	2.30207E-10	6.16545E-12	330.8	12496.3	418.8	65.7	0.25	
9.33624E-12	0.02110	4.53657E-10	9.33624E-12	500.9	24039.1	595.8	75.3	0.50	
9.07737E-12	0.02050	4.53657E-10	9.07737E-12	487.1	24534.8	581.9	134.7	0.50	
9.41508E-12	0.02128	4.53657E-10	9.41508E-12	505.2	24449.6	600.1	74.7	0.50	
8.36971E-12	0.01342	6.34895E-10	8.36971E-12	449.1	33620.6	599.0	137.7	0.70	
9.77087E-12	0.01570	6.34895E-10	9.77087E-12	524.3	33973.6	674.2	168.3	0.70	
1.33783E-11	0.02162	6.34895E-10	1.33783E-11	717.8	34602.5	867.7	143.7	0.70	
1.02405E-11	0.01160	8.97229E-10	1.02405E-11	549.5	47529.2	771.0	216.0	1.00	
9.52206E-12	0.01078	8.97229E-10	9.52206E-12	510.9	49129.0	732.4	169.9	1.00	
8.75997E-12	0.00991	8.97229E-10	8.75997E-12	470.0	47765.4	691.5	278.6	1.00	
9.44844E-12	0.00701	1.362E-09	9.44844E-12	507.0	71666.7	770.8	264.5	1.50	
9.14763E-12	0.00679	1.362E-09	9.14763E-12	490.8	74826.7	754.7	285.9	1.50	
9.95501E-12	0.00739	1.362E-09	9.95501E-12	534.1	72743.2	798.0	241.1	1.50	
2.05347E-12	0.00089	2.33172E-09	2.05347E-12	110.2	122110.2	998.5	672.3	2.50	
1.18706E-11	0.00515	2.33172E-09	1.18706E-11	636.9	122262.3	1525.3	824.7	2.50	
5.21867E-12	0.00226	2.33172E-09	5.21867E-12	280.0	130955.4	1168.4	1168.0	2.50	
2.51954E-11	0.00548	4.64086E-09	2.51954E-11	1351.9	245627.3	2124.9	774.8	5.00	
3.65618E-11	0.00797	4.64086E-09	3.65618E-11	1961.7	248112.2	2734.8	831.5	5.00	
1.92201E-11	0.00417	4.64086E-09	1.92201E-11	1031.3	253281.8	1804.3	712.7	5.00	
2.69658E-11	0.00295	9.18604E-09	2.69658E-11	1446.9	490699.6	3084.1	1479.7	10.00	
2.43422E-10	0.02731	9.18604E-09	2.43422E-10	13060.9	495123.7	14698.1	1917.1	10.00	
1.19535E-10	0.01323	9.18604E-09	1.19535E-10	6413.7	492819.2	8050.9	1514.9	10.00	

Saturation Data for Experiment #1 (continued)

D1-8/11/05

One site binding (hyperbola)	Does not converge	Bmax molar	0.00E+00	KD molar	0.00E+00
Best-fit values		mole to molar conversion value	0.0003	molar to nM conversion	1.00E+09
BMAX		DPM/mole = (DPM/mmol)*1000	1.79E+17	kd nM =	0.00E+00
KD		Bmax molar to Bmax moles	0		
Std. Error		= DPM/((DPM/mmol)*1000)	0		
BMAX		=Bmax DPM	0		
KD					
95% Confidence Intervals					
BMAX		assay date	8/11/2005		
KD		Bmax(dpm)	0		
Goodness of Fit		DPM/Ci (definition)	2.22E+12		
Degrees of Freedom		Ci/mmol	80.56		
R ² (unweighted)		DPM/mmol	1.79E+14		
Weighted Sum of Squares (1/Y ²)		DPM/pmol	1.79E+05		
Absolute Sum of Squares		1/(DPM/mmol)	5.59E-15		
Sy.x		1/(DPM/pmol)	5.59E-06		
Data		SA(dpm/pmol)	1.79E+05		
Number of X values		protein/tube (ug)	600		
Number of Y replicates		protein./tube(mg)	0.6		
Total number of values		bmax pmole	0.000000		
Number of missing values		bmax pmole/mg	0		
		Bmax fmole/mg	0		
		Bmax (fmole/100 ug)	0		
		Bmax(fmole/100 ug)/Bmax molar	#DIV/0!		

Saturation Experiment #2

Laboratory D

AR Saturation Assay (cold R1881 and WP supplied by Sequim)

72 assay tubes

Please return by eMail to n.a.Holter@pnl.gov

Provide information in all blue cells in columns O and DK

If the DPM value for a tube was judged unreliable,

Include the DPM value in column O

Provide a reason in column R

The value in column Q will automatically change to FALSE

For your convenience, data reduction is performed in columns

U through BZ, and the values needed for analysis are presented

in columns CF through CN

Cells in column S are presented with a grey background

if the total binding exceeds 10% of the hot added at that concentration, the cytosol concentration is probably too high for good competitive assays

Laboratory Code: D

Run identification: 8162005

Assay start date: 8/16/2005

Tracer lot number: 3559-507

Specific activity on day of assay: 80.50 Ci/mmole

Cytosol lot or vial number:

protein (cytosol) per tube: 600 ug

protein (cytosol) per tube: 0.6 mg

KD 7.39E-01 nM

Bmax 0.83 fmole/100 ug

total volume in tubes 300 uL

volume of ethanol counted: 2 mL

multiply DPM in sample by : 3

Receptor_Notes
diluted to 2 mg/ml for use (0.6 mg/300 ul)

protocol calls for counting decanted EtOH supe
reflects 100ul of reaction mixture processed

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (uM)	Cold R1881 Volume (uL)	Cold Final Concentration (nM)	Triamelenone Acetate (uL)	Cytosol (ul)	Significant portion of label on Vial	dpm as counted	corrected DPM for 2mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
1	1	H	10.0	7.5	0.25	—	—	—	50	300	—	113.60	340.8	TRUE	
2	2	H	10.0	7.5	0.25	—	—	—	50	300	—	138.30	414.9	TRUE	
3	3	H	10.0	7.5	0.25	—	—	—	50	300	—	140.25	420.75	TRUE	
4	1	H	10.0	15	0.50	—	—	—	50	300	—	168.30	504.9	TRUE	
5	2	H	10.0	15	0.50	—	—	—	50	300	—	215.83	647.49	TRUE	
6	3	H	10.0	15	0.50	—	—	—	50	300	—	192.06	576.18	TRUE	
7	1	H	10.0	21	0.70	—	—	—	50	300	—	252.80	758.4	TRUE	
8	2	H	10.0	21	0.70	—	—	—	50	300	—	224.01	672.03	TRUE	
9	3	H	10.0	21	0.70	—	—	—	50	300	—	235.88	707.64	TRUE	
10	1	H	10.0	30	1.00	—	—	—	50	300	—	367.60	1102.8	TRUE	
11	2	H	10.0	30	1.00	—	—	—	50	300	—	291.85	875.55	TRUE	
12	3	H	10.0	30	1.00	—	—	—	50	300	—	393.25	1179.75	TRUE	
13	1	H	10.0	45	1.50	—	—	—	50	300	—	310.29	930.87	TRUE	
14	2	H	10.0	45	1.50	—	—	—	50	300	—	295.81	887.43	TRUE	
15	3	H	10.0	45	1.50	—	—	—	50	300	—	308.87	926.61	TRUE	
16	1	H	100.0	7.5	2.50	—	—	—	50	300	—	446.62	1339.86	TRUE	
17	2	H	100.0	7.5	2.50	—	—	—	50	300	—	499.48	1498.44	TRUE	
18	3	H	100.0	7.5	2.50	—	—	—	50	300	—	454.83	1364.49	TRUE	
19	1	H	100.0	15	5.00	—	—	—	50	300	—	695.62	2086.86	TRUE	
20	2	H	100.0	15	5.00	—	—	—	50	300	—	741.65	2224.95	TRUE	
21	3	H	100.0	15	5.00	—	—	—	50	300	—	690.07	2070.21	TRUE	
22	1	H	100.0	30	10.00	—	—	—	50	300	—	3043.65	9130.95	TRUE	
23	2	H	100.0	30	10.00	—	—	—	50	300	—	1860.73	5582.19	TRUE	
24	3	H	100.0	30	10.00	—	—	—	50	300	—	3455.90	10367.7	TRUE	
25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	62.43	187.29	TRUE	

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Saturation Experiment #2 (continued)

Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (uL)	Hot Final Concentration (nM)	Cold Initial Concentration (uM)	Cold R1881 Volume (uL)	Cold Final Concentration (nM)	Triamcelonone Acetate (uL)	Cyrosol (uL)	Significant portion of label on Vial	dpm as counted	corrected DPM for 2mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	49.83	149.49	TRUE	
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	38.40	115.2	TRUE	
28	1	HC	10.0	15	0.5	1.00	15	50	50	300	C7	66.80	200.4	TRUE	
29	2	HC	10.0	15	0.5	1.00	15	50	50	300	C7	87.41	262.23	TRUE	
30	3	HC	10.0	15	0.5	1.00	15	50	50	300	C7	83.70	251.1	TRUE	
31	1	HC	10.0	21	0.7	1.00	21	70	50	300	C6	83.05	249.15	TRUE	
32	2	HC	10.0	21	0.7	1.00	21	70	50	300	C6	88.89	266.67	TRUE	
33	3	HC	10.0	21	0.7	1.00	21	70	50	300	C6	81.58	244.74	TRUE	
34	1	HC	10.0	30	1	1.00	30	100	50	300	C5	124.71	374.13	TRUE	
35	2	HC	10.0	30	1	1.00	30	100	50	300	C5	125.46	376.38	TRUE	
36	3	HC	10.0	30	1	1.00	30	100	50	300	C5	103.11	309.33	TRUE	
37	1	HC	10.0	45	1.5	1.00	45	150	50	300	C4	141.86	425.58	TRUE	
38	2	HC	10.0	45	1.5	1.00	45	150	50	300	C4	122.61	367.83	TRUE	
39	3	HC	10.0	45	1.5	1.00	45	150	50	300	C4	192.67	578.01	TRUE	
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	193.29	579.87	TRUE	
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	205.97	617.91	TRUE	
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	180.76	542.28	TRUE	
43	1	HC	100.0	15	5	10.00	15	500	50	300	C2	421.65	1264.95	TRUE	
44	2	HC	100.0	15	5	10.00	15	500	50	300	C2	514.63	1543.89	TRUE	
45	3	HC	100.0	15	5	10.00	15	500	50	300	C2	492.48	1477.44	TRUE	
46	1	HC	100.0	30	10	10.00	30	1000	50	300	C1	798.37	2395.11	TRUE	
47	2	HC	100.0	30	10	10.00	30	1000	50	300	C1	861.83	2585.49	TRUE	
48	3	HC	100.0	30	10	10.00	30	1000	50	300	C1	1143.12	3429.36	TRUE	
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12262.38	12262.38	TRUE	
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12570.89	12570.89	TRUE	
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—	—	12187.35	12187.35	TRUE	
52	1	Hot	10.0	15	0.06	—	—	—	—	—	—	25361.83	25361.83	TRUE	
53	2	Hot	10.0	15	0.06	—	—	—	—	—	—	25598.13	25598.13	TRUE	
54	3	Hot	10.0	15	0.06	—	—	—	—	—	—	26000.92	26000.92	TRUE	
55	1	Hot	10.0	21	0.08	—	—	—	—	—	—	34291.32	34291.32	TRUE	
56	2	Hot	10.0	21	0.08	—	—	—	—	—	—	35160.85	35160.85	TRUE	
57	3	Hot	10.0	21	0.08	—	—	—	—	—	—	37403.21	37403.21	TRUE	
58	1	Hot	10.0	30	0.10	—	—	—	—	—	—	58027.12	58027.12	TRUE	
59	2	Hot	10.0	30	0.10	—	—	—	—	—	—	48064.03	48064.03	TRUE	
60	3	Hot	10.0	30	0.10	—	—	—	—	—	—	49643.66	49643.66	TRUE	
61	1	Hot	10.0	45	0.30	—	—	—	—	—	—	74345.88	74345.88	TRUE	
62	2	Hot	10.0	45	0.30	—	—	—	—	—	—	72949.51	72949.51	TRUE	
63	3	Hot	10.0	45	0.30	—	—	—	—	—	—	72443.30	72443.3	TRUE	
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—	—	119031.40	119031.4	TRUE	
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—	—	120032.60	120032.6	TRUE	
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—	—	119425.30	119425.3	TRUE	
67	1	Hot	100.0	15	1.00	—	—	—	—	—	—	240015.60	240015.6	TRUE	
68	2	Hot	100.0	15	1.00	—	—	—	—	—	—	245155.20	245155.2	TRUE	
69	3	Hot	100.0	15	1.00	—	—	—	—	—	—	253835.30	253835.3	TRUE	
70	1	Hot	100.0	30	3.00	—	—	—	—	—	—	497270.30	497270.3	TRUE	
71	2	Hot	100.0	30	3.00	—	—	—	—	—	—	482522.70	482522.7	TRUE	
72	3	Hot	100.0	30	3.00	—	—	—	—	—	—	490549.10	490549.1	TRUE	

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Saturation Experiment #2 (continued)

