DRAFT REPORT

Preparation and Characterization of Rat Ventral Prostate Cytosol (WA 4-11–Task 3)

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Study Director:	Aruna Koganti, Ph.D.
EPA Contract Number:	68-W-01-023 (Battelle Prime Contractor)
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This report contains 138 pages, including eight appendices.

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Participation

The following principal staff participated in the conduct of this study:

Study Director:	Aruna Koganti, Ph.D.
Scientists:	LaShawn Brown, B.Sc.
	Rebecca McGee, B.Sc.
Technical Writers:	Blaise Considine, B.A.
	Steven Sorrow, B.Sc.

Study Dates and Data Retention

Study initiation date:	27 April 2005
Date protocol signed by Sponsor:	14 April 2005
Date test articles received:	22, 29 June 2005
Experimental start date:	07 July 2005
Experimental end date:	02 September 2005
Study completion date:	

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following submission of the final report to Battelle Memorial Institute. At the end of this period, Battelle will be notified to determine whether the data will be transferred, retained, or destroyed.

Statement of Compliance

This study was conducted to the standards of U.S. FDA 21 CFR Part 58 with the exceptions stated below. This study was conducted under my scientific guidance and management.

Exceptions: 1) The computer systems at In Vitro Technologies, Inc. are not validated. 2) The dosing solutions used in the study were not analyzed and the stability has not been determined. However, the stability of the methyltrienolone and dexamethasone in ethanol were evaluated and reported separately by Battelle. 3) The graphing and data analysis to determine B_{max}, K_d, IC_{50}, and RBA were conducted by Battelle and reported to In Vitro Technologies, but a signed report was not provided. However, this information was audited by Battelle QAU and a QA statement is provided in Appendix 8.

Aruna Koganti, Ph.D.		
Study Director	Signature	Date

Quality Assurance Statement

This study was inspected in accordance with In Vitro Technologies standard operating procedures. Based on audits conducted, the results reported herein accurately reflect the methods used and the data collected for this study.

All findings were reported to the Study Director and In Vitro Technologies Management.

Inspection/Audit Dates:	Study Phase Audited:	Date(s) reported to Study Director and Management:
06 July 2005	Saturation Assay	06 July 2005
12 July 2005	Saturation Assay	12 July 2005
20 July 2005	Saturation Assay	21 July 2005
27 July 2005	Competitive Assay	28 July 2005
02 August 2005	Competitive Assay	02 August 2005
03 August 2005	Competitive Assay	04 August 2005
10 August 2005	Competitive Assay	11 August 2005
02 September 2005	Competitive Assay	02 September 2005
29, 30 November 2005	Data and Report	30 November 2005

Sharon Isbell		
Director, Quality Systems	Signature	Date

Glossary of Abbreviations

AR ..... androgen receptor
B_{max} ..... binding maximum
DMSO ..... dimethyl sulfoxide
EPA ..... Environmental Protection Agency
HAP ..... hydroxylapatite
IC_{50} ..... concentration at which 50% of specific activity is inhibited
K_d ..... dissociation constant
K_i ..... inhibitory constant
NSB ..... non-specific binding
RBA ..... relative binding affinity
TEDG + PMSF buffer ..... buffer with Tris, ethylene diamine tetraacetic acid, dithiothreitol, glycerol, and phenylmethylsulfonyl fluoride

Summary

The objective of this study was to prepare rat ventral prostate cytosol and characterize it by evaluating the inhibition of androgen receptor binding of [^{3}H]-R1881 by known chemicals. This study was part of a multi-laboratory effort for the evaluation of the androgen receptor-binding assay. In Vitro Technologies prepared rat ventral prostate cytosol and characterized it by conducting competitive binding receptor assays of known chemicals, using the radiolabeled and inert R1881, dexamethasone, protocol, and reporting criteria provided by Battelle. This *in vitro* test method involved combining cytosol, substrate, and test articles in a common reaction vessel. The inhibitory effect of the test articles on androgen receptor binding of R1881 was evaluated by measuring the amount of bound [^{3}H]-R1881 (substrate).

The cytosol (Lot #: 0725-06-01) used in these experiments was prepared and characterized at In Vitro Technologies. The protein concentration was determined to be 4.005 mg protein/mL.

A total of three saturation experiments were conducted and the amount of cytosolic protein used for each incubation in the saturation binding experiments was 0.6 mg. The K_d observed across the three saturation experiments was 0.83, 0.93, and 1.03 nM and the B_{max} observed was 14.26, 16.76, and 15.29 fmole/100 µg cytosolic protein.

A total of three competitive binding experiments were conducted and the amount of cytosolic protein used for each incubation in the competitive binding experiments was 1.0 mg. The IC_{50} values for R1881 were determined to be 0.886, 1.95, and 0.695 nM. The IC_{50} values for dexamethasone were determined to be 57.7, 76.9, and 69.8 µM. The RBA values for dexamethasone in comparison to R1881 were, 0.0015, 0.0025, and 0.0010%. The K_i values for R1881 and dexamethasone were 0.427, 0.940, 0.335, and 27804, 37055, 33634 nM, respectively.

Introduction

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the Environmental Protection Agency (EPA) to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of in vivo tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Method Validation Committee will provide advice and counsel on the validation assays.

One potential endocrine target for environmental chemicals is the androgen receptor. The objective of this study was to evaluate the inhibition of androgen receptor (AR) binding of R1881 in rat ventral prostate cytosol by known chemicals, thus validating androgen receptor binding as a potential tool for screening environmental chemicals. Rat ventral prostate cytosol was selected because it provided a biological source of the androgen receptor. Since the assay was evaluated for its potential to serve as a screening assay, the use of rat tissue enhanced its availability.

Experimental Methods

Test Article Information and Preparation

Battelle provided the following test materials at the necessary dilutions:

- Substrate: ^{3}H-R1881 (CAS No. 965-93-5)
- Reference Article: Unlabelled R1881–Methyltrienolone (CAS No. 965-93-5)
- Test Article: Dexamethasone (CAS No. 50-02-2)
- Rat ventral prostate tissue

These test and reference articles were used in the androgen receptor-binding assay to determine relative binding affinities (RBAs).

The following were prepared at In Vitro Technologies or were supplied by In Vitro Technologies:

- Buffer with Tris, sodium molybdate, ethylene diamine tetraacetic acid, dithiothreitol, glycerol, and phenylmethylsulfonyl fluoride (Low Salt TEDG-PMSF Buffer) (pH 7.4) (Refer to In Vitro Technologies method B065.A and B066.A)
- Absolute ethanol (Sigma, lot 03648PC)

- Hydroxylapatite (HAP; Lot Number: 83862B)
- Liquid scintillation cocktail (Formula 989, Perkin Elmer; Lot Number: 138-050201)
- Rat ventral prostate cytosol (Lot Number: 0725-06-01)

R1881 Preparation

Battelle provided the R1881 stock solutions prepared in absolute ethanol. Battelle was responsible for the preparation and analysis of the R1881 stocks.

Fresh dilutions of the R1881 stock solutions were prepared in assay buffer on the day of use. The final concentrations for R1881 were 3.3×10^{-7} (NSB), 10^{-7}, 3.3×10^{-8}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} M. The total volume of solvent used in each assay was no more than 3.33% of the total assay volume.

Dexamethasone Preparation

Battelle provided the dexamethasone stock solutions prepared in absolute ethanol. Battelle was responsible for the preparation and analysis of the dexamethasone stocks.

Fresh dilutions of the dexamethasone stock solutions were prepared in assay buffer on the day of use. The final concentrations for dexamethasone were 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M. The total volume of solvent used in each assay was no more than 3.33% of the total assay volume.

Substrate Preparation

The substrate solution was prepared from the radiolabeled R1881 received from Battelle and stored at 4 ± 3°C in the original container. The [^{3}H]-R1881 was diluted with TEDG + PMSF buffer to achieve a substrate solution at a concentration of 10 nM. The substrate solution (30 µL) was added to the incubation mixtures to achieve a final concentration of 1 nM [^{3}H]-R1881 in the assay.

Preparation of Rat Ventral Prostate Cytosol

Rat ventral prostate cytosol was prepared as specified in the In Vitro Technologies biological method B067.A. The method is described in brief below:

The rat ventral prostate tissue was collected by Battelle and shipped to In Vitro Technologies on dry ice. The prostate tissue was stored at –70 ± 10°C until cytosol preparation.

The prostate tissue was weighed and placed in ice-cold low salt TEDG buffer at a ratio of 0.1 g of tissue per 1.0 mL buffer. The tissue was minced with Metzenbaum scissors until all pieces were small 1- to 2-mm cubes. The cubes were homogenized using an appropriate homogenizer (5-second bursts).

Note: The homogenizer probe was cooled prior to homogenizing each sample by placing the probe in ice-cold TEDG buffer. The homogenization tube was kept in an ice-cold water bath during the homogenizing process.

The homogenate was transferred to pre-cooled centrifuge tubes and spun in a centrifuge for 30 minutes at 30,000 × g at 4°C. The pellet contained the nuclear fraction and the supernatant contained the cytosol.

The cytosol from all the prostates collected the same day was pooled and divided into aliquots. The aliquots were used immediately in the AR binding assay or were stored at –70 ± 10°C until use.

Note: The cytosol can be stored frozen at –70 ± 10°C for 6 months prior to use in the AR binding assay.

The protein content for each batch of cytosol was determined using an appropriate method.

Assays

Protein Assay

The protein concentration of the cytosol preparation was determined on each day of use as described in In Vitro Technologies method B020.A. In brief, a six-point standard curve was prepared, ranging from 0.13 to 1.5 mg protein/mL. The protein standards were made from bovine serum albumin. Protein was determined using a BioRad® Protein Assay Kit II (Hercules, CA). To a 10 µL aliquot of standard or unknown, 200 µL of dye reagent was added and mixed. The samples were placed at room temperature for at least 5 minutes and up to an hour to allow for color development. The absorbance (600 nm) was measured using a plate reader. The protein concentration of the cytosol sample was determined by extrapolation of the absorbance value using the standard curve developed using the protein standards.

Evaluation of the Cytosol for Conducting Androgen Receptor Competitive Binding Assays

Prior to conducting the androgen receptor competitive binding assays with unknown chemicals, the cytosol was characterized at In Vitro Technologies. This was accomplished in two steps as follows:

1) A series of saturation radioligand binding assays were conducted to demonstrate androgen receptor specificity and saturation. Nonlinear regression analysis of these data and subsequent Scatchard plots document androgen receptor binding affinity (K_d) and the number of receptors (B_{max}).

2) A series of androgen receptor competitive binding assays were conducted using R1881 and dexamethasone, substances with known affinities for the androgen receptor. Comparison of IC_{50} values (i.e., the concentration of a substance that inhibits [^{3}H]-R1881 binding by 50%) from

these assays with reported values in the literature assisted in documenting that the cytosol was appropriate for routine use in the laboratory.

Saturation Radioligand Binding Assay

Androgen receptor saturation binding experiments measured total, non-specific, and specific binding of increasing concentrations of [^{3}H]-R1881 under conditions of equilibrium.

The experimental setup and details of the saturation-binding assay are specified in the In Vitro Technologies biological method B065.A. The saturation binding experiments were conducted as three independent replicates. The same technician conducted all three replicate experiments.

Competitive Binding Assay (Inhibition of Androgen Receptor Binding of [^{3}H]-R1881 by Test Articles)

An androgen receptor competitive binding assay measured the binding of a single concentration of [^{3}H]-R1881 in the presence of increasing concentrations of a test article. The experimental setup and details of the competitive binding assay are specified in the In Vitro Technologies biological method B066.A.

These experiments tested the androgen receptor binding of [^{3}H]-R1881 in the presence of multiple concentrations of a test article. The inhibition experiments were conducted as three independent replicates. All three replicate experiments for a given test article were conducted by the same technician and there were three (triplicate) repetitions for each concentration within a given replicate.

Control samples were included for each replicate experiment. These included:

- Vehicle or ethanol control (substrate, buffer, vehicle [used for preparation of test article solutions], and cytosol)
- Non-specific background control (substrate, buffer, R1881, and cytosol).

Six repetitions of each type of control were included with each replicate experiment and were treated the same as the other samples. The control sets were split so that three tubes (of each control type) are run at the beginning and three at the end of each replicate set.

Description of Data Calculations

In Vitro Technologies supplied all raw data to Battelle in electronic format using Microsoft Excel® spreadsheets and Prism templates (developed and provided by Battelle).