Ten Percent Rule	Saturation X values, Bound y values, NSB y values			Run	Position	Total Binding -- Positions 1-24 radiolabeled R1881 plus cytosol (Panel A)											
						Tube Identification			Assay tube contents							Total Volume	
						Rep	Tube Type Code	Conc. Code	Hot Conc. Initial (nM)	Hot R1881 Volume (ul)	Cold R1881 Conc. Initial (mM)	Cold R1881 volume (ul)	Triamcetenone Acetate (ul)	Cytosol (ul)	Hot Conc. Final (nM)		Cold Conc. Final (nM)
2.8%	0.25	340.8	150.7	8E+06	1	1	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
3.4%	0.25	414.9	150.7	8E+06	2	2	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
3.4%	0.25	420.8	150.7	8E+06	3	3	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
2.0%	0.5	504.9	237.9	8E+06	4	1	H	c2	10.0	15	—	—	—	300	0.50	—	300
2.5%	0.5	647.5	237.9	8E+06	5	2	H	c2	10.0	15	—	—	—	300	0.50	—	300
2.2%	0.5	576.2	237.9	8E+06	6	3	H	c2	10.0	15	—	—	—	300	0.50	—	300
2.1%	0.7	758.4	253.5	8E+06	7	1	H	c3	10.0	21	—	—	—	300	0.70	—	300
1.9%	0.7	672.0	253.5	8E+06	8	2	H	c3	10.0	21	—	—	—	300	0.70	—	300
2.0%	0.7	707.6	253.5	8E+06	9	3	H	c3	10.0	21	—	—	—	300	0.70	—	300
2.1%	1	1102.8	353.3	8E+06	10	1	H	c4	10.0	30	—	—	—	300	1.00	—	300
1.7%	1	875.6	353.3	8E+06	11	2	H	c4	10.0	30	—	—	—	300	1.00	—	300
2.3%	1	1179.8	353.3	8E+06	12	3	H	c4	10.0	30	—	—	—	300	1.00	—	300
1.3%	1.5	930.9	457.1	8E+06	13	1	H	c5	10.0	45	—	—	—	300	1.50	—	300
1.2%	1.5	887.4	457.1	8E+06	14	2	H	c5	10.0	45	—	—	—	300	1.50	—	300
1.3%	1.5	926.6	457.1	8E+06	15	3	H	c5	10.0	45	—	—	—	300	1.50	—	300
1.1%	2.5	1339.9	580.0	8E+06	16	1	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
1.3%	2.5	1498.4	580.0	8E+06	17	2	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
1.1%	2.5	1364.5	580.0	8E+06	18	3	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
0.8%	5	2086.9	1428.8	8E+06	19	1	H	c7	100.0	15	—	—	—	300	5.00	—	300
0.9%	5	2225.0	1428.8	8E+06	20	2	H	c7	100.0	15	—	—	—	300	5.00	—	300
0.8%	5	2070.2	1428.8	8E+06	21	3	H	c7	100.0	15	—	—	—	300	5.00	—	300
1.9%	10	9131.0	2803.3	8E+06	22	1	H	c8	100.0	30	—	—	—	300	10.00	—	300
1.1%	10	5582.2	2803.3	8E+06	23	2	H	c8	100.0	30	—	—	—	300	10.00	—	300
2.1%	10	10367.7	2803.3	8E+06	24	3	H	c8	100.0	30	—	—	—	300	10.00	—	300

Saturation Experiment #2 (continued)

Run	Position							Number of molecules					Ratio	
		Total Counts (dpm)	Non Specific Binding (Mean of reps in pos. 25-48) (dpm)	Specific Binding (Total Non Specific) (dpm)	Ratio of NSB/ total binding	Ratio Total binding/ Hot	Total Added (Mean of reps in pos. 49-72) (dpm)	Free (total added - bound) (dpm)	Total Binding molecules (fmole)	Non Specific Binding molecules (fmole)	Specific Binding molecules (fmole)	Total Added (Mean of reps in pos. 49-72) (fmole)	Free (total added - bound) (fmole)	Specific Bound / Free
8E+06	1	340.8	150.7	190.1	44.2%	2.8%	12340.2	11999.4	2	1	1	76	73	0.02
8E+06	2	414.9	150.7	264.2	36.3%	3.4%	12340.2	11925.3	3	1	2	76	73	0.02
8E+06	3	420.8	150.7	270.1	35.8%	3.4%	12340.2	11919.5	3	1	2	76	73	0.02
8E+06	4	504.9	237.9	267.0	47.1%	2.0%	25653.6	25148.7	3	1	2	157	154	0.01
8E+06	5	647.5	237.9	409.6	36.7%	2.5%	25653.6	25006.1	4	1	3	157	153	0.02
8E+06	6	576.2	237.9	338.3	41.3%	2.2%	25653.6	25077.4	4	1	2	157	153	0.01
8E+06	7	758.4	253.5	504.9	33.4%	2.1%	35618.5	34860.1	5	2	3	218	213	0.01
8E+06	8	672.0	253.5	418.5	37.7%	1.9%	35618.5	34946.4	4	2	3	218	214	0.01
8E+06	9	707.6	253.5	454.1	35.8%	2.0%	35618.5	34910.8	4	2	3	218	214	0.01
8E+06	10	1102.8	353.3	749.5	32.0%	2.1%	51911.6	50808.8	7	2	5	318	311	0.01
8E+06	11	875.6	353.3	522.3	40.3%	1.7%	51911.6	51036.1	5	2	3	318	312	0.01
8E+06	12	1179.8	353.3	826.5	29.9%	2.3%	51911.6	50731.9	7	2	5	318	310	0.02
8E+06	13	930.9	457.1	473.7	49.1%	1.3%	73246.2	72315.4	6	3	3	448	442	0.01
8E+06	14	887.4	457.1	430.3	51.5%	1.2%	73246.2	72358.8	5	3	3	448	443	0.01
8E+06	15	926.6	457.1	469.5	49.3%	1.3%	73246.2	72319.6	6	3	3	448	442	0.01
8E+06	16	1339.9	580.0	759.8	43.3%	1.1%	119496.4	118156.6	8	4	5	731	723	0.01
8E+06	17	1498.4	580.0	918.4	38.7%	1.3%	119496.4	117998.0	9	4	6	731	722	0.01
8E+06	18	1364.5	580.0	784.5	42.5%	1.1%	119496.4	118131.9	8	4	5	731	723	0.01
8E+06	19	2086.9	1428.8	658.1	68.5%	0.8%	246335.4	244248.5	13	9	4	1507	1494	0.00
8E+06	20	2225.0	1428.8	796.2	64.2%	0.9%	246335.4	244110.4	14	9	5	1507	1494	0.00
8E+06	21	2070.2	1428.8	641.5	69.0%	0.8%	246335.4	244265.2	13	9	4	1507	1495	0.00
8E+06	22	9131.0	2803.3	6327.6	30.7%	1.9%	490114.0	480983.1	56	17	39	2999	2943	0.01
8E+06	23	5582.2	2803.3	2778.9	50.2%	1.1%	490114.0	484531.8	34	17	17	2999	2965	0.01
8E+06	24	10367.7	2803.3	7564.4	27.0%	2.1%	490114.0	479746.3	63	17	46	2999	2935	0.02

Saturation Experiment #2 (continued)

		Non Specific Binding -- Positions 25-48 radiolabeled R1881 plus 100 X inert R1881 plus cytosol													
Run	Position	Tube Identification			Assay tube contents								Scintillation Results		
		Rep	Tube Type Code	Conc. Code	Hot Conc. R1881 Initial		Cold R1881 Conc. Initial		Triamcelenone Acetate	Cytosol	Hot Conc. Final		Cold Conc. Final	Counts per Scintillation Vial (Total Binding)	Non Specific Binding (Mean of reps in pos. 25-48)
					(nM)	(ul)	(mM)	(ul)			(nM)	(nM)			
8E+06	25	1	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	187.3	150.7	
8E+06	26	2	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	149.5	150.7	
8E+06	27	3	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	115.2	150.7	
8E+06	28	1	HC	c2	10.0	15	1.00	15	50	300	0.5	50	200.4	237.9	
8E+06	29	2	HC	c2	10.0	15	1.00	15	50	300	0.5	50	262.2	237.9	
8E+06	30	3	HC	c2	10.0	15	1.00	15	50	300	0.5	50	251.1	237.9	
8E+06	31	1	HC	c3	10.0	21	1.00	21	50	300	0.7	70	249.2	253.5	
8E+06	32	2	HC	c3	10.0	21	1.00	21	50	300	0.7	70	266.7	253.5	
8E+06	33	3	HC	c3	10.0	21	1.00	21	50	300	0.7	70	244.7	253.5	
8E+06	34	1	HC	c4	10.0	30	1.00	30	50	300	1	100	374.1	353.3	
8E+06	35	2	HC	c4	10.0	30	1.00	30	50	300	1	100	376.4	353.3	
8E+06	36	3	HC	c4	10.0	30	1.00	30	50	300	1	100	309.3	353.3	
8E+06	37	1	HC	c5	10.0	45	1.00	45	50	300	1.5	150	425.6	457.1	
8E+06	38	2	HC	c5	10.0	45	1.00	45	50	300	1.5	150	367.8	457.1	
8E+06	39	3	HC	c5	10.0	45	1.00	45	50	300	1.5	150	578.0	457.1	
8E+06	40	1	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	579.9	580.0	
8E+06	41	2	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	617.9	580.0	
8E+06	42	3	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	542.3	580.0	
8E+06	43	1	HC	c7	100.0	15	10.00	15	50	300	5	500	1265.0	1428.8	
8E+06	44	2	HC	c7	100.0	15	10.00	15	50	300	5	500	1543.9	1428.8	
8E+06	45	3	HC	c7	100.0	15	10.00	15	50	300	5	500	1477.4	1428.8	
8E+06	46	1	HC	c8	100.0	30	10.00	30	50	300	10	1000	2395.1	2803.3	
8E+06	47	2	HC	c8	100.0	30	10.00	30	50	300	10	1000	2585.5	2803.3	
8E+06	48	3	HC	c8	100.0	30	10.00	30	50	300	10	1000	3429.4	2803.3	

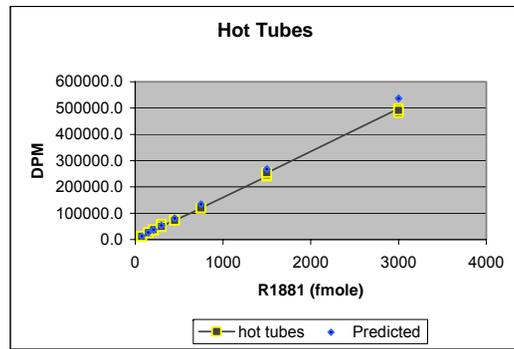
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Saturation Experiment #2 (continued)

Free -- Positions 49-72, radiolabeled R1881 without cytosol										
Run	Position	Rep	Tube Type Code	Conc. Code	Hot R1881 Conc. Initial (nM)	Hot R1881 Volume (ul)	Molecules of R1881 (fmole)	Counts per Scintillation Vial (dpm)	Experimental number of molecules (fmole)	Total Added (Mean of reps in pos. 49-72) (dpm)
8E+06	49	1	Hot	c1	10	7.5	75	12262.4	75	12340.2
8E+06	50	2	Hot	c1	10	7.5	75	12570.9	77	12340.2
8E+06	51	3	Hot	c1	10	7.5	75	12187.4	75	12340.2
8E+06	52	1	Hot	c2	10	15	150	25361.8	155	25653.6
8E+06	53	2	Hot	c2	10	15	150	25598.1	157	25653.6
8E+06	54	3	Hot	c2	10	15	150	26000.9	159	25653.6
8E+06	55	1	Hot	c3	10	21	210	34291.3	210	35618.5
8E+06	56	2	Hot	c3	10	21	210	35160.9	215	35618.5
8E+06	57	3	Hot	c3	10	21	210	37403.2	229	35618.5
8E+06	58	1	Hot	c4	10	30	300	58027.1	355	51911.6
8E+06	59	2	Hot	c4	10	30	300	48064.0	294	51911.6
8E+06	60	3	Hot	c4	10	30	300	49643.7	304	51911.6
8E+06	61	1	Hot	c5	10	45	450	74345.9	455	73246.2
8E+06	62	2	Hot	c5	10	45	450	72949.5	446	73246.2
8E+06	63	3	Hot	c5	10	45	450	72443.3	443	73246.2
8E+06	64	1	Hot	c6	100	7.5	750	119031.4	728	119496.4
8E+06	65	2	Hot	c6	100	7.5	750	120032.6	734	119496.4
8E+06	66	3	Hot	c6	100	7.5	750	119425.3	731	119496.4
8E+06	67	1	Hot	c7	100	15	1500	240015.6	1469	246335.4
8E+06	68	2	Hot	c7	100	15	1500	245155.2	1500	246335.4
8E+06	69	3	Hot	c7	100	15	1500	253835.3	1553	246335.4
8E+06	70	1	Hot	c8	100	30	3000	497270.3	3043	490114.0
8E+06	71	2	Hot	c8	100	30	3000	482522.7	2952	490114.0
8E+06	72	3	Hot	c8	100	30	3000	490549.1	3001	490114.0

predicted dpm

Computation Check	
13404	
13404	
13404	8/16/05 specific activity date
26807	80.50 Ci/mMole 3H R1881
26807	2.22E+12 DPM/Ci (definition)
26807	
37530	1.7871E+14 DPM/mMole
37530	1.7871E+11 DPM/nMole
37530	178.7 DPM/fmole
53614	0.005596 fmole/DPM
53614	



Linear regression results (LINEST function)	
(Regression line forced through 0,0)	
Slope	163.4407836 dpm/fmole
1/slope	0.006118424 fmole/dpm
x	y
origin	0 0
end point	3042.5 497270.3
SLOPE function, used if missing HOT tubes	
Slope	163.1 dpm/fmole
1/slope	0.006131 fmole/dpm
x	y
origin	0 0
end point	3048.9 497270.3

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Saturation Experiment #2 (continued)

Prism input for bound/free		Prism input for specific bound							
specific bound/molar	bound/free	average total added molar	specific bound/molar	specific bound/dpm	total added dpm	total bound dpm	NSB dpm	Hot Final Concentration (nM)	
3.54645E-12	0.01585	2.30167E-10	3.54645E-12	190.1	12262.4	340.8	187.3	0.25	
4.92854E-12	0.02216	2.30167E-10	4.92854E-12	264.2	12570.9	414.9	149.5	0.25	
5.03766E-12	0.02266	2.30167E-10	5.03766E-12	270.1	12187.4	420.8	115.2	0.25	
4.97984E-12	0.01062	4.78486E-10	4.97984E-12	267.0	25361.8	504.9	200.4	0.50	
7.63939E-12	0.01638	4.78486E-10	7.63939E-12	409.6	25598.1	647.5	262.2	0.50	
6.30934E-12	0.01349	4.78486E-10	6.30934E-12	338.3	26000.9	576.2	251.1	0.50	
9.41691E-12	0.01448	6.64347E-10	9.41691E-12	504.9	34291.3	758.4	249.2	0.70	
7.80595E-12	0.01198	6.64347E-10	7.80595E-12	418.5	35160.9	672.0	266.7	0.70	
8.47014E-12	0.01301	6.64347E-10	8.47014E-12	454.1	37403.2	707.6	244.7	0.70	
1.39799E-11	0.01475	9.68243E-10	1.39799E-11	749.5	58027.1	1102.8	374.1	1.00	
9.74126E-12	0.01023	9.68243E-10	9.74126E-12	522.3	48064.0	875.6	376.4	1.00	
1.54151E-11	0.01629	9.68243E-10	1.54151E-11	826.5	49643.7	1179.8	309.3	1.00	
8.8359E-12	0.00655	1.36617E-09	8.8359E-12	473.7	74345.9	930.9	425.6	1.50	
8.02567E-12	0.00595	1.36617E-09	8.02567E-12	430.3	72949.5	887.4	367.8	1.50	
8.75645E-12	0.00649	1.36617E-09	8.75645E-12	469.5	72443.3	926.6	578.0	1.50	
1.41724E-11	0.00643	2.22882E-09	1.41724E-11	759.8	119031.4	1339.9	579.9	2.50	
1.71302E-11	0.00778	2.22882E-09	1.71302E-11	918.4	120032.6	1498.4	617.9	2.50	
1.46318E-11	0.00664	2.22882E-09	1.46318E-11	784.5	119425.3	1364.5	542.3	2.50	
1.22747E-11	0.00269	4.59459E-09	1.22747E-11	658.1	240015.6	2086.9	1265.0	5.00	
1.48504E-11	0.00326	4.59459E-09	1.48504E-11	796.2	245155.2	2225.0	1543.9	5.00	
1.19642E-11	0.00263	4.59459E-09	1.19642E-11	641.5	253835.3	2070.2	1477.4	5.00	
1.18022E-10	0.01316	9.1415E-09	1.18022E-10	6327.6	497270.3	9131.0	2395.1	10.00	
5.18309E-11	0.00574	9.1415E-09	5.18309E-11	2778.9	482522.7	5582.2	2585.5	10.00	
1.41089E-10	0.01577	9.1415E-09	1.41089E-10	7564.4	490549.1	10367.7	3429.4	10.00	

Saturation Experiment #2 (continued)

D2-8/16/05

One site binding (hyperbola)		Bmax molar	1.66E-11	KD molar	7.39E-10
Best-fit values		mole to molar conversion value	0.0003	molar to nM conversion	1.00E+09
BMAX	1.66E-11	DPM/mole = (DPM/mmol)*1000	1.79E+17	kd nM =	7.39E-01
KD	7.39E-10	Bmax molar to Bmax moles	4.986E-15		
Std. Error		= DPM/((DPM/mmol)*1000)	4.986E-15		
BMAX	3.11E-12	=Bmax DPM	891.0680834		
KD	2.96E-10				
95% Confidence Intervals		assay date	8/16/2005		
BMAX	1.018e-011 to 2.30	Bmax(dpm)	891.0680834		
KD	1.252e-010 to 1.35	DPM/Ci (definition)	2.22E+12		
Goodness of Fit		Ci/mmol	80.50		
Degrees of Freedom	22	DPM/mmol	1.79E+14		
R ² (unweighted)	-0.003867	DPM/pmol	1.79E+05		
Weighted Sum of Squares	2.952	1/(DPM/mmol)	5.60E-15		
Absolute Sum of Squares	2.78E-20	1/(DPM/pmol)	5.60E-06		
Sy.x	3.55E-11	SA(dpm/pmol)	1.79E+05		
Data		protein/tube (ug)	600		
Number of X values	24	protein./tube(mg)	0.6		
Number of Y replicates	1	bmax pmole	0.004986		
Total number of values	24	bmax pmole/mg	0.00831		
Number of missing values	0	Bmax fmole/mg	8.31		
		Bmax (fmole/100 ug)	0.831		
		Bmax(fmole/100 ug)/Bmax molar	5.00E+10		

ABC Study No. 49655

CYTOSOL CHECK DATA

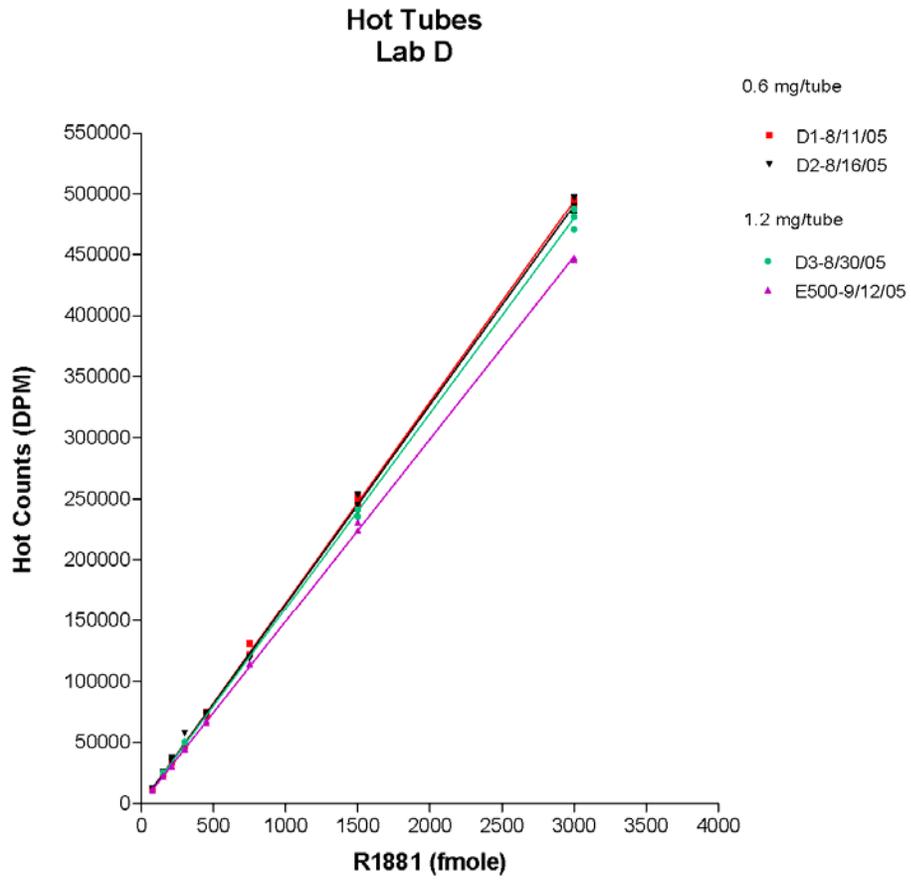
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Bmax-Kd WA4-11-5 or 3 AR.xls

Bmax-Kd

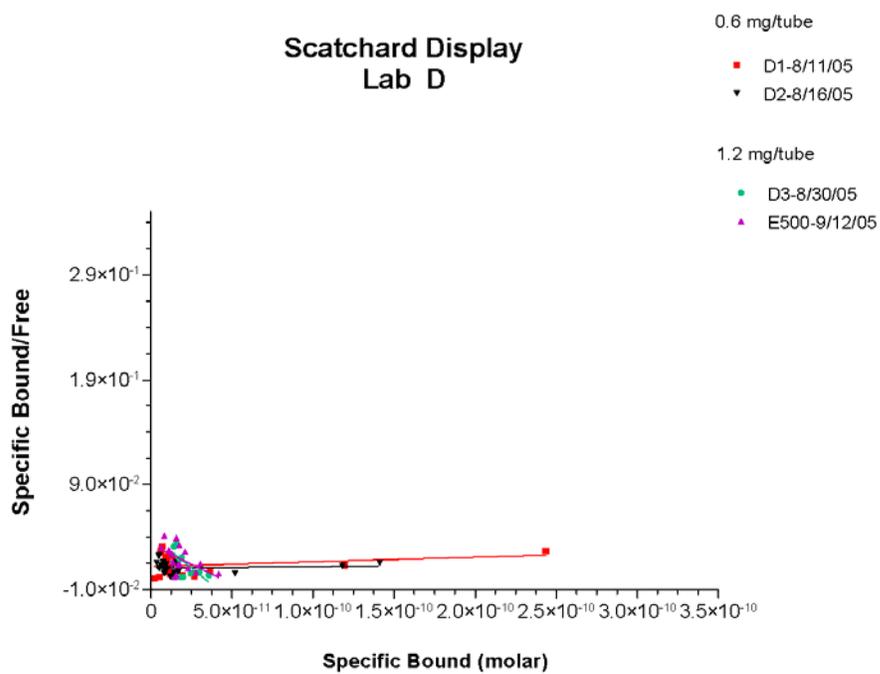
LabB	1	2	3		
date	7/5/2005	7/7/2005	7/11/2005		
Bmax (fmole/100 ug)	5.30	6.67	5.32		
Kd (nM)	0.70	1.00	0.66		
LabE	476	478	480	484	500
date	7/10/2005	7/11/2005	7/13/2005	7/25/2005	9/12/2005
Bmax (fmole/100 ug)	15.03	14.41	14.40	12.26	0.66
Kd (nM)	0.94	0.93	0.93	0.83	0.38
LabC	1a (Seq#6397a)	2 (Seq#6398)	3 (Seq#6399)		
date	7/6/2005	7/12/2005	7/19/2005		
Bmax (fmole/100 ug)	14.26	16.76	15.29		
Kd (nM)	0.83	0.93	1.03		
LabA	S3-071805	S4-081605	S4-081605	S5-082305	
date	7/18/2005	8/16/2005	8/16/2005	8/23/2005	
Bmax (fmole/100 ug)	6.24	7.40	6.10	5.72	
Kd (nM)	1.92	2.65	2.00	2.88	
LabD	8112005	8162005	83005		
date	8/11/2005	8/16/2005	8/30/2005		
Bmax (fmole/100 ug)	0.00	0.83	0.61		
Kd (nM)	0.00	0.74	0.31		

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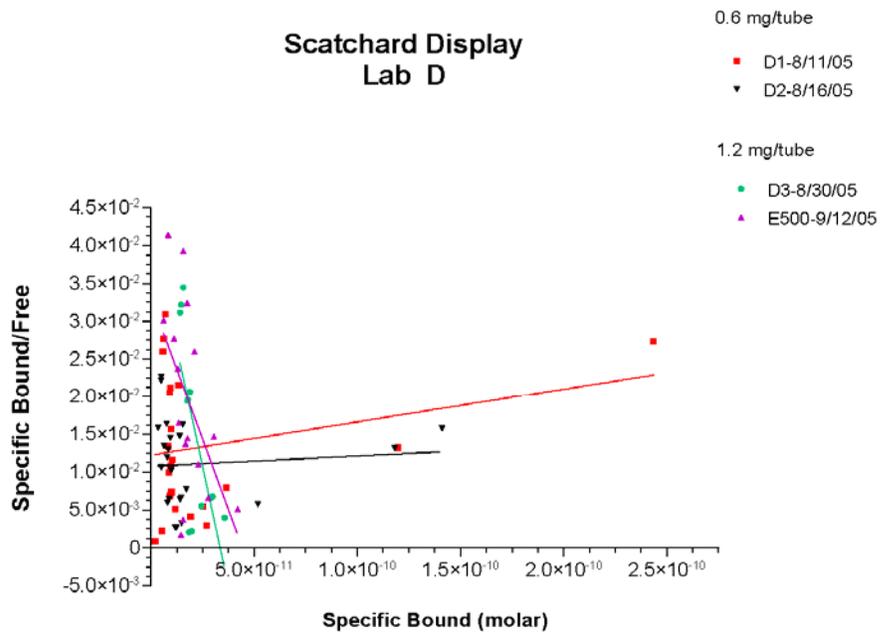


ABC Study No. 49655

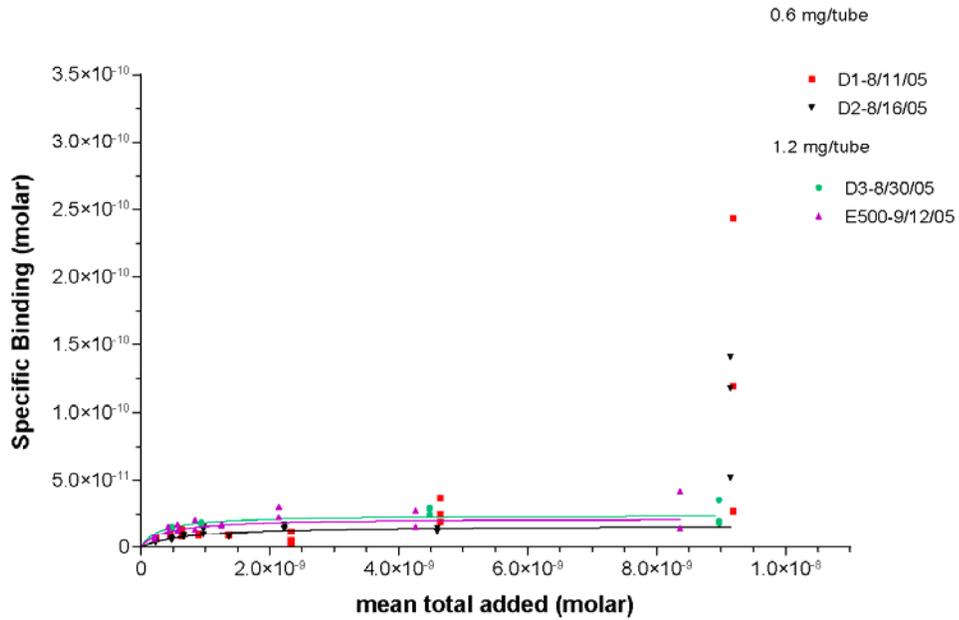
4-11-3-Saturation_D.pzf.D Scatchard (molar) - Thu Sep 15 10:23:07 2005