IC_{50} Calculation

Data for the non-radiolabeled R1881 standard curve and each test article were plotted as the percentage of [^{3}H]-R1881 bound versus the molar concentration (log) of competitor. Estimates of IC_{50} values were determined using appropriate nonlinear curve fitting software to fit a one-site

competitive binding model. The model was constrained to fit the bottom of the curve to 0% and the top to 100%.

Relative Binding Affinity Values

The RBA value for each test article was calculated by dividing the IC_{50} value for R1881 by the IC_{50} of the test article and expressing the value as a percent (e.g., RBA for R1881 = 100%).

K_i Calculation

An estimate of the K_i was calculated using the Cheng-Prusoff equation if the competitive binding curve reflects a pure competition for a single binding site: $K_i = IC_{50}/(1 + (L/K_d))$ where L = radioligand concentration and K_d = equilibrium dissociation constant of radioligand.

Reporting of Ambiguities

1. The shaker stopped working during saturation Run 1. It may have been sitting in the refrigerator too long.
2. Sample tubes 30 and 36 had white residue present after inverting to decant supernatant in saturation Run 2.
3. Sample 5 was cloudy compared to others after Formula 989 scintillation cocktail was added in saturation Run 2.
4. Samples 3, 30, and 40 appeared cloudier than normal in saturation Run 3 after Formula 989 scintillation cocktail was added.
5. The scintillation vial for sample 14 broke during competitive Run 2. The sample was transferred to a different vial and analyzed.
6. A lot of HAP slurry left over during competitive Run 4. Pipet clogged while dispensing/aspirating.

None of these observations appeared to impact the data.

Criteria for Data Acceptance

All data obtained are reported.

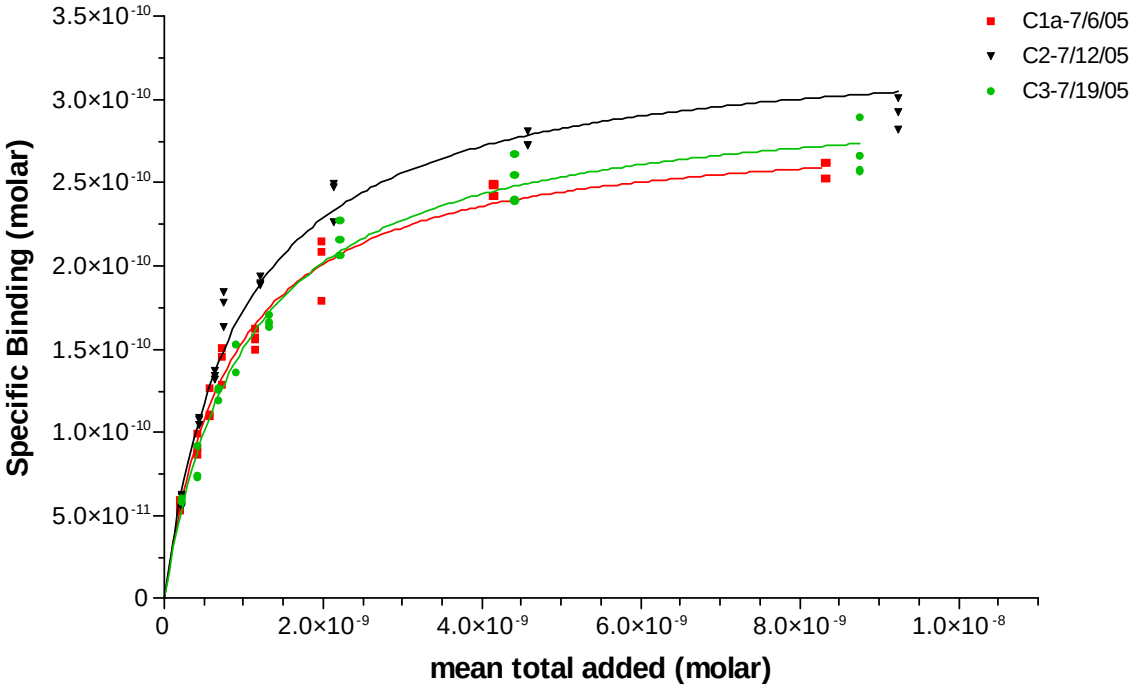
Results

The cytosol (Lot #: 0725-06-01) used in these experiments was prepared and characterized at In Vitro Technologies. The protein concentration was determined to be 4.005 mg protein/mL.

A total of three saturation experiments were conducted and the amount of cytosolic protein used for each incubation in the saturation binding experiments was 0.6 mg. The K_d observed across the three saturation experiments was 0.83, 0.93, and 1.03 nM and the B_{max} observed was 14.26, 16.76, and 15.29 fmole/100 µg cytosolic protein.

A total of three competitive binding experiments were conducted and the amount of cytosolic protein used for each incubation in the competitive binding experiments was 1.0 mg. The IC_{50} values for R1881 were determined to be 0.886, 1.95, and 0.695 nM. The IC_{50} values for dexamethasone were determined to be 57.7, 76.9, and 69.8 µM. The RBA values for dexamethasone in comparison to R1881 were 0.0015, 0.0025, and 0.0010%. The K_i values for R1881 and dexamethasone were 0.427, 0.940, 0.335, and 27804, 37055, 33634 nM, respectively.

Figure 1: Saturation Binding of ^{3}H-R1881 to the Androgen Receptor



Specific bound	C1a-7/6/05	C2-7/12/05	C3-7/19/05
BMAX	2.852e-010	3.351e-010	3.057e-010
KD	8.348e-010	9.300e-010	1.029e-009
Std. Error			
BMAX	8.160e-012	1.031e-011	1.203e-011
KD	4.740e-011	5.555e-011	7.795e-011
95% Confidence Intervals			
BMAX	2.683e-010 to 3.022e-010	3.138e-010 to 3.565e-010	2.808e-010 to 3.307e-010
KD	7.365e-010 to 9.331e-010	8.148e-010 to 1.045e-009	8.677e-010 to 1.191e-009
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9811	0.9747	0.9811
Weighted Sum of Squares (1/Y²)	0.09067	0.1109	0.1628
Absolute Sum of Squares	2.109e-021	3.619e-021	2.427e-021
Sy.x	9.791e-012	1.283e-011	1.050e-011
Data			
Number of X values	24	24	24
Number of Y replicates	1	1	1
Total number of values	24	24	24
Number of missing values	0	0	0

Figure 2: Competitive Binding of R1881 and Dexamethasone (Weak Positive) to the Androgen Receptor

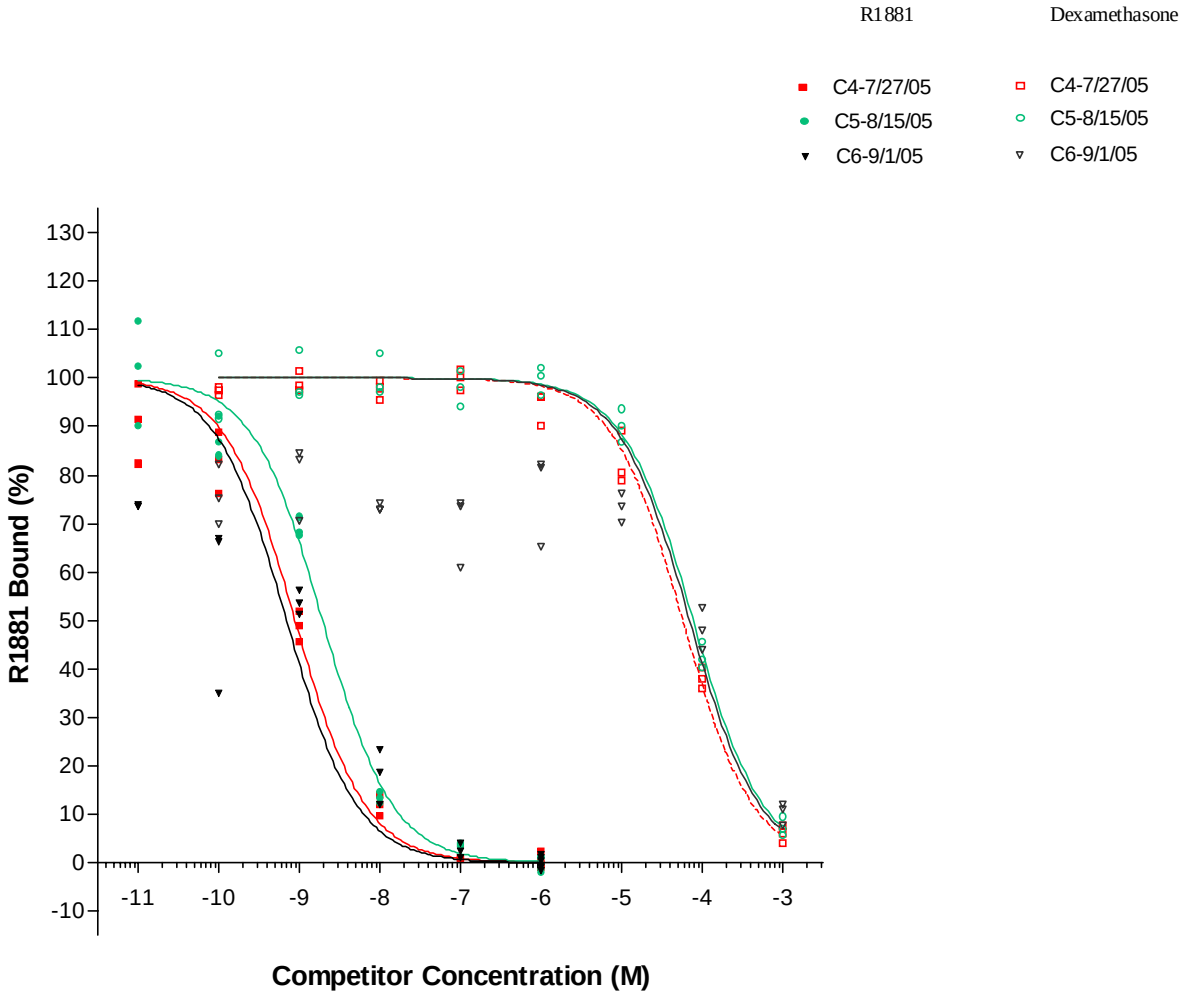


Table 1: Saturation Binding Experiments

Run number	Assay date	Technician	K_d (nM)	B_{max} (fmole/100 µg)
1	06 Jul 2005	LSB	0.83	14.26
2	12 Jul 2005	LSB	0.93	16.76
3	19 Jul 2005	LSB	1.03	15.29
Mean			0.93	15.44

Table 2: Competitive Binding Experiments

Run Number	Assay Date	Technician	IC_{50} (nM)	RBA (%)	Ki (nM)
R1881					
1	27 Jul 2005	LSB	0.886	100	0.427
2	15 Aug 2005	LSB	1.95	100	0.940
3	01 Sep 2005	LSB	0.695	100	0.335
Dexamethasone					
1	27 Jul 2005	LSB	57700	0.0015	27804
2	15 Aug 2005	LSB	76900	0.0025	37055
3	01 Sep 2005	LSB	69800	0.0010	33634

Abbreviations: IC_{50}, concentration at which 50% of activity is inhibited; RBA, relative binding affinity; K_i, inhibitory constant

RBA of dexamethasone = (IC_{50} of R1881 / IC_{50} of dexamethasone) × 100

$K_i = IC_{50} /(1 + (L/Kd))$; L = 1 nM; mean K_d = 0.93 nM

Appendix 1: Copy of In Vitro Technologies Protocol No. 1147

In Vitro Technologies, Inc.
Protocol No. 1147
Version: Final (14 April 2005)

Preparation and Characterization of Rat Ventral Prostate Cytosol (WA 4-11–Task 3)

Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2639
Sponsor Representative:	David P. Houchens, Ph.D. E-mail: houchensd@battelle.org Tel.: (614) 424-3564 Fax: (614) 458-3564
Testing Facility:	In Vitro Technologies, Inc. 1450 South Rolling Road Baltimore, Maryland 21227 Tel.: (410) 455-1242 Fax: (410) 455-1245 E-mail: contractservices@invitrotech.com
Study Director:	Aruna Koganti, Ph.D.
EPA Contract Number:	68-W-01-023 (Battelle Prime Contractor)
In Vitro Technologies Study Number:	270-1147-10
Proposed Experimental Start Date:	17 May 2005
Proposed Experimental End Date:	18 June 2005

Objective

The objective of this study is to prepare rat ventral prostate cytosol and characterize it by evaluating the inhibition of androgen receptor (AR) binding of [^{3}H]-R1881 by known chemicals. This study is part of a multi-laboratory effort for the evaluation of the androgen receptor binding assay. This protocol is specific to the study to be conducted at In Vitro Technologies, Inc.