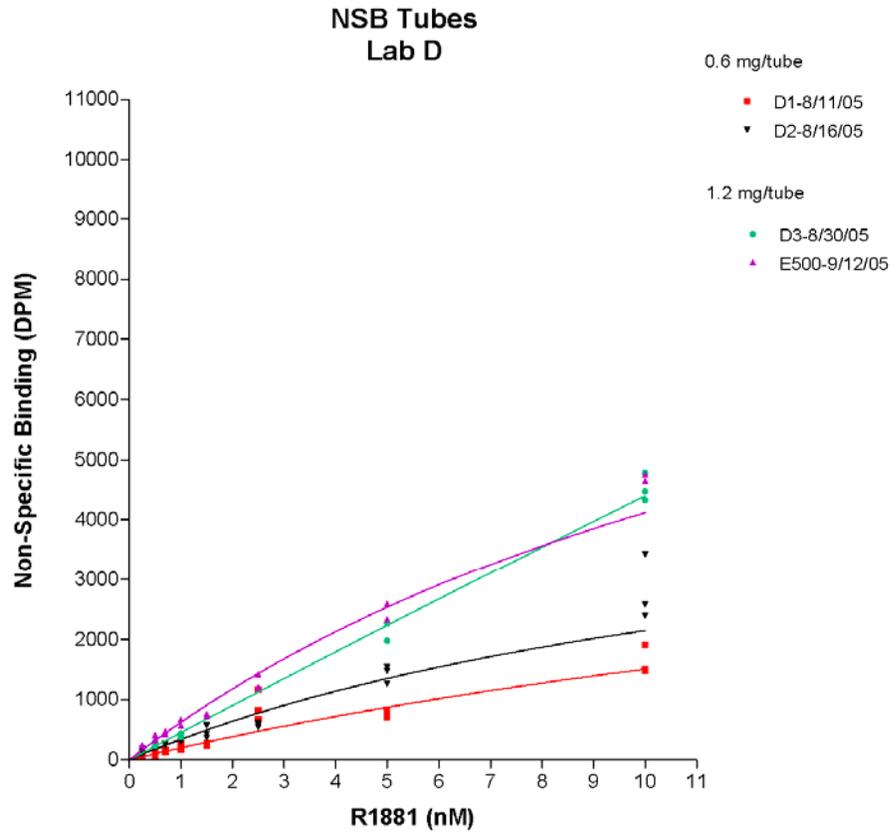
CONFIDENTIAL



Lab D



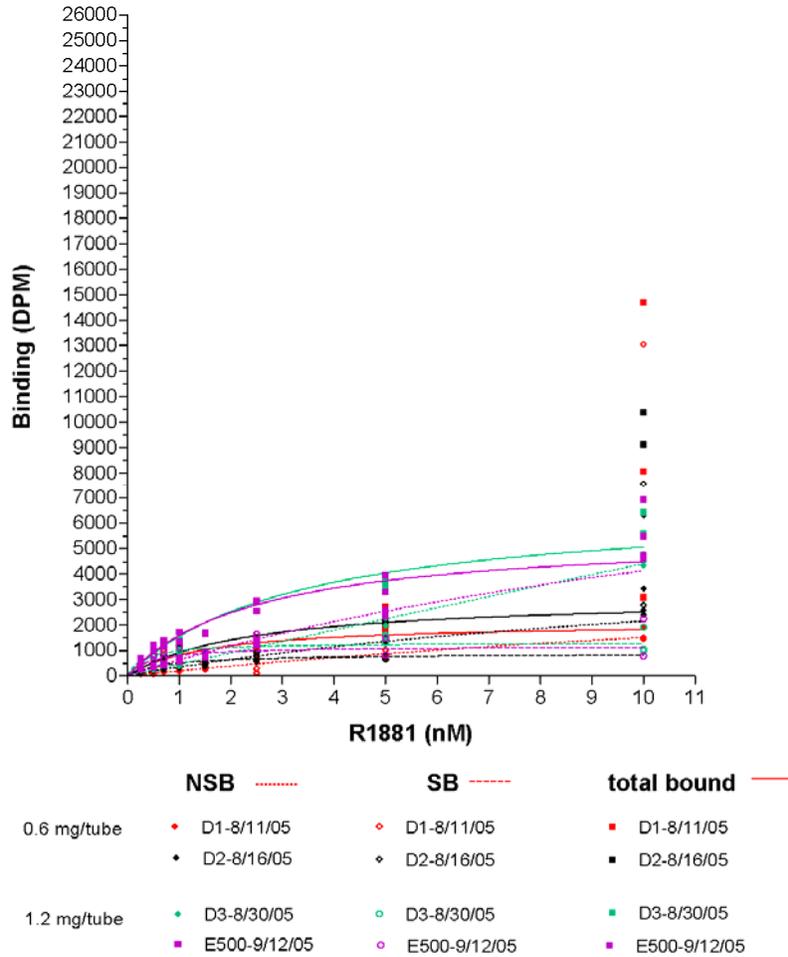
Specific bound	D1-8/11/05			
One site binding (hyperbola)	Does not converge.			
Best-fit values		D2-8/16/05	D3-8/30/05	E500-9/12/05
BMAX		1.662e-011	2.442e-011	2.184e-011
KD		7.389e-010	3.074e-010	3.770e-010
Std. Error				
BMAX		3.107e-012	2.142e-012	2.776e-012
KD		2.959e-010	1.128e-010	1.318e-010
95% Confidence Intervals				
BMAX		1.018e-011 to 2.306e-011	1.964e-011 to 2.919e-011	1.589e-011 to 2.780e-011
KD		1.252e-010 to 1.353e-009	5.619e-011 to 5.587e-010	9.430e-011 to 6.596e-010
Goodness of Fit				
Degrees of Freedom		22	10	14
R ² (unweighted)		-0.003867	0.4622	0.3348
Weighted Sum of Squares (1/Y ²)		2.952	0.3411	1.103
Absolute Sum of Squares		2.779e-020	2.789e-022	8.050e-022
Sy.x		3.554e-011	5.281e-012	7.583e-012
Data				
Number of X values		24	24	23
Number of Y replicates		1	1	1
Total number of values		24	12	16
Number of missing values		0	12	7



ABC Study No. 49655

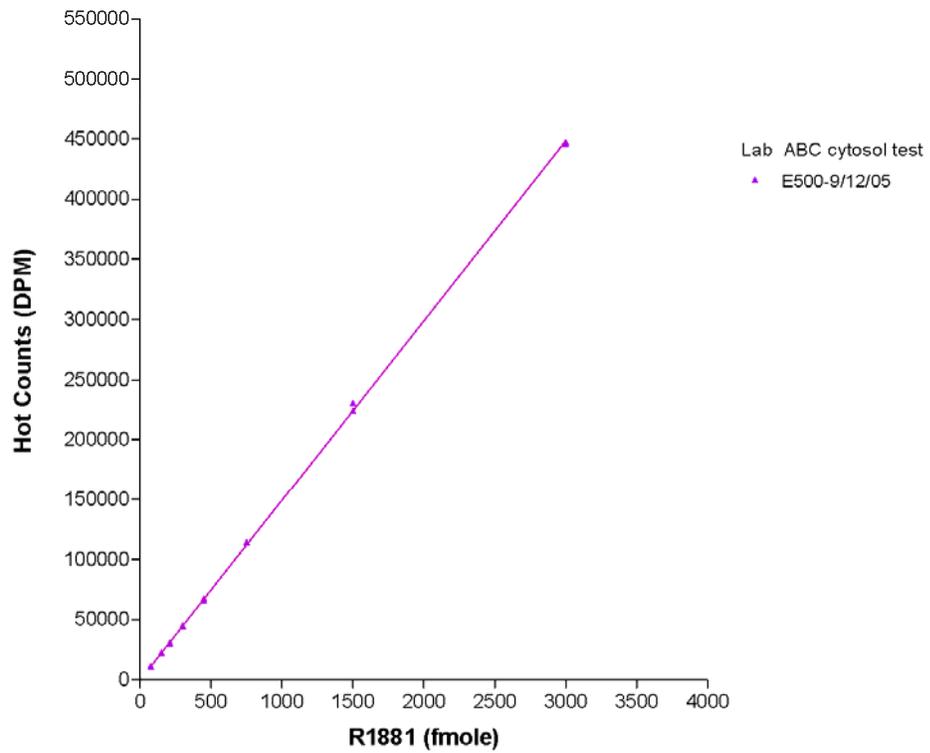
4-11-3-Saturation_D.pzf.D data (DPM) - Thu Sep 15 10:23:07 2005

bound counts
Lab D

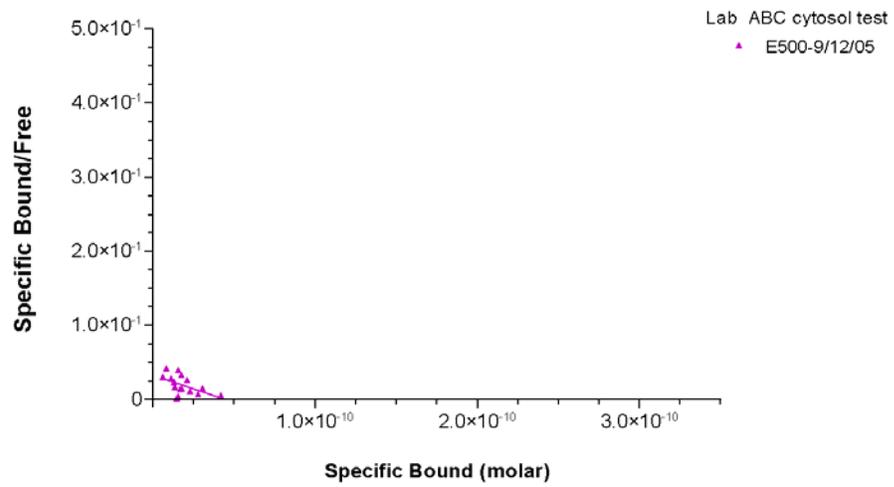


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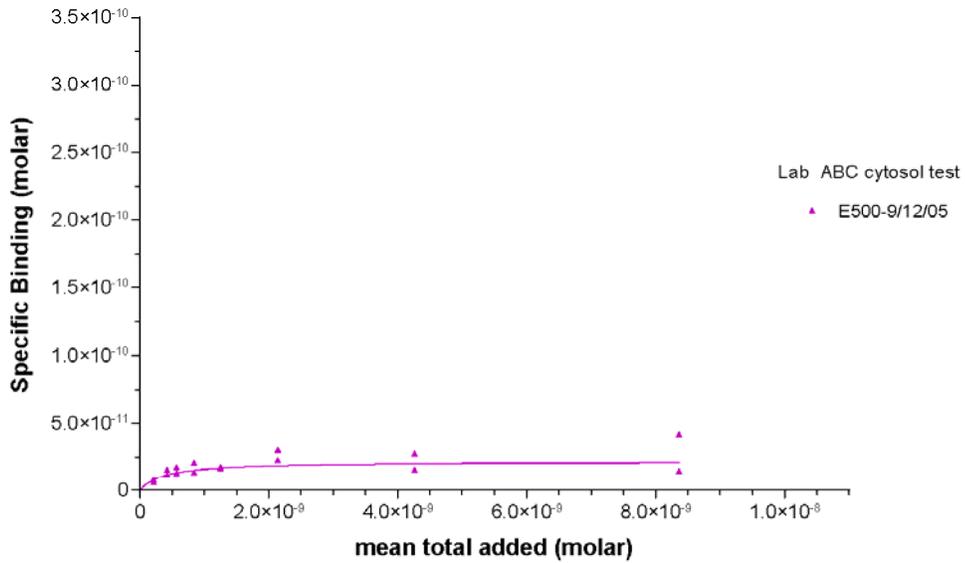
**Hot Tubes
Lab E Run 500
1.0 mg/tube**



Scatchard Display
Lab E Run 500
1.0 mg/tube

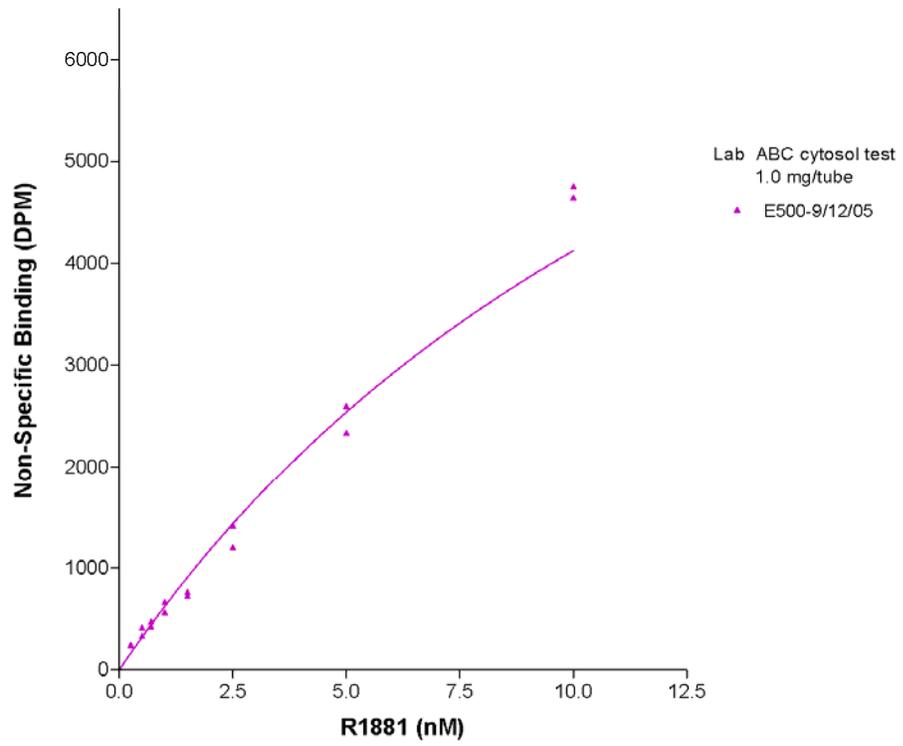


Lab E run 500
1.0 mg/tube



E500-9/12/05	
BMAX	2.184e-011
KD	3.770e-010
Std. Error	
BMAX	2.776e-012
KD	1.318e-010
95% Confidence Intervals	
BMAX	1.589e-011 to 2.780e-011
KD	9.430e-011 to 6.596e-010
Goodness of Fit	
Degrees of Freedom	14
R ² (unweighted)	0.3348
Weighted Sum of Squares (1/Y ²)	1.103
Absolute Sum of Squares	8.050e-022
Syx	7.583e-012
Data	
Number of X values	23
Number of Y replicates	1
Total number of values	16
Number of missing values	7

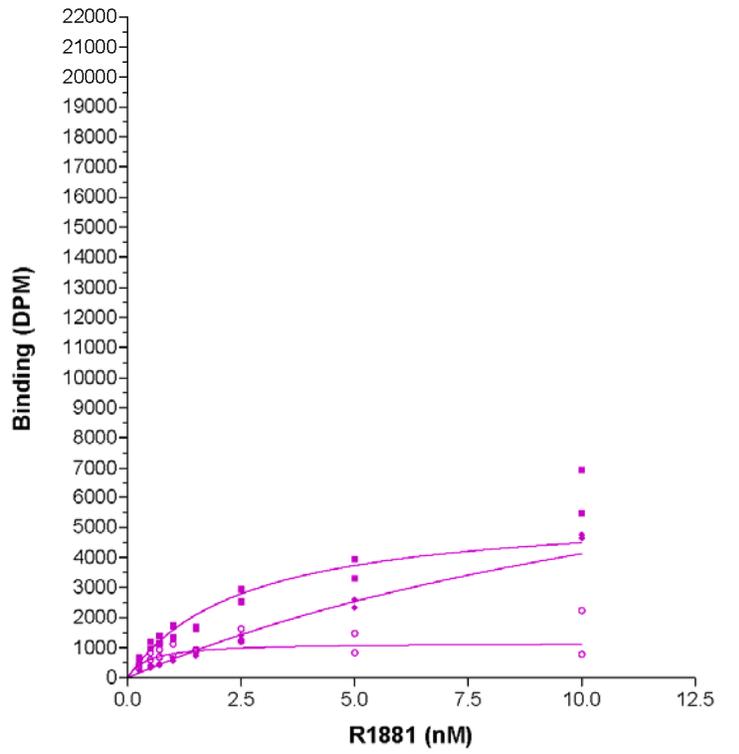
NSB Tubes
Lab E Run 500
1.0 mg/tube



ABC Study No. 49655

4-11-5-Saturation_E.pzf:E500 data (DPM) - Thu Sep 15 10:17:51 2005

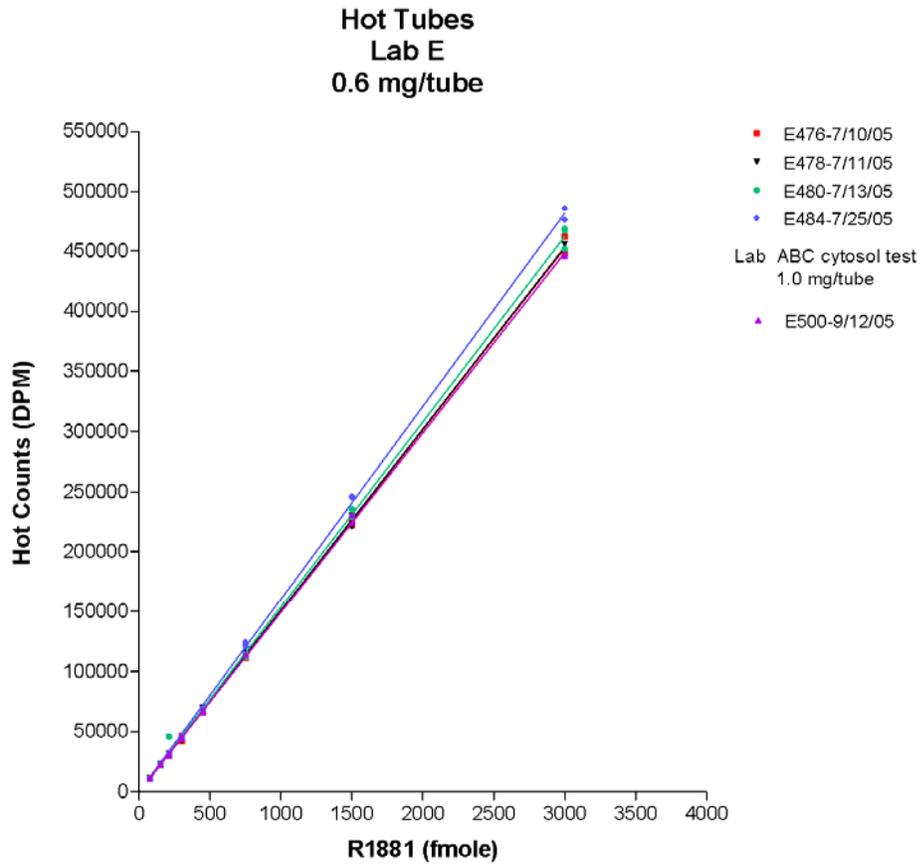
bound counts
Lab E Run 500
1.0 mg/tube



NSB SB total bound

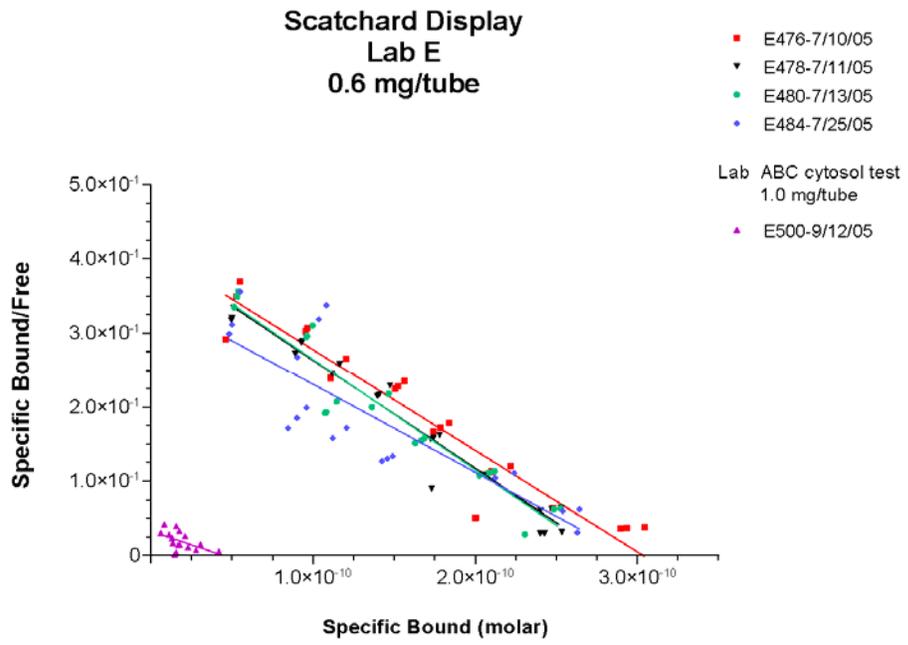
Lab ABC cytosol test
1.0 mg/tube

• E500-9/12/05 ◊ E500-9/12/05 ■ E500-9/12/05



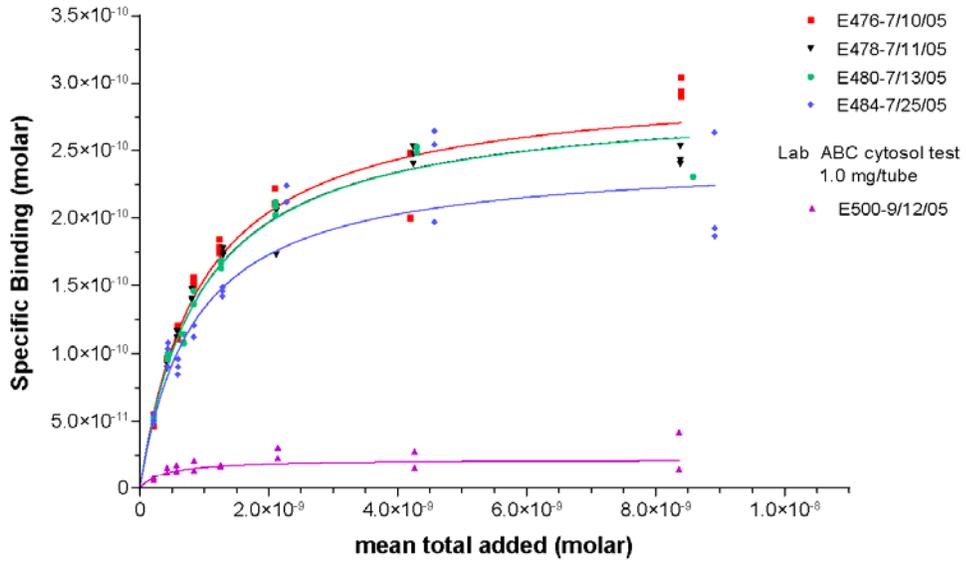
ABC Study No. 49655

4-11-5-Saturation_E.pzf.E Scatchard (molar) - Thu Sep 15 10:17:51 2005

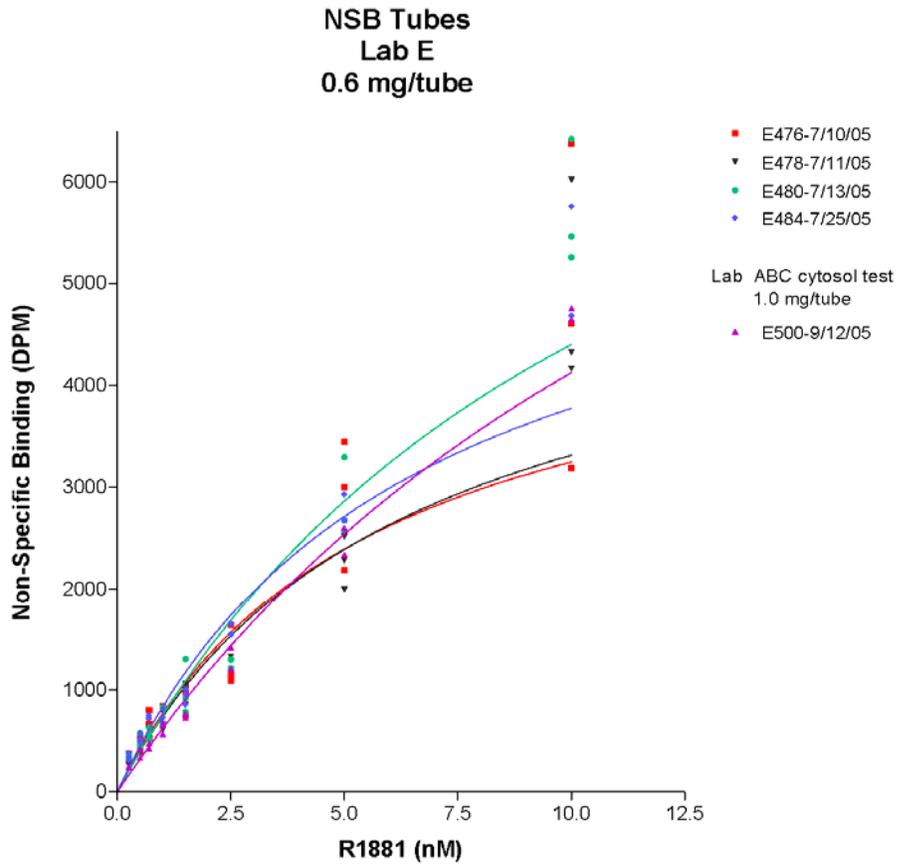


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Lab E
0.6 mg/tube

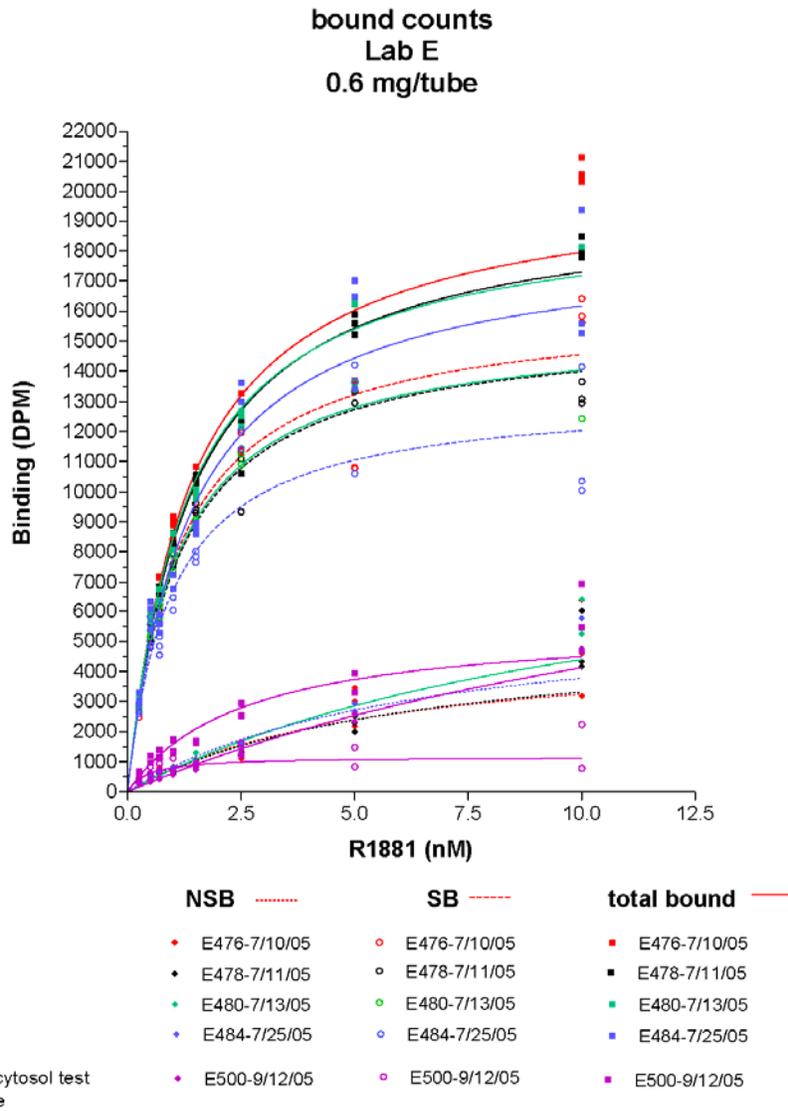


Specific bound	E476-7/10/05	E478-7/11/05	E480-7/13/05	E484-7/25/05	E500-9/12/05
BMAX	3.007e-010	2.881e-010	2.880e-010	2.452e-010	2.184e-011
KD	9.417e-010	9.265e-010	9.302e-010	8.337e-010	3.770e-010
Std. Error					
BMAX	1.283e-011	7.899e-012	1.109e-011	1.554e-011	2.776e-012
KD	7.723e-011	4.914e-011	6.621e-011	1.064e-010	1.318e-010
95% Confidence Intervals					
BMAX	2.741e-010 to 3.273e-011	2.717e-010 to 3.045e-011	2.649e-010 to 3.112e-011	2.128e-010 to 2.776e-011	1.589e-011 to 2.780e-011
KD	7.815e-010 to 1.102e-009	8.246e-010 to 1.028e-009	7.916e-010 to 1.089e-009	6.119e-010 to 1.056e-009	9.430e-011 to 6.596e-010
Goodness of Fit					
Degrees of Freedom	22	22	19	20	14
R ² (unweighted)	0.9436	0.9768	0.9711	0.8601	0.3348
Weighted Sum of Squares (1/Y ²)	0.2034	0.08688	0.08935	0.4048	1.103
Absolute Sum of Squares	7.290e-021	2.487e-021	2.279e-021	1.411e-020	8.050e-022
Sy.x	1.820e-011	1.063e-011	1.095e-011	2.656e-011	7.583e-012
Data					
Number of X values	24	24	24	24	23
Number of Y replicates	1	1	1	1	1
Total number of values	24	24	21	22	16
Number of missing values	0	0	3	2	7



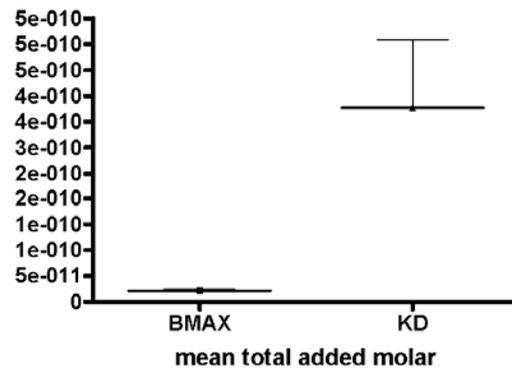
ABC Study No. 49655

4-11-5-Saturation_E.pzf.E data (DPM) - Thu Sep 15 10:17:51 2005



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E500-sp_b: nonlinear regression:Summary table



Miscellaneous Information

EPA Androgen Receptor (AR) Cytosol Planner

EPA AR Cytosol Planner

Planner Overview: The calculations on this page are presented as a guideline only and are based on experimental assumptions made in our laboratory (e.g.; 1 - cytosol protein yield/rat will be at least 20 mg, 2 - the cytosol is aliquoted such that there is minimal waste, minimal needed to repeat assays and the cytosol is stored properly, 3 - cytosol required for Saturation assay is 0.6 mg/tube yielding linear Scatchard plots and acceptable Kd and Bmax values, 4 - cytosol required for competitive assay is 1.2 mg per tube). Note: At present we are using 1.0 mg of cytosol protein to acquire acceptable data with this set of competitive assays.

NOTE: Changing any **Bold Shaded** number will change the calculated amount of rats needed. Saturation default is for 1 run @ 0.6 mg cytosol/tube. Competitive default is 1 run with a weak positive and no unknowns @ 1.2 mg cytosol/tube.

ANIMALS NEEDED	Saturation	Competitive	
	Enter Number of runs (w/extras) below	Enter Number of runs (w/extras) below	
Run Breakdown			
	Unknowns/run	0	
Total Runs	4	Total Runs	3
Cytosol tubes/run	48	Cytosol tubes/run	51
Total tubes	192	Total tubes	153
Cytosol/tube (mg)	0.6	Cytosol/tube (mg)	1.2
Total Cytosol (mg)	115.2	Total Cytosol (mg)	184
mg Cytosol/rat	20	mg Cytosol/rat	20
actual # of rats needed	5.8	actual # of rats needed	9.2
Total rats/task (+20%)	7	Total rats/task (+20%)	12
		COMBINED TOTALS	
			299 ml
			19 rats

Cytosol Tube Breakdown

Saturation (triplicate)	number of tubes
Hots	24
Hot/Cold	24
Competitive (triplicate)	number of tubes
Zero	6
NSB	6
STDs (5)	15
WP (8 dilutions)	24
Unknown (8 dilutions)	24

order 19 rats
SD males 90-95 DO

Data Basic Information

Introduction	This workbook contains a series of Androgen Receptor binding assay data collection templates. Each template is presented as a worksheet. The worksheet names and contents are defined below. The initial rows (1 through 17 or so, depending on the number of unknowns in a competitive assay) define the assay run. The remaining rows of the template define the volumes and concentrations of the ingredients of each tube in the assay.
Recommended reading	See the extensive collection of relevant material at: http://www.graphpad.com/articles/library.cfm
Defining an assay run	Assign a unique identifier to each assay run. A run consists of a definition of contents of each assay tube and the associated DPM values obtained from the scintillation counter. Keep track of the start date of the assay run, the identification of the person performing the assay, the lot number of the cytosol and radiotracer and the purpose (saturation, competitive, Ki, etc.) of the run. There are cells provided for assay run identification in the upper rows of column O.
Adding DPM values from each tube	Most scintillation counters will produce an Excel spreadsheet as one form of electronic data. Therefore, the templates are designed so that all DPM values may be entered directly into column O by cut and paste from the electronic output of the scintillation counter. Care must be taken to insure that the DPM values from the scintillation counter are aligned correctly with the assay tube definitions in the template.
Annotating results judged to be unusable	Occasionally the laboratory procedure review or data review indicates that one or more DPM values should be discarded for cause. In this case, add an explanation in column R. The cell in column Q will automatically change to FALSE. DPM values marked "FALSE" will be ignored in the data reduction portion of the template.
10% rule	These templates facilitate checking for reasonable assay behavior. The "10% rule" (total binding DPM should not exceed 10% of total DPMs added) is indicated in column S on saturation templates and a cell in column Q on competitive templates. Values that exceed 10% are color coded. It may be acceptable for some saturation values to exceed 10% at the higher concentrations. However, there may be something wrong (usually too much protein) with a competitive assay that exceeds the 10% rule. See the Saturation Checklist and Competitive Checklist spreadsheets for additional guidance.
Competitive assay characterization values	Summary values for the competitive assay are shown in cells S4 through V13. These should be reviewed before submission of the data.
Extracting data for curve fitting	These spreadsheets provide for definition of the run, input of the DPM values and perform data reduction to the point where values are ready to be analyzed by nonlinear regression. For saturation assays, these values are presented in CF through CI, with additional variables you might wish to analysis in columns CJ through CN. For competitive assays, these values are in color coded cells in columns T and U. It is generally safest to cut and paste these values into the nonlinear curve fitting software (if it supports a spreadsheet format).