Test / Reference Article Identification

Test / Reference Article	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection
Dexamethasone	50-02-2	$C_{22}H_{29}FO_5$	392.47	Known weak inhibitor (Test Article)
R1881	965-93-5	$C_{19}H_{24}O_2$	284.40	Known inhibitor (Reference Article)

Test System Identification

The test system for this study is rat ventral prostate cytosol prepared by In Vitro Technologies. The lot or batch number of the cytosol preparation and any other unique identifier assigned by In Vitro Technologies will be recorded in study documentation and will be reported in the study report. Each incubation tube will be labeled with a unique identifier.

Test System Justification

This test system was selected because it provides a biological source of the androgen receptor. Since the assay is being evaluated for its potential to serve as a screening assay, the use of rat tissue enhances its availability.

Description of Study

In Vitro Technologies will prepare rat ventral prostate cytosol and characterize it by conducting competitive binding receptor assays of known chemicals, using the radiolabeled and inert R1881, dexamethasone, protocol, and reporting criteria provided by Battelle. This *in vitro* test method involves combining cytosol, substrate, and test articles in a common reaction vessel. The inhibitory effect of the test articles on androgen receptor binding of R1881 will be evaluated by measuring the amount of bound [^{3}H]-R1881 (substrate). In Vitro Technologies will conduct three separate experiments to evaluate the inhibition of androgen receptor binding of [^{3}H]-R1881 by the test articles.

There is no applicable route of administration since this is an *in vitro* study.

Experimental Methods

Materials

Battelle will provide the following materials:

- [^{3}H]-R1881
- R1881 stocks
- Dexamethasone stocks
- Rat ventral prostate tissue

The following will be prepared at In Vitro Technologies or will be supplied by In Vitro Technologies:

- Buffer with Tris, sodium molybdate, ethylene diamine tetraacetic acid, dithiothreitol, glycerol, and phenylmethylsulfonyl fluoride (Low Salt TEDG-PMSF Buffer) (pH 7.4) (Refer to In Vitro Technologies method B065.A and B066.A)
- Absolute ethanol
- Dimethyl sulfoxide (DMSO)
- Hydroxylapatite (HAP)
- Liquid scintillation cocktail (Formula 989, Perkin Elmer)
- Rat ventral prostate cytosol

The supplier, lot numbers, and the purity of the materials received and used in this study will be included in the study report.

R1881 Preparation

Battelle will provide the R1881 stock solutions prepared in absolute ethanol. Battelle will be responsible for the preparation and analysis of the R1881 stocks.

Fresh dilutions of the R1881 stock solutions will be prepared in assay buffer on the day of use. The final target concentrations for R1881 are 3.3×10^{-7} (NSB), 10^{-7}, 3.3×10^{-8}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} M. The total volume of solvent used in each assay will be no more than 3.33% of the total assay volume.

Dexamethasone Preparation

Battelle will provide the dexamethasone stock solutions prepared in absolute ethanol. Battelle will be responsible for the preparation and analysis of the dexamethasone stocks.

Fresh dilutions of the dexamethasone stock solutions will be prepared in assay buffer on the day of use. The final target concentrations for dexamethasone are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M. The total volume of solvent used in each assay will be no more than 3.33% of the total assay volume.

Substrate Preparation

The substrate solution will be prepared from the radiolabeled R1881 received from Battelle and stored at 4 to 5°C in the original container. The [^{3}H]-R1881 will be diluted with TEDG + PMSF buffer to achieve a substrate solution at a concentration of 10 nM. The substrate solution (30 µL) will be added to the incubation mixtures to achieve a final concentration of 1 nM [^{3}H]-R1881 in the assay.

Preparation of Rat Ventral Prostate Cytosol

Rat ventral prostate cytosol will be prepared as specified in the In Vitro Technologies biological method B067.A. The method is described in brief below:

The rat ventral prostate tissue will be collected by Battelle and shipped to In Vitro Technologies on dry ice. The prostate tissue will be stored at –70 ± 10°C until cytosol preparation.

The prostate tissue will be weighed and placed in ice-cold low salt TEDG buffer at a ratio of 0.1 g of tissue per 1.0 mL buffer. The tissue will be minced with Metzenbaum scissors until all pieces are small 1-2 mm cubes and then homogenized using an appropriate homogenizer (5-second bursts).

Note: The homogenizer probe will be cooled prior to homogenizing each sample by placing the probe in ice-cold TEDG buffer. The homogenization tube will be kept in an ice-cold water bath during the homogenizing process.

The homogenate will be transferred to pre-cooled centrifuge tubes and spun in a centrifuge for 30 minutes at 30,000 × *g* at 4°C. The pellet contains the nuclear fraction and the supernatant contains the cytosol.

The cytosol from all the prostates collected the same day will be pooled and divided into aliquots. The aliquots will be used immediately in the AR binding assay or will be stored at –70 ± 10°C until use.

Note: The cytosol can be stored frozen at –70 ± 10°C for 6 months prior to use in the AR binding assay. Do not thaw and re-freeze the cytosol.

The protein content for each batch of cytosol will be determined using an appropriate method.

Note: The dithiothreitol in the buffer is not compatible with the Pierce BCA Protein Assay. Typical protein values are 5.5 to 8 mg/mL.

Assays

Protein Assay

The protein concentration of the cytosol preparation will be determined on each day of use as described in In Vitro Technologies method B020.A. In brief, a six-point standard curve will be prepared, ranging from 0.13 to 1.5 mg protein/mL. The protein standards will be made from bovine serum albumin. Protein will be determined using a BioRad® Protein Assay Kit II (Hercules, CA). To a 10 µL aliquot of standard or unknown, 200 µL of dye reagent will be added and mixed. The samples will be placed at room temperature for at least 5 minutes and up to an hour to allow for color development. The absorbance (600 nm) will be measured using a plate reader. The protein concentration of the cytosol sample will be determined by extrapolation of the absorbance value using the standard curve developed using the protein standards.

Evaluation of the Cytosol for Conducting Androgen Receptor Competitive Binding Assays

Prior to conducting the androgen receptor competitive binding assays with unknown chemicals, the cytosol will be characterized at In Vitro Technologies. This will be accomplished in two steps as follows:

1) A series of saturation radioligand binding assays will be conducted to demonstrate androgen receptor specificity and saturation. Nonlinear regression analysis of these data and subsequent Scatchard plots should document androgen receptor binding affinity (K_d) and the number of receptors (B_{max}).

2) A series of androgen receptor competitive binding assays will be conducted using R1881 and dexamethasone, substances with known affinities for the androgen receptor. Comparison of IC_{50} values (i.e., the concentration of a substance that inhibits [^{3}H]-R1881 binding by 50%) from these assays with reported values in the literature will assist in documenting that the cytosol is appropriate for routine use in the laboratory.

Saturation Radioligand Binding Assay

Androgen receptor saturation binding experiments measure total, non-specific, and specific binding of increasing concentrations of ^{3}H-R1881 under conditions of equilibrium.

The experimental setup and details of the saturation binding assay are specified in the In Vitro Technologies biological method B065.A. The saturation binding experiments will be conducted as three independent replicates. The same technician will conduct all three replicate experiments.

Competitive Binding Assay (Inhibition of Androgen Receptor Binding of [^{3}H]-R1881 by Test Articles)

An androgen receptor competitive binding assay measures the binding of a single concentration of [^{3}H]-R1881 in the presence of increasing concentrations of a test article. The experimental setup and details of the competitive binding assay are specified in the In Vitro Technologies biological method B066.A.

These experiments will test the androgen receptor binding of [^{3}H]-R1881 in the presence of multiple concentrations of a test article. The inhibition experiments will be conducted as three independent replicates. All three replicate experiments for a given test article will be conducted by the same technician and there will be three (triplicate) repetitions for each concentration within a given replicate.

Control samples will be included for each replicate experiment. These include:

- Vehicle or ethanol control (substrate, buffer, vehicle [used for preparation of test article solutions], and cytosol)
- Non-specific background control (substrate, buffer, R1881, and cytosol).

Six repetitions of each type of control are included with each replicate experiment and are treated the same as the other samples. The control sets will be split so that three tubes (of each control type) are run at the beginning and three at the end of each replicate set.

Description of Data Calculations

In Vitro Technologies will supply all raw data to Battelle in electronic format using Microsoft Excel® spreadsheets and Prism templates (to be developed and provided by Battelle).

IC_{50} Calculation

Data for the non-radiolabeled R1881 standard curve and each test article will be plotted as the percentage of [^{3}H]-R1881 bound versus the molar concentration (log) of competitor. Estimates of IC_{50} values will be determined using appropriate nonlinear curve fitting software to fit a one-site competitive binding model. The model will be constrained to fit the bottom of the curve to 0% and the top to 100%.

Relative Binding Affinity Values

The RBA value for each test article is calculated by dividing the IC_{50} value for R1881 by the IC_{50} of the test article and expressing the value as a percent (e.g., RBA for R1881 = 100%).

K_i Calculation

An estimate of the K_i can be calculated using the Cheng-Prusoff equation if the competitive binding curve reflects a pure competition for a single binding site: $K_i = IC_{50}/(1 + (L/K_d))$ where

L = radioligand concentration and K_d = equilibrium dissociation constant of radioligand. Alternatively, the K_i can be determined experimentally.

Reporting of Ambiguities

Ambiguities or unclear directions in the written protocol and a list of all problems that are encountered will be reported to Battelle.

Criteria for Data Acceptance

All data obtained will be reported.

Study Report

At completion of Task 3, tabular and graphical summaries of data will be prepared using the Excel spreadsheet and Prism document templates provided by Battelle. These electronic files will be submitted to Battelle within 7 days after completion of the task. Data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total DPM – background DPM, RBA, IC_{50}, and K_i.

Data Retention

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following issuance of the final report. At the end of this period, Battelle will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed. Study records to be maintained will include:

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used.
- Protocol and any amendments
- List of any protocol deviations
- List of standard operating procedures

Protocol Approval

This protocol has been reviewed and approved by the following:

Sponsor Representatives

David P. Houchens, Ph.D.	David P. Houchens	4/14/05
Program Manager Endocrine Disruptor Screening Program Battelle Memorial Institute	Signature	Date

James E. Morris, Ph.D.	James E. Morris	4-16-05
Principal Investigator Endocrine Disruptor Screening Program Battelle Memorial Institute	Signature	Date

Study Director

The study will be conducted to the standards of U.S. FDA 21 CFR Part 58. The study will be conducted under my scientific guidance and management. I have reviewed the procedures outlined in this protocol.

Aruna Koganti, Ph.D.	K. Aruna Prabha	27 April 2005
Study Director In Vitro Technologies	Signature	Date

Review

Terri L. Pollock, B.A.	Terri L Pollock	4-14-05
Quality Assurance Manager Battelle Memorial Institute	Signature	Date