Adding summary values	The nonlinear curve fitting software will estimate parameters of the model (IC50, Kd, Bmax, etc). Use cut and paste to copy these values to the indicated cells in the upper rows of column O for competitive runs, and to the appropriate cells in column DK for saturation runs (cells other than the bmax and Kd are optional, they are not included in any analysis). For saturation runs, the conversion from molar values is done in the spreadsheet, and the converted numbers are placed automatically in column O.
Submitting results to Battelle	Fill in the blue cells in column O and submit an electronic copy of the worksheet to Battelle.

Worksheet name	Contents
Battelle Supplied Reagents Saturation	Tabulation of concentrations and codes for diluted cold R1881 and Weak Positive vials to be sent to each participating laboratory
Competitive_SC	Saturation assay tube layout for initial study using dilutions of cold R1881 supplied by Battelle.
	Competitive assay tube layout for initial study using dilutions of cold R1881 and Weak Positive supplied by Battelle.

Data Basic Information (continued)

Checklist for competitive binding

from "Fitting Models to Biological Data using Linear and Nonlinear Regression", Harvey Motulsky & Arthur Christopoulos, GraphPad Software, 2003, page 213.

Checklist for competitive binding results

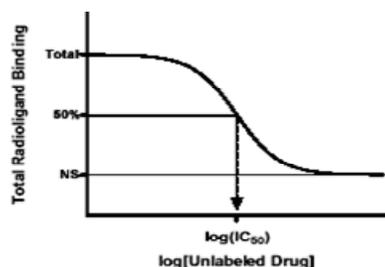
When evaluating results of competitive binding, ask yourself these questions:

Question	Comment
Is the $\log IC_{50}$ reasonable?	The IC_{50} should be near the middle of the curve, with at least several concentrations of unlabeled competitor on either side of it.
Are the standard errors too large? Are the confidence intervals too wide.	The SE of the $\log IC_{50}$ should be less than 0.5 log unit (ideally a lot less).
Are the values of <i>Top</i> and <i>Bottom</i> reasonable?	<i>Top</i> should be near the binding you observed in the absence of competitor. <i>Bottom</i> should be near the binding you observed in the presence of a maximal concentration of competitor. If the best-fit value of <i>Bottom</i> is negative, consider fixing it to a constant value equal to nonspecific binding determined in a control tube.
Has binding reached equilibrium?	Competitive binding incubations take longer to equilibrate than saturation binding incubations. You should incubate for 4-5 times the half-life for radioligand dissociation.
Does only a small fraction of the radioligand bind?	The equations are based on the assumption that the free concentration of labeled ligand is essentially identical to the concentration you added. Compare the total binding in the absence of competitor in cpm, to the amount of ligand added in cpm. If the ratio is greater than 10% at any concentration, then you've violated this assumption. Try to revise your experimental protocol, perhaps using a large incubation volume.
Does the curve have the expected steepness?	The competitive binding curve has a Hill slope (or slope factor) of -1. If your data form a curve shallower than this, see "Shallow competitive binding curves" on page 215.

Typically, the *Top* is constrained to equal 100% and the *Bottom* is constrained to 0%. See the indicated cell in column Q where this ratio is tabulated on the competitive assay worksheets.

What is a competitive binding curve?

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations of unlabeled ligand. Ideally, the concentration of unlabeled ligand varies over at least six orders of magnitude.



The top of the curve is a plateau at a value equal to radioligand binding in the absence of the competing unlabeled drug. The bottom of the curve is a plateau equal to nonspecific binding. The concentration of unlabeled drug that produces radioligand binding half way between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%) or EC_{50} (effective concentration 50%).

If the radioligand and competitor both bind reversibly to the same binding site, binding at equilibrium follows this equation (where *Top* and *Bottom* are the Y values at the top and bottom plateau of the curve).

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(N - 1) \log IC_{50}}}$$

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Data Basic Information (continued)

Checklist for saturation binding

from "Fitting Models to Biological Data using Linear and Nonlinear Regression", Harvey Motulsky & Arthur Christopoulos, GraphPad Software, 2003, page 204-205.

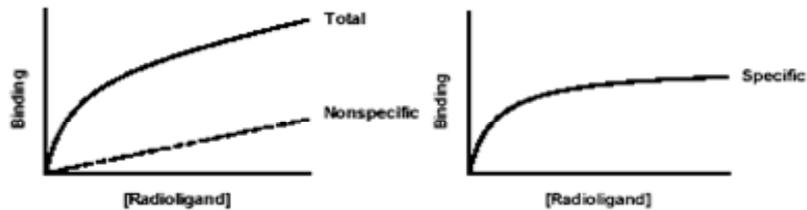
Checklist for saturation binding

When evaluating results of saturation binding analyses, ask yourself these questions:

Question	Comment
Did only a small fraction of the radioligand bind?	The analysis assumes that the free concentration is almost identical to the concentration you added. You can test this by comparing the total counts that bound to the total counts added to the tube. If more than 10% of the ligand bound (at any ligand concentration), then the standard analysis won't work. Either change the experimental protocol (increase the volume) or use a method that accounts for depletion of radioligand -- see "Analyzing saturation binding with ligand depletion" on page 208.
Did the binding equilibrate?	The tubes with the lowest concentration of radioligand take the longest to equilibrate. So test equilibration time with a low concentration of radioligand.
Did you use high enough concentrations of radioligand?	Calculate the ratio of the highest radioligand concentration you used divided by the K_d reported by the program (both in nM or pM). The highest concentration should be at least 10 times the K_d , so that occupancy exceeds 90%.
Is the B_{max} reasonable?	Typical values for B_{max} are 10-1000 fmol binding sites per milligram of membrane protein, 100-10000 sites per cell or 1 receptor per square micron of membrane. If you use cells transfected with receptor genes, then the B_{max} may be many times larger than these values.

Question	Comment
Is the K_d reasonable?	Typical values for K_d of useful radioligands range between 10 pM and 10 nM. If the K_d is much lower than 10 pM, the dissociation rate is probably very slow and it will be difficult to achieve equilibrium. If the K_d is much higher than 10 nM, the dissociation rate will probably be fast, and you may be losing binding sites during separation of bound ligand from free radioligand.
Are the standard errors too large? Are the confidence intervals too wide?	Divide the SE of the B_{max} by the B_{max} , and divide the SE of the K_d by the K_d . If either ratio is much larger than about 20%, look further to try to find out why.
Is the nonspecific binding too high?	Divide the nonspecific binding at the highest concentration of radioligand by the total binding at the highest concentration. Nonspecific binding should usually be less than 50% of the total binding.

Data Basic Information (continued)

Introduction to saturation binding experiments

Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations of the radioligand. Nonlinear regression analysis of saturation binding data allows you to determine receptor number and affinity. Because this kind of experiment used to be analyzed with linear Scatchard plots (more accurately attributed to Rosenthal), they are sometimes called "Scatchard experiments".

Analyses of saturation binding data depend on the assumption that you have allowed the incubation to proceed to equilibrium. This can take anywhere from a few minutes to many hours, depending on the ligand, receptor, temperature, and other experimental conditions. The lowest concentration of radioligand will take the longest to equilibrate. When testing equilibration time, therefore, use a low concentration of radioligand (perhaps 10-20% of the K_d).

Data Basic Information (continued)

AR Chemical Aliquots Code for Laboratory D

<i>Material</i>	<i>Initial Concentration (M)</i>	<i>Initial Concentration (nM)</i>	<i>Labels on set 1 vials</i>	<i>Labels on set 2 vials</i>	<i>Labels on set 3 vials</i>	<i>Labels on set 4 vials</i>	<i>Labels on set 5 vials</i>
Cold R-1881 for saturation, 4 vials	1.00E-05	10000.0	D-1-C1	D-2-C1			
	1.00E-06	1000.0	D-1-C2	D-2-C2			
Cold R-188 for Competitive Standard Curve, 12 vials	1.00E-05	10000.0	D-1-S0	D-2-S0			
	3.00E-06	3000.0	D-1-S1	D-2-S1			
	3.00E-07	300.0	D-1-S2	D-2-S2			
	3.00E-08	30.0	D-1-S3	D-2-S3			
	3.00E-09	3.0	D-1-S4	D-2-S4			
	3.00E-10	0.3	D-1-S5	D-2-S5			
Weak Positive (dexamethazone) 16 vials	3.00E-02	30000000.0	D-1-P1	D-2-P1			
	3.00E-03	3000000.0	D-1-P2	D-2-P2			
	3.00E-04	300000.0	D-1-P3	D-2-P3			
	3.00E-05	30000.0	D-1-P4	D-2-P4			
	3.00E-06	3000.0	D-1-P5	D-2-P5			
	3.00E-07	300.0	D-1-P6	D-2-P6			
	3.00E-08	30.0	D-1-P7	D-2-P7			
	3.00E-09	3.0	D-1-P8	D-2-P8			
Tracer Hot R-1881 for saturation and	1.00E-07	100.0	D-1-H1	D-2-H1	D-3-H1	D-4-H1	
	1.00E-08	10.0	D-1-H2	D-2-H2	D-3-H2	D-4-H2	D-5-H2

Cytosol vials will be sent on dry ice. Each container will have a unique identifier. The lot number and protein concentration will be supplied in the accompanying documentation.

Cytosol

Tracer

R1881		
lot number	3538-497	
specific activity	82 Ci/mmole	
certification date	9/16/2004	
Physical Characteristics of 3H		
	Half life	lambda
years	12.35	0.05613
days	4510.84	0.00015

APPENDIX II
PROTOCOL

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PROTOCOL

1. **Title:** Androgen Receptor Competitive Binding Protocol Using Rat Ventral Prostate Cytosol

2. **Sponsor:** Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

3. **Testing Facility:** ABC Laboratories
7200 East ABC Lane
Columbia, Missouri 65202

4. **Objective:** To provide data to establish laboratory variability among five laboratories using the same assay and common preparations of rat prostrate cytosol, R1881 and dexamethasone. This protocol is specific to the study to be conducted at ABC Laboratories.

5. **Duration:** approximately 45 days

6. **Proposed Study Dates:**
 - a. Initiation of Task: November 15, 2004
 - b. Completion of Task: December 31, 2004

7. **Protocol Approval:**
 - a. Study Director: C. Gliser Date: Nov 17 '04
Camelia Gliser, B.S.

 - b. Management: Amy Mize Date: 17 NOV 04
Amy Mize, Ph.D.

 - c. Sponsor: _____ Date: _____
David P. Houchens, Ph.D.

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Reviewed By:

- a. ABC QA
Representative: _____ Date: _____
Michelle Haines, B.A.
- b. EDSP Battelle
QAM: _____ Date: _____
Terri L. Pollock, B.A.

8. **Test, control and reference substances:**

- a.1 Test Substances: Dexamethasone (CAS 50-28-2) will be prepared and supplied by Battelle.
- a.2 Reference substance: R1881 (Methyltrienolone) (CAS 965-93-5) will be supplied by Battelle.
- a.3 Marker: Radiolabeled-R1881 (³H-Methyltrienolone) (CAS 68-23-5) Tracer.
- b. Storage: Upon receipt from the supplier, ³H-R1881 will be stored at -20⁰C. Other test substances will be stored according to conditions specified by the supplier.
- c. Disposition: All quantities of the test substances which are dispensed will be documented.

9. **Test System:**

- a. Identification: The test system is rat prostrate cytosol. Each tube within the assay will be labeled as defined in the assay. Upon receipt from the supplier, cytosol will be stored at -80⁰C.
- b. Justification for selection of the test system: Rat prostate cytosol is used because of the extensive data that exist for comparison and the inability to identify appropriate recombinant system(s) without false negative and positive acting chemicals in the *in vitro* binding assay.

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- c. Source: The rat prostate cytosol will be prepared by Battelle – Richland per Battelle SOPs. The cytosol will be shipped to ABC Laboratories for use in the assays.
10. **Experimental Design:**
- a. Assay to be Performed: Saturation binding experiments and competitive binding experiments with R1881 will be run according to the updated assay protocol (see Appendix 1). Each experiment shall be conducted once each day on three separate days by the same technician. In addition, competitive binding experiments will be run with the weak AR binder dexamethasone (at this time assume 8 dilutions). Each experiment shall be conducted once each day on three separate days by the same technician.
 - b. Frequency of Tests: Three tubes will be run per concentration and each assay will be run three times for the saturation and competitive binding assay. The assay is described in detail in Appendix 1.
 - c. Route of administration and Reason for its choice: The test and control substances are added directly to the cytosol in assay tubes in the appropriate sequence with the other reagents in the assay. The direct application and sequence is required for this assay type.
 - d. Analysis of Data: Graph pad prism will be used to calculate K_d and number of receptors for the saturation binding experiment. Also, the IC_{50} for R1881 and dexamethasone and Relative Binding Affinity (RBA) for dexamethasone as compared to R1881 from the competitive binding studies will be calculated.
 - e. Method for control of bias: Replicate incubations and aliquot numbers for analysis along with simultaneous incubation of positive controls side by side with the test incubations will control experimental bias.
11. **Quality Assurance**: This study will be audited by the Quality Assurance Unit to assure adherence to Good Laboratory Practice Regulations, adherence to the study protocol and compliance with ABC Labs Standard Operating Procedures. The Quality Assurance Unit will conduct a review of the raw data for accuracy and traceability and will audit the final report.
12. **Reports**: A Report will be prepared at the completion of the study. The report will include, but not be limited to, the following:
- a. Design of the study and the results obtained.
 - b. Name and address of the facility performing the study.
 - c. Copy of the approved protocol, including all changes and revisions.

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- d. Date of the completed report.
 - e. Instances of ambiguity or unclear direction.
 - f. Prism or other data files.
 - g. Description of all circumstances that may have affected the quality or integrity of data.
 - h. Name, chemical structure, Chemical Abstract Service Registry Number (if known), physical nature and purity (if known) of the test, control and reference substances.
 - i. Justification for choice of solvent/vehicle if other than water or ethanol, and information to demonstrate that the solvent/vehicle, if other than an established solvent does not bind to or otherwise affect the Androgen Receptor (AR).
 - j. Type and source of AR, its isolation from tissues, protein concentration of AR preparation and method of storage.
 - k. Test conditions.
 - l. Results including extent of precipitation of test substance(s), solvent control response compared to the negative control, K_d and IC_{50} values with confidence limits for R1881, and RBA values for dexamethasone as compared to R1881.
13. **Alteration of Design:** Alterations of the protocol may be made as the study progresses. Changes will be documented as required by ABC SOPs.
14. **Data Notebooks:** All original data will be maintained in data notebooks. These will include, but not necessarily be limited to the following:
- a. The original signed protocol and all amendments.
 - b. Test system records.
 - c. Test substances receipt and use records.
 - d. Test substances preparation data.
 - e. Sample preparation data.
 - f. Scintillation counting data.
 - g. Calculations to determine final reported values.
15. **Records to be Maintained:** The protocol, any amendments, the final report and all raw data collected as a result of this study will be archived by Battelle-Richland. The associated facility records will also be archived as required by Battelle-Richland SOPs.
16. **Personnel:** *Curricula vitae* for all personnel involved in the execution of the study are on file at ABC, Columbia, Missouri.
17. **Compliance Statement:** This study will be conducted in compliance with the U.S. EPA Good Laboratory Practice Regulations as set forth in Part 160 Title 40 of the Code of Federal Regulations.

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APPENDIX 1

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APPENDIX 1

Androgen Receptor Competitive Binding Protocol Using Rat Ventral Prostate Cytosol

OP No. NHEERL-H/RTD/EB/VW/2002-03-000

1.0 Purpose and Applicability

Determine ability of compound to compete with [³H] ligand for binding in rat ventral prostate tissue homogenate.

2.0 Safety and Operating Precautions

All procedures with radioisotopes should follow the regulations and procedures as described in the Hazardous Agent Protocol and in the Radiation Safety Manual and Protocols for US EPA.

3.0 Animal Use

Follow U.S. EPA approved animal use protocols

4.0 Equipment and Materials**4.1 Equipment**

- Corning Stir/hot Plates
- Pipets
- 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 refrigerator
- Speed-vac
- 20⁰ freezer

4.2 Chemicals

- Tris HCL & Tris Base
- Phenylmethylsulfonyl Fluoride (PMSF)
- Glycerol 99%+
- Sodium Molybdate
- Ethylenediaminetetraacetic acid (EDTA); Disodium salt
- Dithiothreitol (DTT)
- Potassium Chloride
- Hydroxylapatite (HAP; BIO-RAD)
- Scintillation Cocktail (Flow Scint III)
- Ethyl Alcohol, anhydrous
- Negative Control (Corticosterone)
- [³H]-R1881 (NEN; Purity >97%)
- Radioinert R1881 (NEN)

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- Triamcinolone Acetonide
- Steroids (Steraloids - recrystallized)
- scintillation cocktail (we use optifluor)

4.3 Supplies

- 20 ml Polypropylene Scintillation Vials
- 12 x 75 mm Borosilicate Glass Test Tubes
- 1 000 ml graduated cylinders
- 500 ml Erlenmeyer flasks
- pipet tips
- other glassware as appropriate

5.0 Stock Preparations5.1. Preparation of Stock Solutions for making TEDG Buffer

5.1.1. EDTA Stock Solution: Add 7.444g disodium EDTA to 100 ml ddH₂O = 200mM. Store at 4°C. Use 750 µl/100ml TEDG buffer = 1.5 mM.

5.1.2. PMSF Stock Solution: Add 1.742 g PMSF to 100 ml ethanol = 100 mM. Store at 4°C. Use 1.00 ml/100ml TEDG buffer = 1.0 mM.

5.1.3. Sodium Molybdate Stock: Add 2.419 g sodium molybdate to 8.0 ml ddH₂O in a 10 ml volumetric flask; bring the total volume to 10 mls = 1.0 M. Store at 4°C. Use 100 µl/100ml TEDG buffer = 1.0 mM.

5.1.4. 1 M Tris Buffer: Add 147.24 g Tris-HCL + 8.0 g Tris base to 800mls ddH₂O in a volumetric flask; bring the final volume to 1.0 liter. Refrigerate to 4°C and pH (using 4°C pH standardizing solutions) the cooled solution to 7.4. Store at 4°C. Use 1.0 ml/100 ml TEDG buffer = 10mM. (50 mM Tris = 50 ml 1 M Tris/1 L ddH₂O)

5.1.5. Potassium Chloride Stock Solution: Add 298.2 g KCL to 600 ml ddH₂O 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 = 0.4 M.

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

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 ddH₂O
- 1.0 ml 1M TRIS
- 10.0 ml glycerol
- 100 :1 1 M sodium molybdate
- 7 50 :1 200mM EDTA
- 1.0 ml 100mM PMSF
- 15.4 mg DTT (add immediately before use)

Check pH of the final solution to make sure it is 7.4 at 4°C.

5.3 Preparation of 50 mM TRIS Buffer

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Add 50.0 ml of 1.0 M TRIS to 950 ml ddH₂O. Store at 4°C. Check pH of the final solution to make sure it is 7.4 at 4°C.

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). The evening before the receptor extraction, pour 100 ml (or an appropriate volume) into a 100 ml graduated cylinder, parafilm seal the top and place in the refrigerator for at least 2h.

5.4.2. Pour off the phosphate buffer supernatant, and bring the volume to 100 ml 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.

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

5.4.4. 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).

5.4.5. Store at 4°C until ready for use in the extraction.

5.5 Preparation of [³H-17α-Methyl]-R1881 Stock Solutions

Dilute the original 1.0 mCi/ml stock of [³H-17α-methyl]-R1881 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 mls with ethanol. Thus, if the specific activity of the stock vial is 86 Ci/mmol, then pipet 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 10⁻⁷M.

Note: [³H-17α-Methyl]-R1881 stock solution and dilutions should be stored at -20°C. Store stock solution in original protective vial and store dilutions in amber glass vials. This product is light-sensitive; care should be taken to minimize exposure to light.

5.6 Calculation Check and Dilutions

$$\begin{aligned} 86 \mu\text{l} \times 1.0 \text{ mCi}/1000\mu\text{l} &= 86 \times 10^3 \text{ mCi R1881} = 86 \times 10^6 \text{ Ci R1881} \\ 86 \times 10^6 \text{ Ci} \div 86.0 \text{ Ci/mmol} &= 1 \times 10^6 \text{ mmol R1881} = 1 \times 10^9 \text{ moles R1881} \\ 1 \times 10^9 \text{ moles R1881} \div 0.010 \text{ liters} &= 1 \times 10^7 \text{ moles/liter} = 0.1 \mu\text{M} \end{aligned}$$

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., pipet 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).