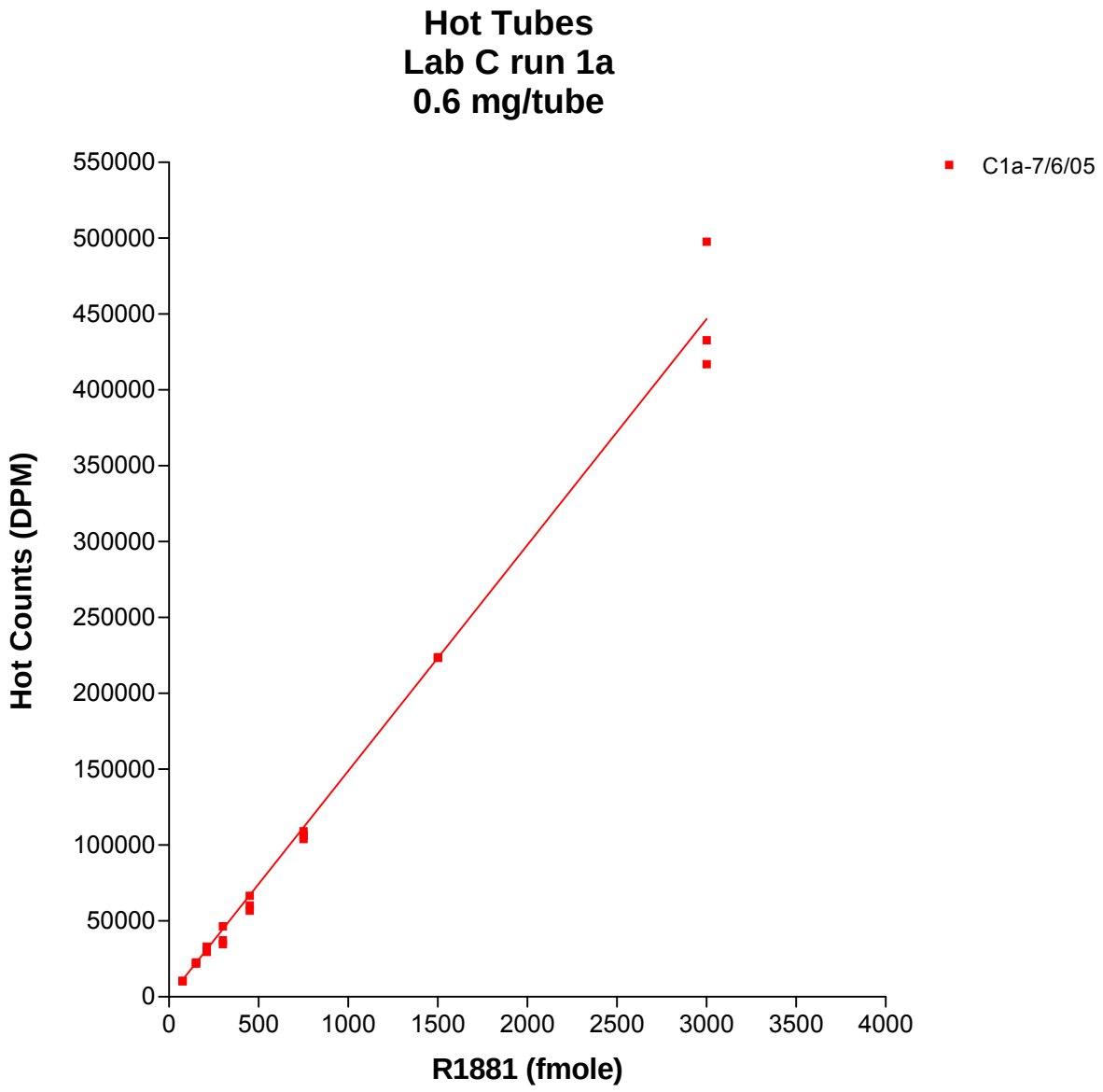
Sharon Isbell	Sharon Isbell	27April2005
Director, Quality Systems In Vitro Technologies	Signature	Date

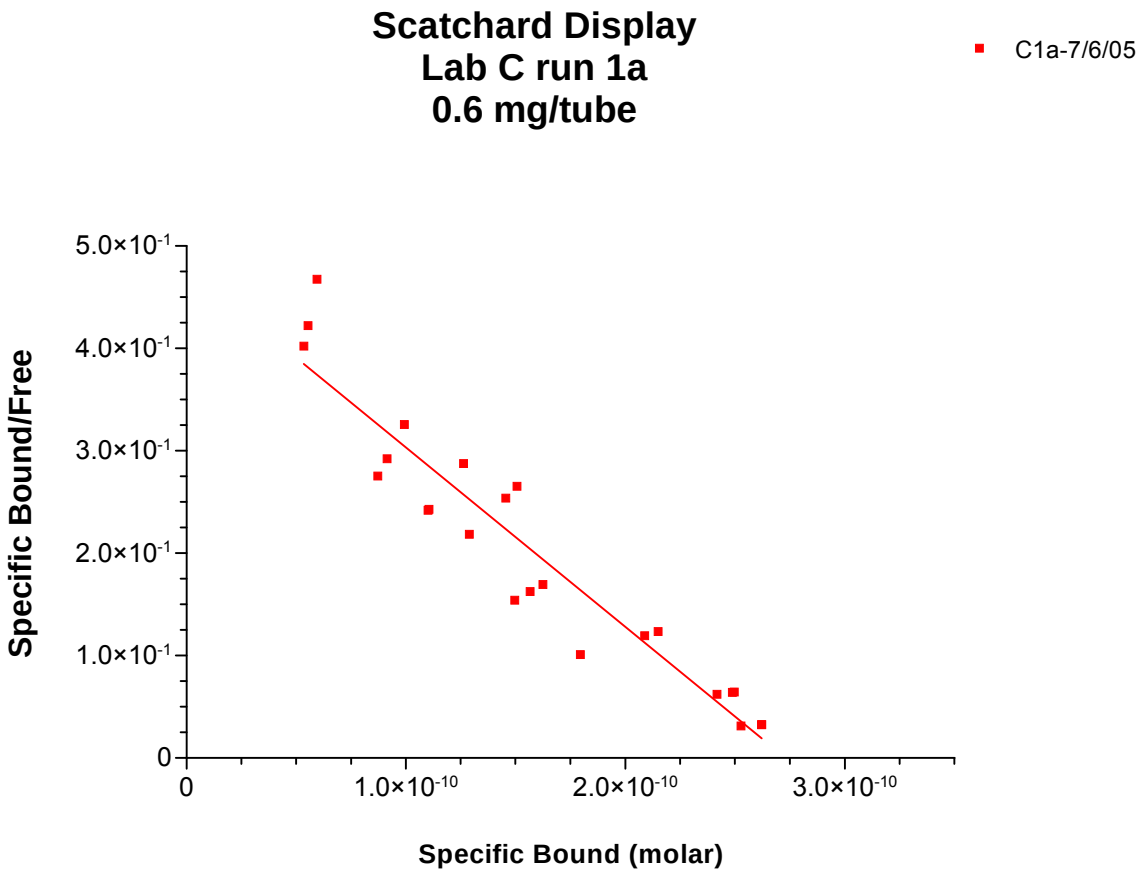
Appendix 2: Protocol Deviation

The following deviation occurred during the conduct of this study:

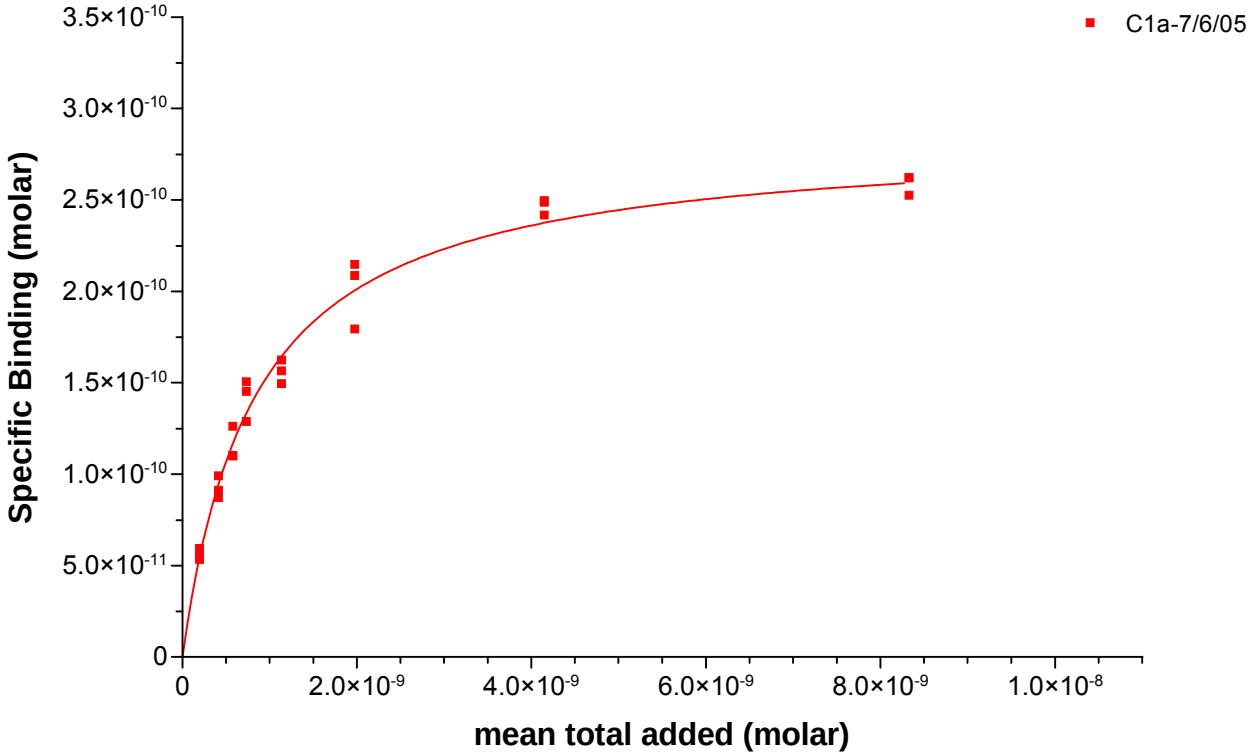
The protocol states that the protein concentration of the cytosol preparation is to be determined on each day of use. The protein concentration was determined at the time the cytosol was prepared and not on each day of use. The study director has correspondence from Battelle stating that the protein concentration determination on each day of use was not necessary. Thus, this deviation did not impact the outcome of the study.

Appendix 3: Prism Files for Saturation Runs

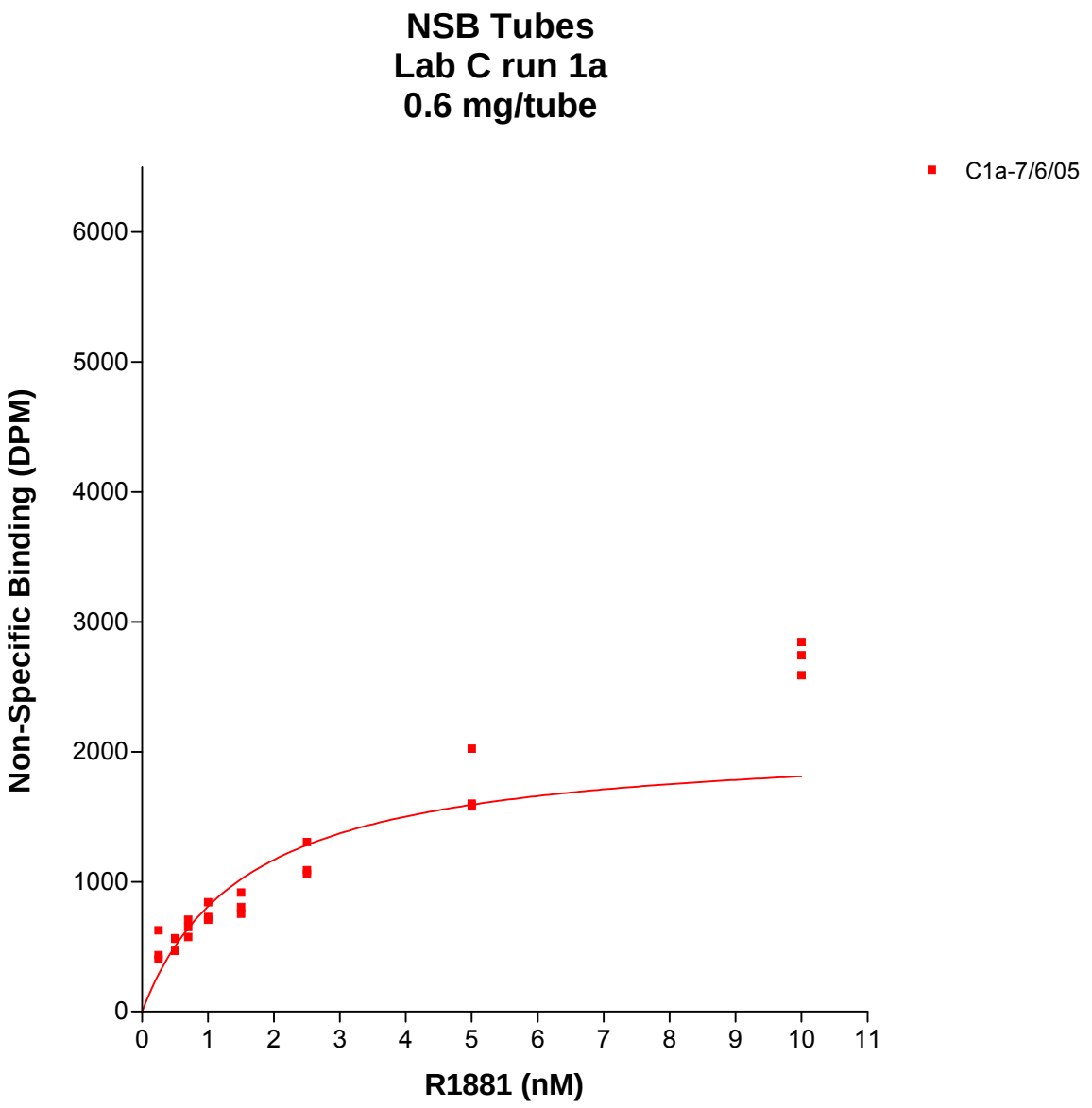

Hot Tubes
Lab C run 1a
0.6 mg/tube
C1a-7/6/05
Hot Counts (DPM)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
500
1000
1500
2000
2500
3000
3500
4000
R1881 (fmole)