To prepare the 1 x 10⁻⁹ M stock simply make a 10-fold dilution of the 1 x 10⁻⁸ M stock (i.e., pipet 1.0 ml of the 1 x 10⁻⁸ M stock into a clean amber colored vial and add 9 mls ethanol = 0.001 μM).

5.7 Preparation of 100X Radioinert R1881 Solutions

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 R1881 stock.

To make the 1.0 μM radioinert R1881 stock, pipet 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 radioinert R1881 stock. To make the 0.10 μM

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radioinert R1881 stock, pipet 2 ml of the 1 μ M stock into an amber vial and dilute to 20 ml with ethanol = 1×10^{-7} M = 0.10 μ M radioinert R1881 stock.

5.8 Compound Stock Preparations

5.8.1. Make stocks 30X above desired final concentration (this accounts for the use of 10 μ l stock in 300 μ l cytosol). Initial Stock of each test chemical solution will be diluted in 100% ethanol at a concentration of 3.0×10^2 M (i.e., 30 mM).

12.37mg/2ml ethanol

EXAMPLE:

4 (t) octyl phenol FW 206.33

1M = 206.33g/L

1mM = .20633mg/ml

x 30 (30 mM desired final stock conc.) = 6.1899 mg/ml

2 ml Stock = 6.1899 mg x 2 = 12.3798 mg

5.8.2. Prepare serial dilutions of R1881 for standard curve in ethanol (100%) 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	
0	0 (EtOH)	0
NSB	1×10^{-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}

* Final concentration = 10 μ l of each standard is added to the assay tube ,except for NSB tubes which are 30 μ l.

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

Serial Dilutions of the Test Chemical	Initial Concentration (Molar)	*Final Concentration (Molar) in AR assay tube
Concentration 1	3×10^{-3}	1×10^{-4}
Concentration 2	3×10^{-4}	1×10^{-5}
Concentration 3	3×10^{-5}	1×10^{-6}
Concentration 4	3×10^{-6}	1×10^{-7}
Concentration 5	3×10^{-7}	1×10^{-8}
Concentration 6	3×10^{-8}	1×10^{-9}
Tube 7	0 (vehicle only)	0

* Final concentration = 10 μ l of each Initial Concentration of test chemical is added to the assay tube along with 300 μ l of ventral prostate cytosol.

6.0 Tissue Homogenate Collection

- 6.1. Castrate 90 day old rats (60-90 day old acceptable; 90 day old preferred) as per laboratory animal protocols.
- 6.2. 24 hours after castration, make low salt TEDG buffer and place in an ice-water bucket.
- 6.3. Kill rat and excise ventral prostate. Tissue should be trimmed of fat, weighed and the weights recorded.
- 6.4. Add low-salt TEDG buffer at 10ml/g tissue.
- 6.5. Mince tissues with Metzenbaum scissors until all pieces are small 1-2 mm cubes. Then homogenize the tissues at 4°C with a Polytron homogenizer using 5-sec bursts of the Polytron. [Note: place probe of the Polytron in TEDG buffer in an ice-water bath to cool it down prior to its use for homogenization. Recool probe as needed.]
- 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 contains the low-salt cytosolic receptor. Pool the supernatant from all rats. Aliquot into 5 ml and store -80°C until needed for assay. Discard after 6 months.
- 6.8. Determine the protein content for each batch of cytosol according to the method by Bradford (1976) using the commercially available BioRad Protein Assay Kit (BioRad Chemical Division, Richmond, CA). Protein concentrations usually range from 5.5 - 8 mg/ml in undiluted cytosol.

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7.0. Assay procedure for chemicals: Day 1

- 7.1. Set up tubes: 12x75 mm glass tubes
 - 7.1.1. Label sufficient glass tubes as needed for the assay.
 - 7.1.2. Add 30µl of 0.01µM [3H] R1881 (1×10^{-8} M) and 50µl triamcinolone acetonide (60µM stock) to ALL tubes
 - 7.1.3. For 3 tubes at beginning of assay and at end of assay, also add 100x inert R1881 (30µl of 10.0 µM, ie 1×10^{-6} M). These tube are for determining nonspecific binding.
 - 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 5.8 for concentrations 1-7 in duplicate)
- 7.3. Remove aliquot of prostate cytosol and thaw on ice. Cytosol should be diluted with ice-cold low-salt TEDG buffer to give a protein concentration of 1.2 mg per 300 µl assay tube. (In our lab this is usually about a 1:1 dilution or 150 µl cytosol:150 µl TEDG buffer)
- 7.4. Add 300µl of diluted 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 5.4 above.
- 7.6. Label the HAP tubes and the scintillation vials to be used the following day - see underlines below.

8.0 Assay procedure: Day 2

- 8.1. The following morning, wash the HAP as described in section 5.4 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, pipet 500 µl of the HAP slurry into clean pre-labelled 12 x 75 mm glass test 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 HAP tube should be prepared for each incubation tube.
- 8.4. Take the incubation tubes from the refrigerator and place them in an ice-water bath with the HAP tubes. Pipet 100 µl from each of the incubation tubes into the appropriate pre-labelled 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 4°C and 600 x g (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 pipet connected to an aspiration apparatus as per the radiation safety protocol.

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- 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 4°C at all times.
- 8.9. Following the last wash and decanting, add 2 mls 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-labelled 20 ml scintillation vials. Add 14 ml of Optifluor scintillation cocktail and count samples using the single label DPM program with quench correction.

9.0. Saturation Radioligand Binding Assay

Prior to routinely conducting the AR competitive binding assays, the methods should be standardized within each laboratory. A series of saturation radioligand binding assays should be conducted to demonstrate AR specificity and saturation. Nonlinear regression analysis of these data and subsequent Scatchard plots will document AR binding affinity (K_d) and maximum specific binding number (B_{max}). Scatchard assay is to be conducted as follows:

Day 1

- 9.1. Set up tubes: 12x75 glass tubes and label for 8 concentrations in duplicate each with and without 100X inert (48 tubes total 1 through 48 below).
- 9.2. Add [³H] R1881 from the appropriate stock solutions to tubes as listed below:
- 9.3. Place 50 µl of 60 mM stock triamcinolone acetonide to ALL tubes.
- 9.4. An aliquot of each concentration of [³H]R1881 should also be counted on scintillation counter to determine total counts added (tube # 49-72 below).

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Saturation Assay Tube Layout

Position	Replicate	Tube Type Code	Hot Initial Concentration (nM)	Hot R1881 Volume (µL)	Hot Final Concentration (nM)	Cold Initial Concentration (nM)	Cold Volume (µL)	Cold Final Concentration (nM)	Triamcelenone Acetate (µL)	Cytosol (µl)
1	1	H	10.0	7.5	0.25	—	—	—	50	300
2	2	H	10.0	7.5	0.25	—	—	—	50	300
3	3	H	10.0	7.5	0.25	—	—	—	50	300
4	1	H	10.0	15	0.50	—	—	—	50	300
5	2	H	10.0	15	0.50	—	—	—	50	300
6	3	H	10.0	15	0.50	—	—	—	50	300
7	1	H	10.0	21	0.70	—	—	—	50	300
8	2	H	10.0	21	0.70	—	—	—	50	300
9	3	H	10.0	21	0.70	—	—	—	50	300
10	1	H	10.0	30	1.00	—	—	—	50	300
11	2	H	10.0	30	1.00	—	—	—	50	300
12	3	H	10.0	30	1.00	—	—	—	50	300
13	1	H	10.0	45	1.50	—	—	—	50	300
14	2	H	10.0	45	1.50	—	—	—	50	300
15	3	H	10.0	45	1.50	—	—	—	50	300
16	1	H	100.0	7.5	2.50	—	—	—	50	300
17	2	H	100.0	7.5	2.50	—	—	—	50	300
18	3	H	100.0	7.5	2.50	—	—	—	50	300
19	1	H	100.0	15	5.00	—	—	—	50	300
20	2	H	100.0	15	5.00	—	—	—	50	300
21	3	H	100.0	15	5.00	—	—	—	50	300
22	1	H	100.0	30	10.00	—	—	—	50	300
23	2	H	100.0	30	10.00	—	—	—	50	300
24	3	H	100.0	30	10.00	—	—	—	50	300
25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300
28	1	HC	10.0	15	0.5	1.00	15	50	50	300
29	2	HC	10.0	15	0.5	1.00	15	50	50	300
30	3	HC	10.0	15	0.5	1.00	15	50	50	300
31	1	HC	10.0	21	0.7	1.00	21	70	50	300
32	2	HC	10.0	21	0.7	1.00	21	70	50	300
33	3	HC	10.0	21	0.7	1.00	21	70	50	300
34	1	HC	10.0	30	1	1.00	30	100	50	300
35	2	HC	10.0	30	1	1.00	30	100	50	300
36	3	HC	10.0	30	1	1.00	30	100	50	300
37	1	HC	10.0	45	1.5	1.00	45	150	50	300
38	2	HC	10.0	45	1.5	1.00	45	150	50	300

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39	3	HC	10.0	45	1.5	1.00	45	150	50	300
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300
43	1	HC	100.0	15	5	10.00	15	500	50	300
44	2	HC	100.0	15	5	10.00	15	500	50	300
45	3	HC	100.0	15	5	10.00	15	500	50	300
46	1	HC	100.0	30	10	10.00	30	1000	50	300
47	2	HC	100.0	30	10	10.00	30	1000	50	300
48	3	HC	100.0	30	10	10.00	30	1000	50	300
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—
52	1	Hot	10.0	15	0.06	—	—	—	—	—
53	2	Hot	10.0	15	0.06	—	—	—	—	—
54	3	Hot	10.0	15	0.06	—	—	—	—	—
55	1	Hot	10.0	21	0.08	—	—	—	—	—
56	2	Hot	10.0	21	0.08	—	—	—	—	—
57	3	Hot	10.0	21	0.08	—	—	—	—	—
58	1	Hot	10.0	30	0.10	—	—	—	—	—
59	2	Hot	10.0	30	0.10	—	—	—	—	—
60	3	Hot	10.0	30	0.10	—	—	—	—	—
61	1	Hot	10.0	45	0.30	—	—	—	—	—
62	2	Hot	10.0	45	0.30	—	—	—	—	—
63	3	Hot	10.0	45	0.30	—	—	—	—	—
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—
67	1	Hot	100.0	15	1.00	—	—	—	—	—
68	2	Hot	100.0	15	1.00	—	—	—	—	—
69	3	Hot	100.0	15	1.00	—	—	—	—	—
70	1	Hot	100.0	30	3.00	—	—	—	—	—
71	2	Hot	100.0	30	3.00	—	—	—	—	—
72	3	Hot	100.0	30	3.00	—	—	—	—	—

- 9.5. Place tubes in speed-vac (Tubes 1-48) and dry the tubes according to instructions. Remove when dry and place on ice.
- 9.6. Cytosol should be diluted with the low salt TEDG buffer to a protein concentration of 1.2 mg per 300 µl assay (in our laboratory this was about a 1:1 dilution). Add 300 µl of diluted prostate cytosol to all tubes (1-48). Keep tubes and cytosol on ice at all times during this procedure. Gently vortex and place tubes in refrigerator overnight in rotor (20hr).
- 9.7. Before leaving for the day, prepare the first wash of the HAP slurry as described in section 5.4 above. If desired, label the HAP tubes and the scintillation vials to be used the following day.

Day 2

- 9.8. Continue as with Day 2 protocol for binding assay above in section 8.0.

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10.0 Data Processing10.1 Free Concentration of [³H]-R1881

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

Calculation Check -

$$\frac{X \text{ DPM}}{2.22 \times 10^{-14} \text{ dpm/Ci}} = \frac{4.5045 \times 10^{-13} \text{ Ci}}{*83.2 \text{ Ci/mmol}} = \frac{5.4141 \times 10^{-15} \text{ mmole}}{1000 \text{ mmole/mole}} = \frac{5.4141 \times 10^{-18} \text{ moles}}{0.0003 \text{ liters}}$$

$$= \frac{1.8047 \times 10^{-14} \text{ moles/liter}}{1 \times 10^{-9} \text{ moles/nmole}} = X (1.8047 \times 10^{-5}) \text{ nm}$$

*Note this value will be the Specific activity of the radioligand ([³H]R1881) used in the assay.

10.2 Calculation of Total, Nonspecific and Specific [³H]-R1881 Binding

- 10.2.1. Total binding is calculated by multiplying the DPM from the tubes that contained only radiolabelled R1881 $\times (1.6242 \times 10^{-2})$. This value will be total binding in fmoles.
- 10.2.2. Nonspecific binding is calculated by multiplying the DPM from the tubes containing radiolabelled R1881 + 100-fold molar excess of radioinert R1881 $\times (1.6242 \times 10^{-2})$. This value will be nonspecific binding in fmoles.
- 10.2.3. Specific binding is calculated by subtracting nonspecific binding from total binding i.e., fmoles total binding - fmoles nonspecific binding = specific binding in fmoles.

10.3 Graphical Presentation of the Data

- 10.3.1. Standard Curve and Test Chemical Competitive Binding Curves: Data for the standard curve and each test chemical will be plotted as the percent ³H_R1881 bound versus the molar concentration. Estimates of the IC₅₀s 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 performed for the standard curve using R1881 to demonstrate that the assay meets acceptable QA standards.
- 10.3.2. Relative Binding Affinity: The RBA for each competitor should be calculated by dividing the IC₅₀ for R1881 by the IC₅₀ of the competitor and expressing as a percent (e.g., RBA for R1881 =100 %).
- 10.3.3. Maximal binding capacity (B_{max}) and association/dissociation constants (K_a / K_d) 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, P.J., and Rodbard, D. (1980) Anal. Biochem. 107, 220-239).

10.4 References

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