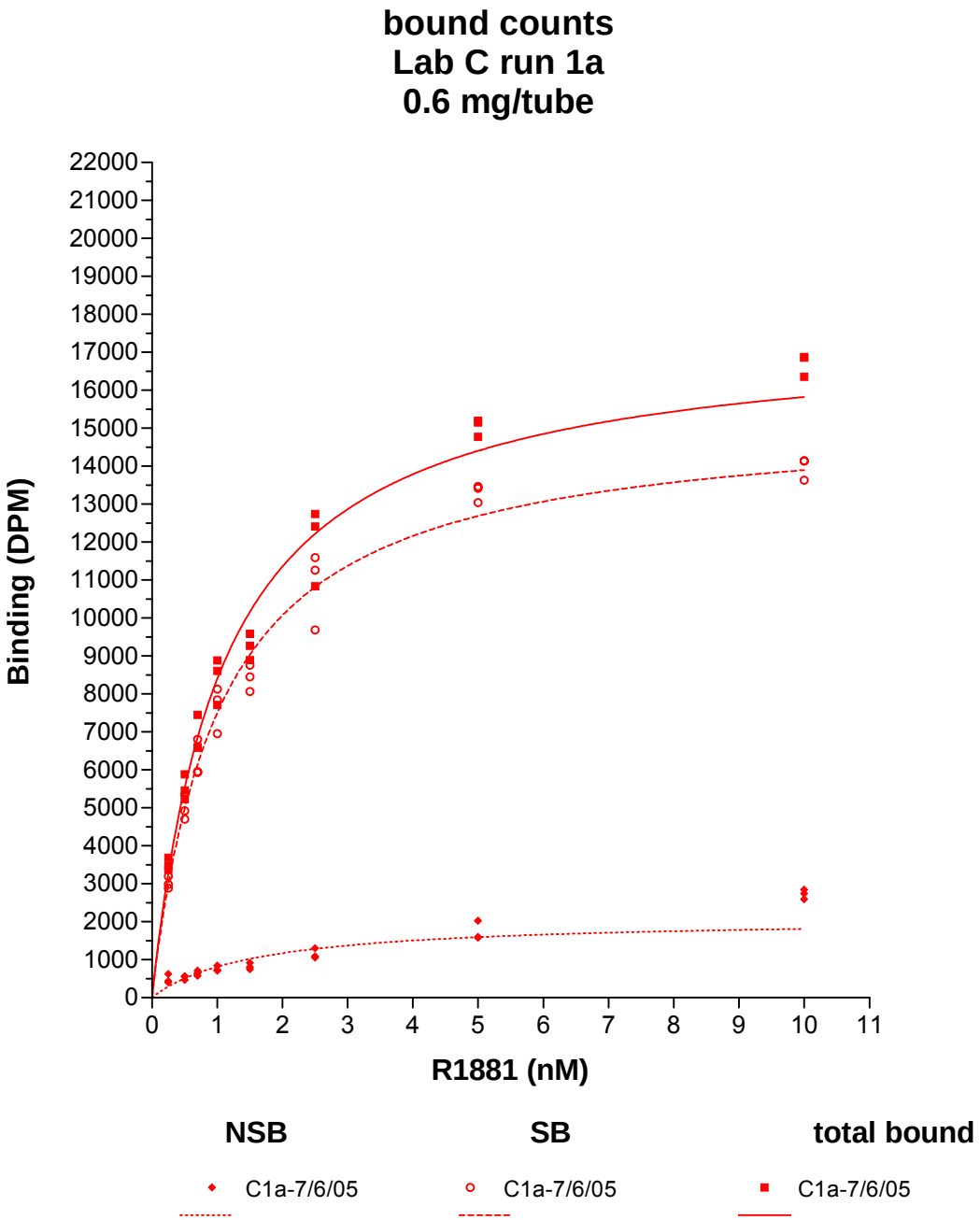

Scatchard Display
Lab C run 1a
0.6 mg/tube
C1a-7/6/05
Specific Bound/Free
5.0×10-1
4.0×10-1
3.0×10-1
2.0×10-1
1.0×10-1
0
0
1.0×10-10
2.0×10-10
3.0×10-10
Specific Bound (molar)

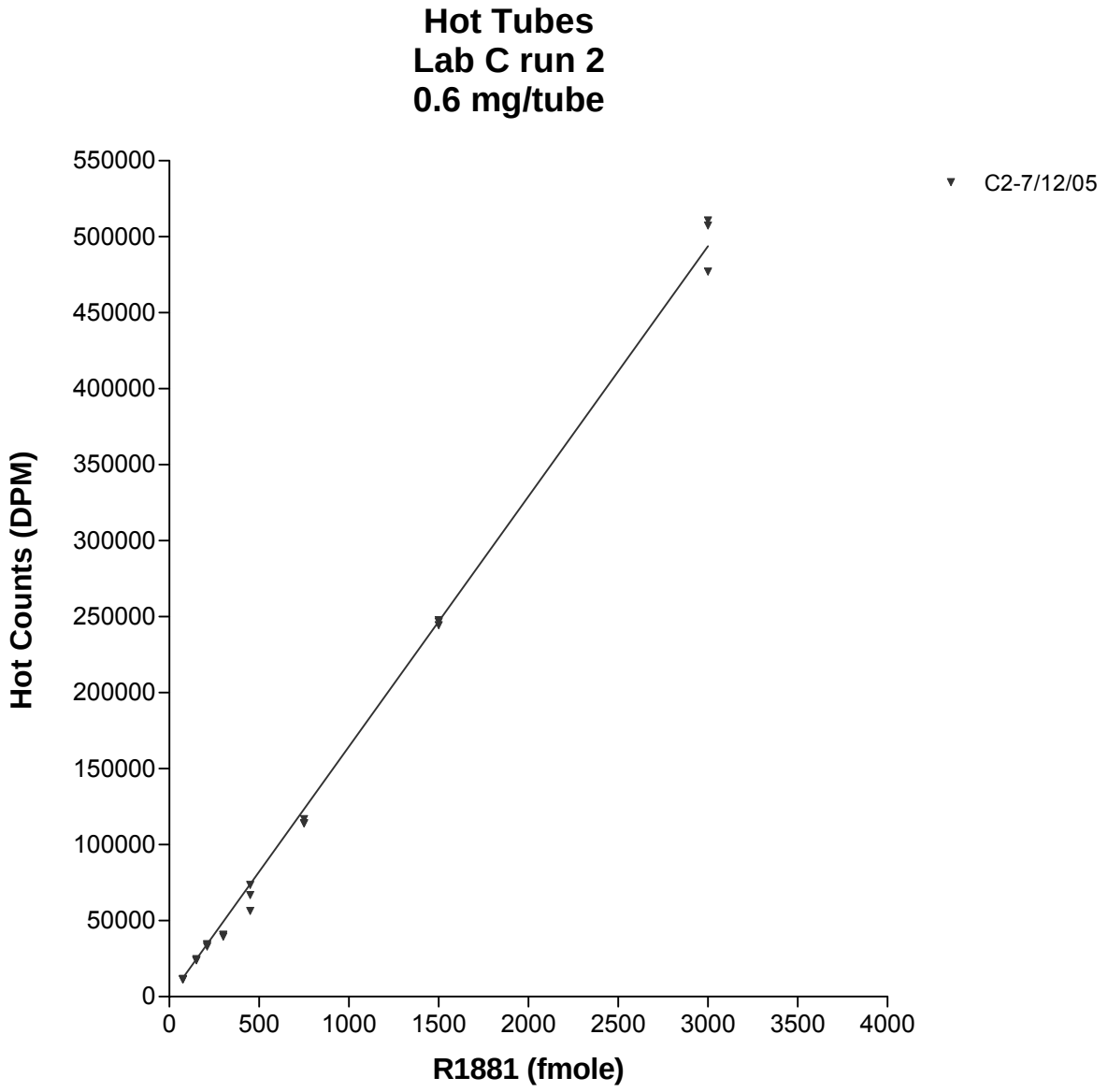
Lab C run 1a
0.6 mg/tube

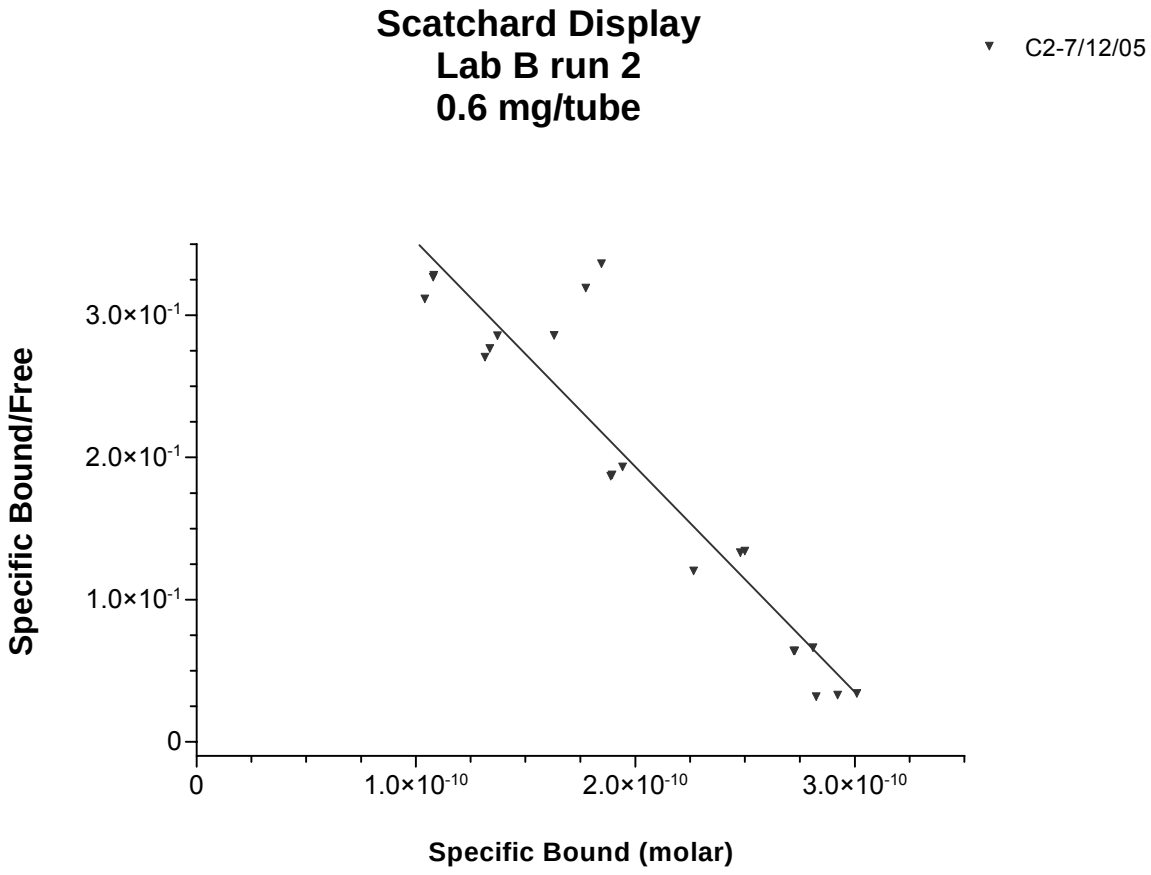


Specific bound	C1a-7/6/05
BMAX	2.852e-010
KD	8.348e-010
Std. Error	
BMAX	8.160e-012
KD	4.740e-011
95% Confidence Intervals	
BMAX	2.683e-010 to 3.022e-010
KD	7.365e-010 to 9.331e-010
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9811
Weighted Sum of Squares ($1/Y^2$)	0.09067
Absolute Sum of Squares	2.109e-021
Sy.x	9.791e-012
Data	
Number of X values	24
Number of Y replicates	1
Total number of values	24
Number of missing values	0

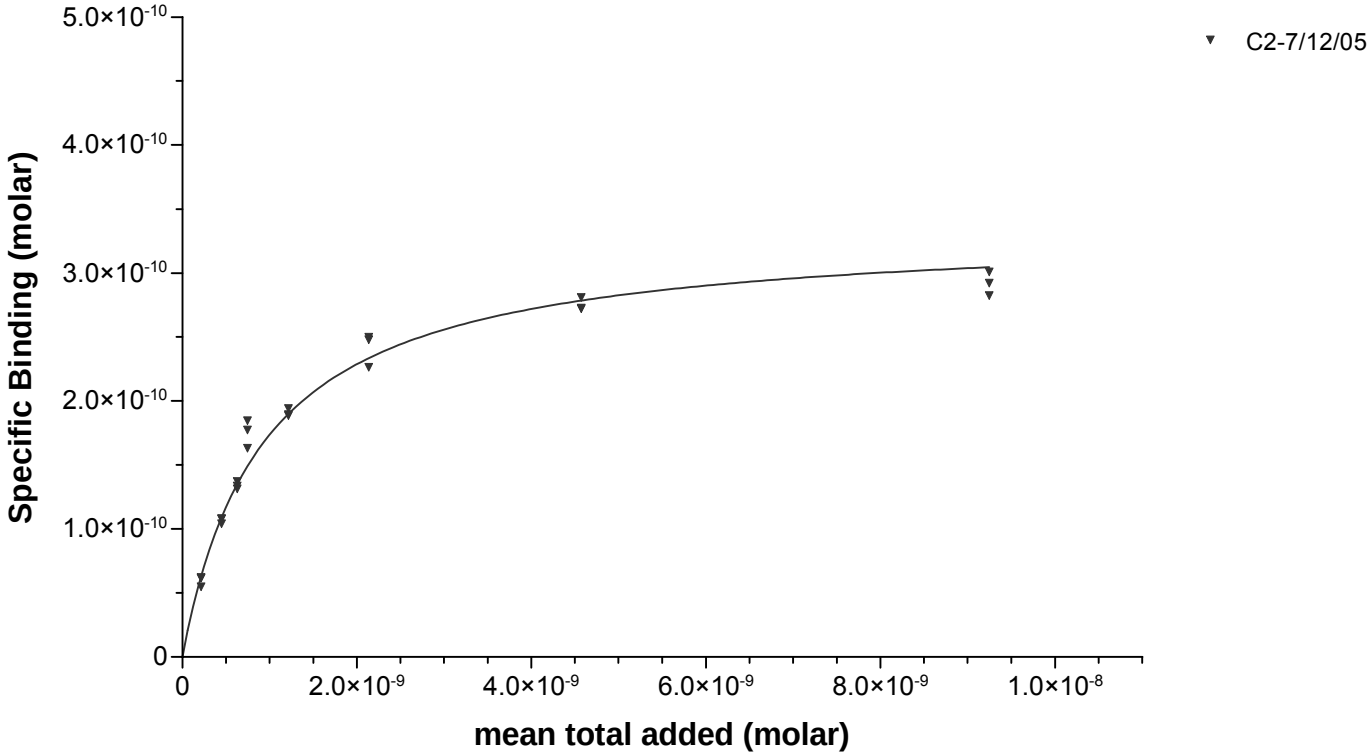

NSB Tubes
Lab C run 1a
0.6 mg/tube
C1a-7/6/05
Non-Specific Binding (DPM)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)


bound counts
Lab C run 1a
0.6 mg/tube
Binding (DPM)
22000
21000
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)
NSB
SB
total bound
C1a-7/6/05
C1a-7/6/05
C1a-7/6/05


Hot Tubes
Lab C run 2
0.6 mg/tube
Hot Counts (DPM)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
500
1000
1500
2000
2500
3000
3500
4000
R1881 (fmole)
C2-7/12/05

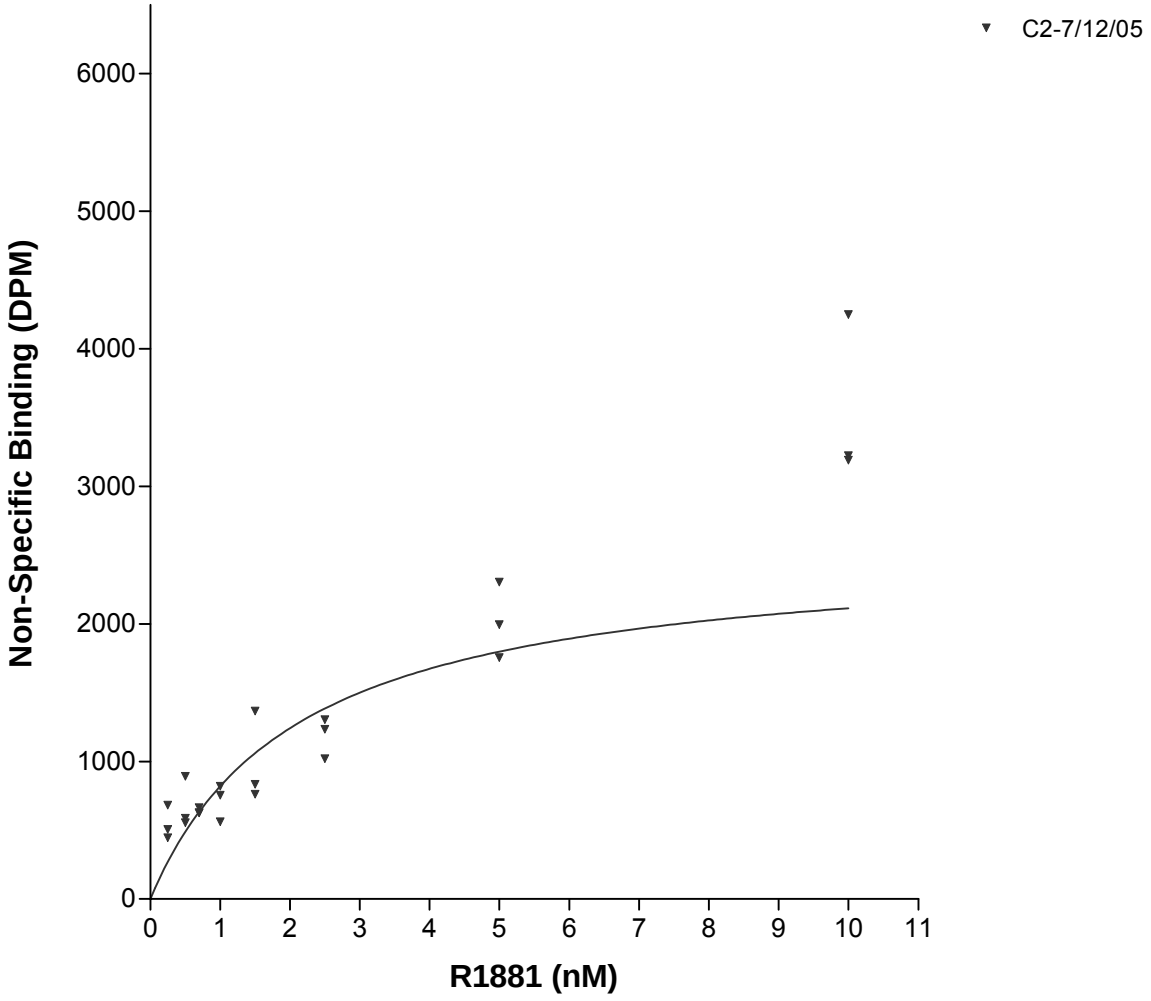

Scatchard Display
Lab B run 2
0.6 mg/tube
C2-7/12/05
Specific Bound/Free
3.0×10-1
2.0×10-1
1.0×10-1
0
0
1.0×10-10
2.0×10-10
3.0×10-10
Specific Bound (molar)

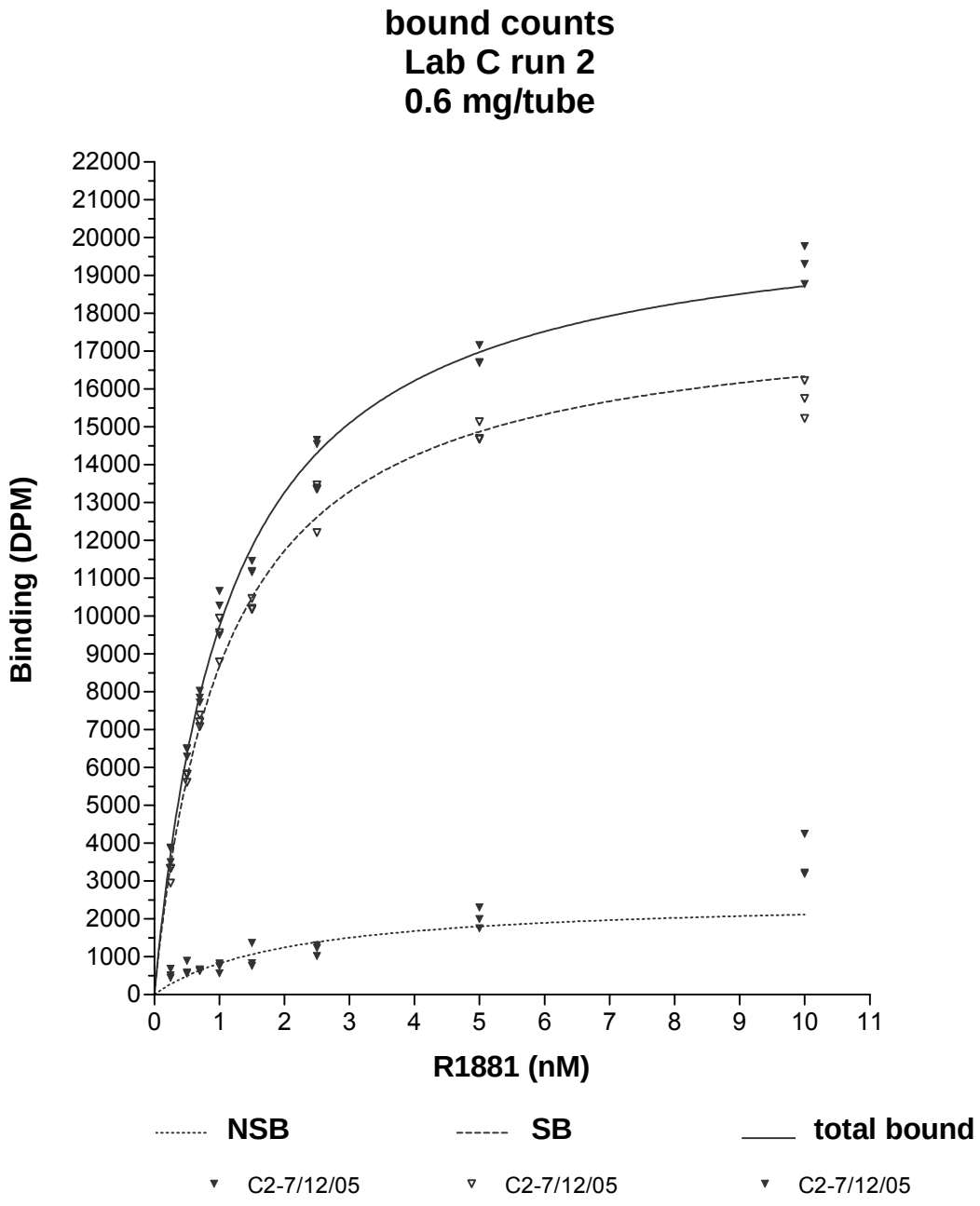
Lab C run 2
0.6 mg/tube

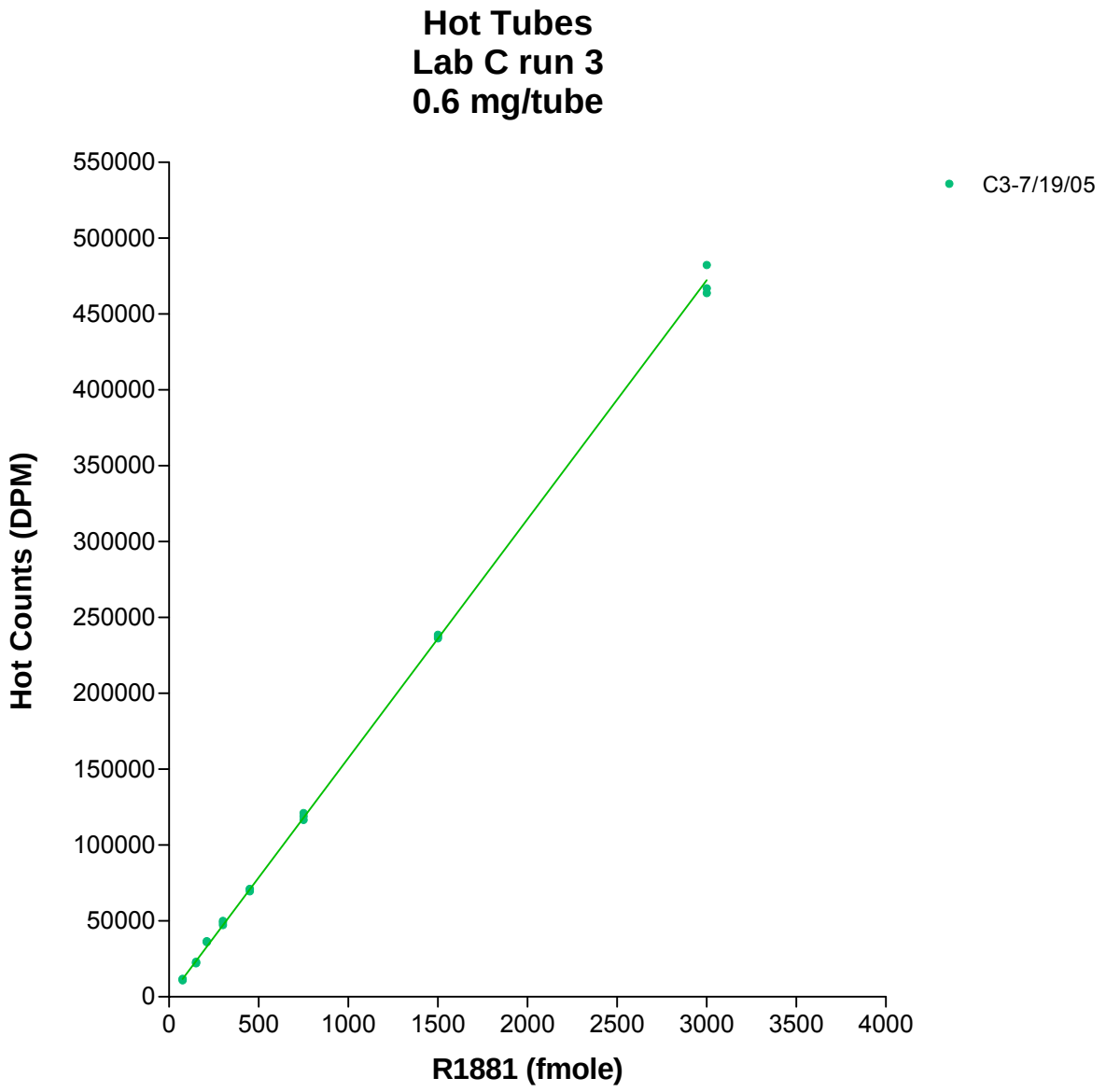


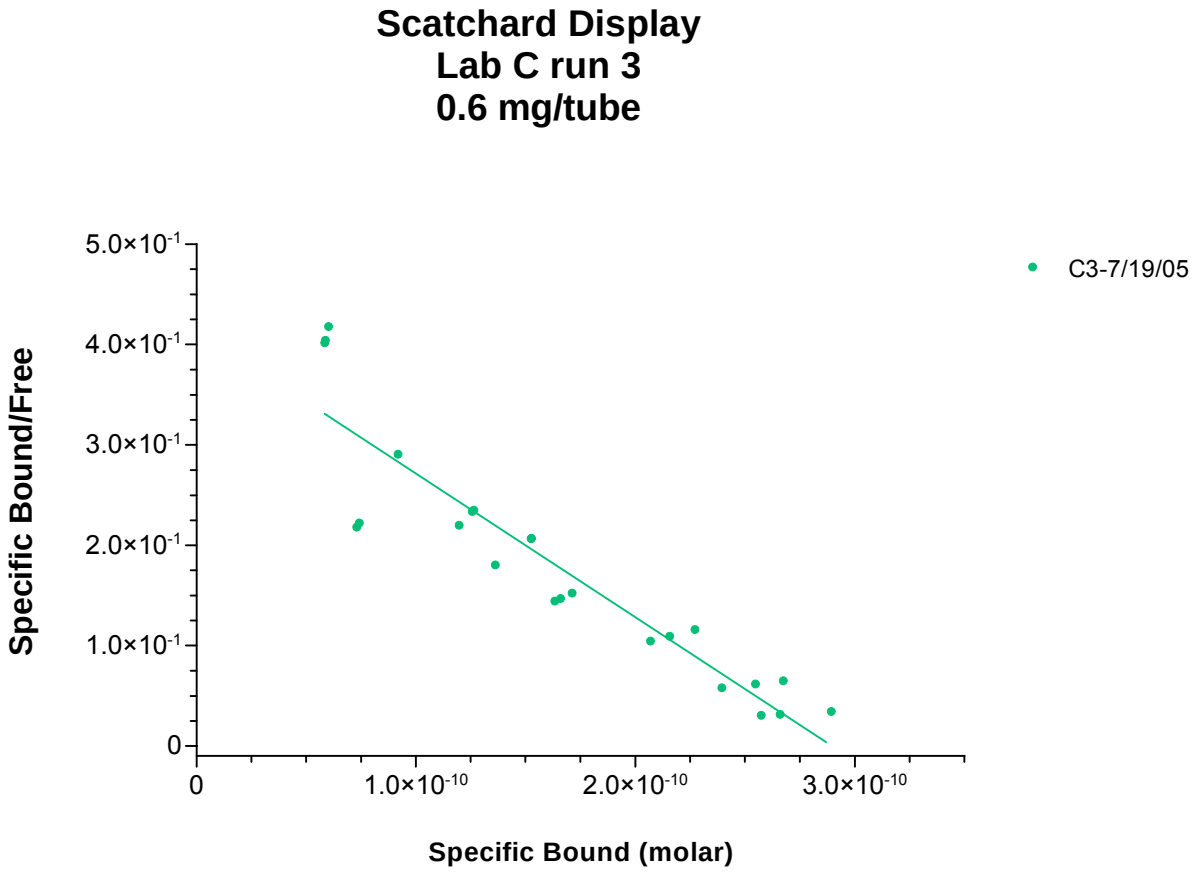
	C2-7/12/05
BMAX	3.351e-010
KD	9.300e-010
Std. Error	
BMAX	1.031e-011
KD	5.555e-011
95% Confidence Intervals	
BMAX	3.138e-010 to 3.565e-010
KD	8.148e-010 to 1.045e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9747
Weighted Sum of Squares ($1/Y^2$)	0.1109
Absolute Sum of Squares	3.619e-021
Sy.x	1.283e-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 C run 2
0.6 mg/tube

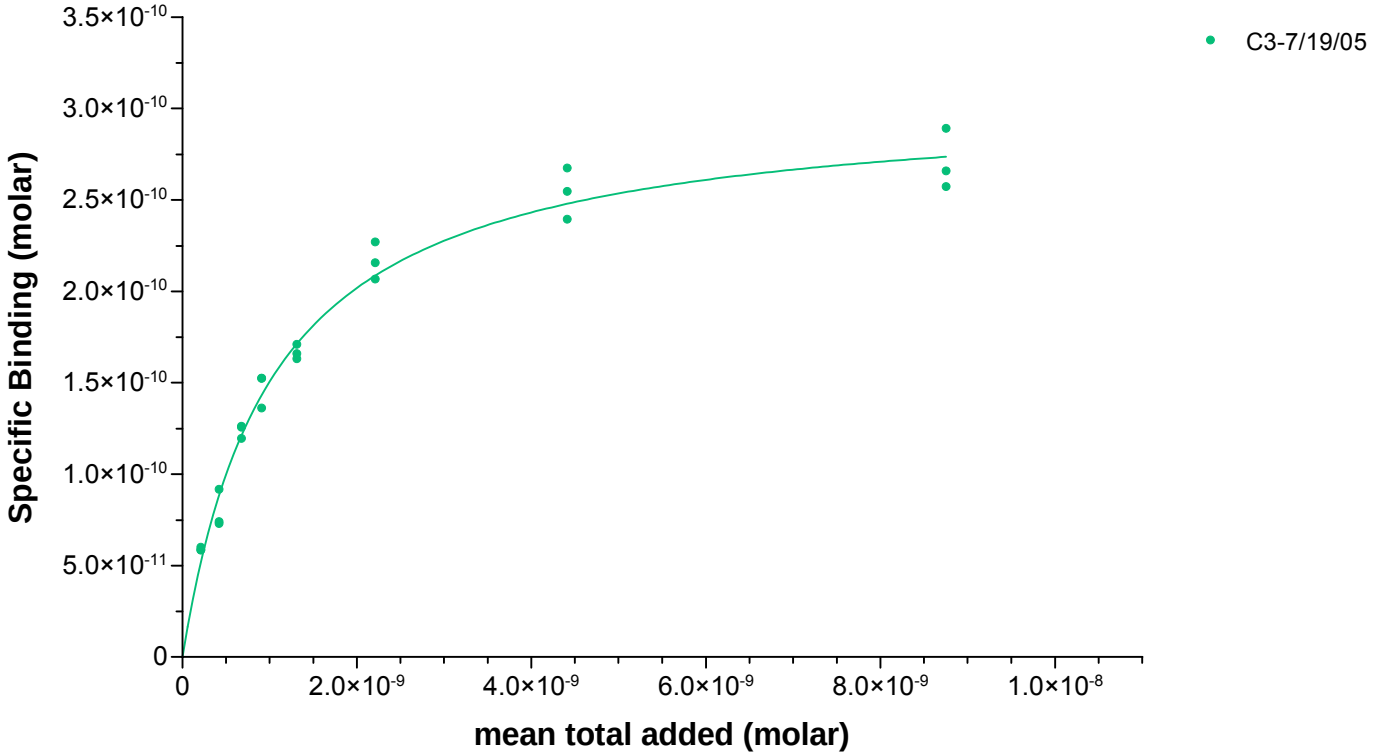



bound counts
Lab C run 2
0.6 mg/tube
Binding (DPM)
22000
21000
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)
NSB
SB
total bound
C2-7/12/05
C2-7/12/05
C2-7/12/05

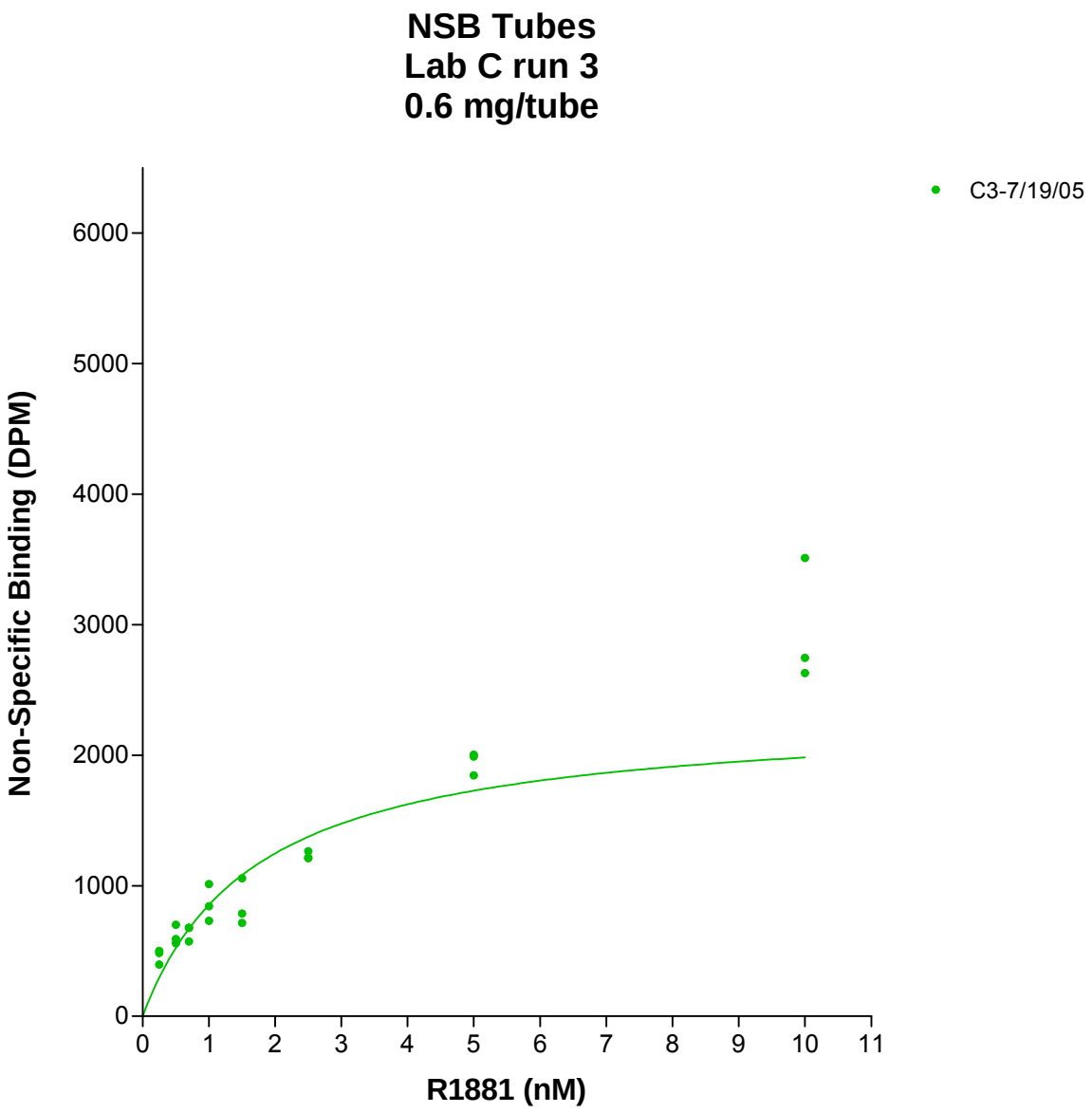

Hot Tubes
Lab C run 3
0.6 mg/tube
Hot Counts (DPM)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
500
1000
1500
2000
2500
3000
3500
4000
R1881 (fmole)
C3-7/19/05

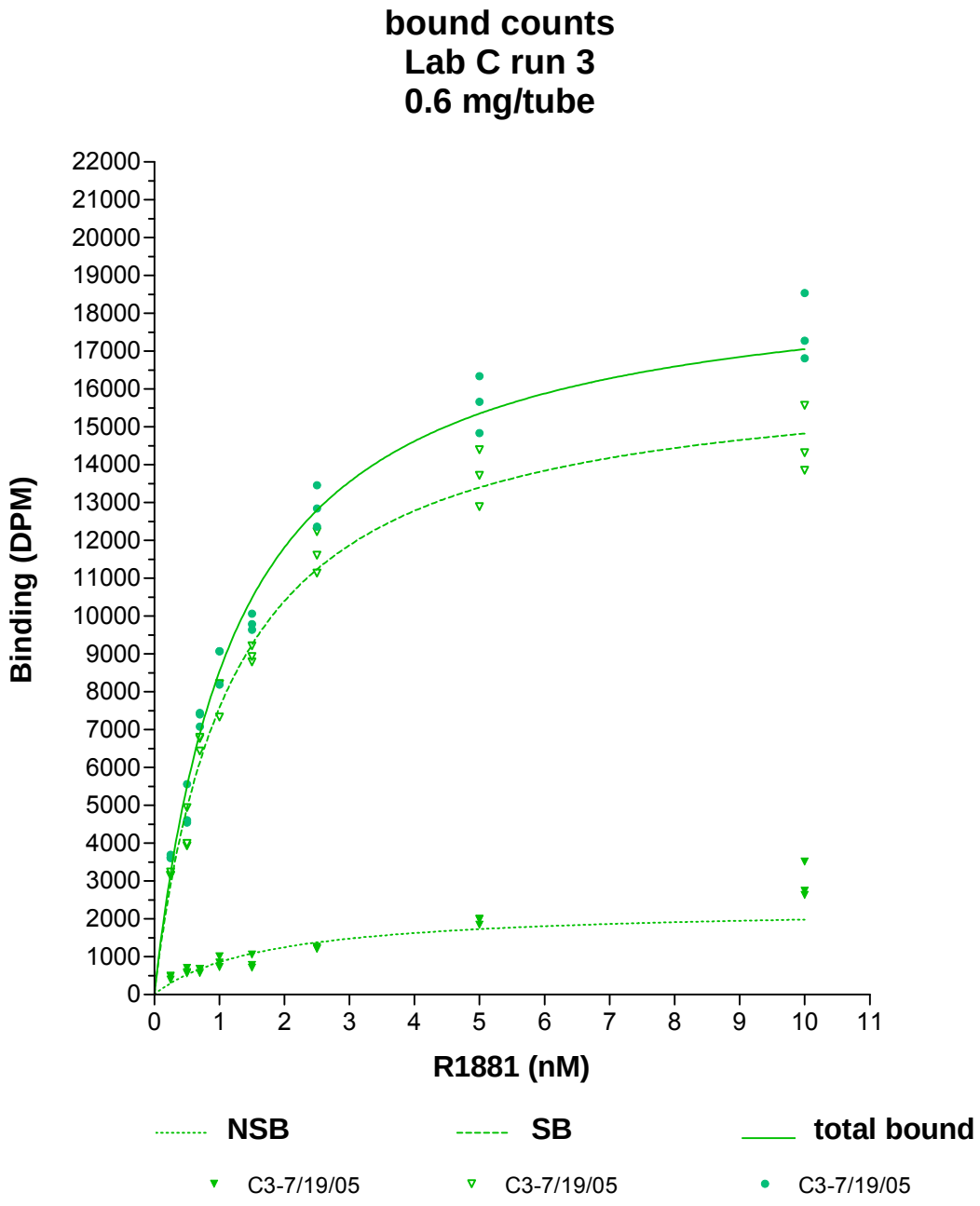

Scatchard Display
Lab C run 3
0.6 mg/tube
Specific Bound/Free
5.0×10-1
4.0×10-1
3.0×10-1
2.0×10-1
1.0×10-1
0
0
1.0×10-10
2.0×10-10
3.0×10-10
Specific Bound (molar)
C3-7/19/05

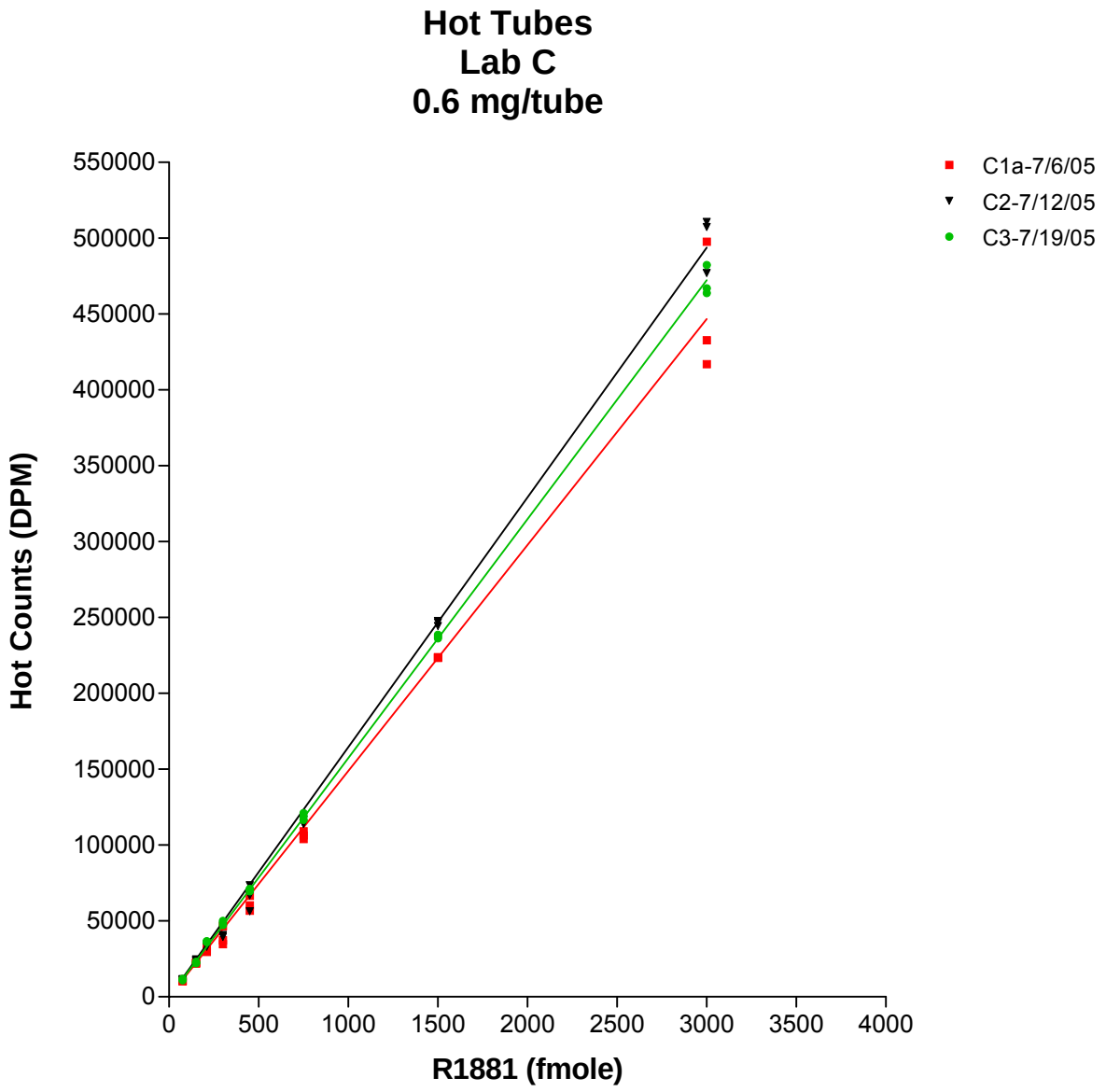
Lab C run 3
0.6 mg/tube

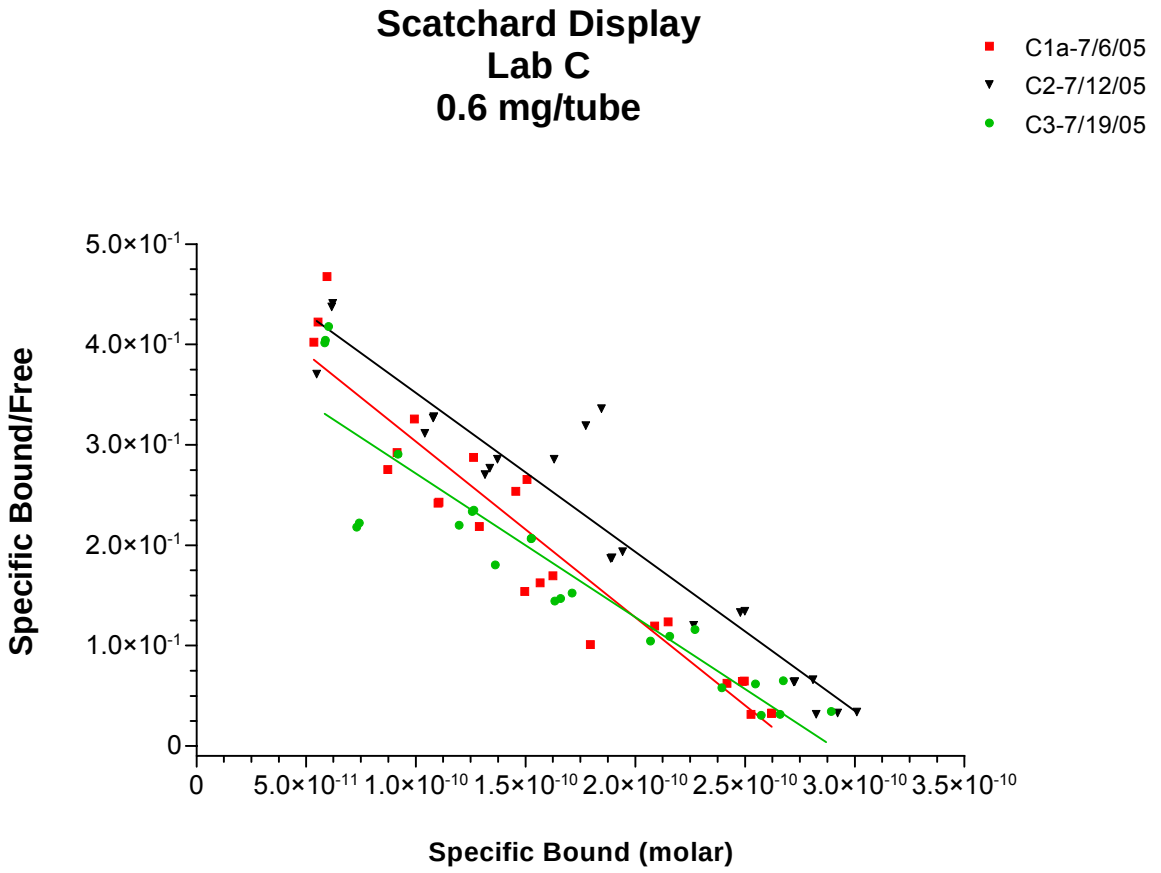


	C3-7/19/05
BMAX	3.057e-010
KD	1.029e-009
Std. Error	
BMAX	1.203e-011
KD	7.795e-011
95% Confidence Intervals	
BMAX	2.808e-010 to 3.307e-010
KD	8.677e-010 to 1.191e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9811
Weighted Sum of Squares ($1/Y^2$)	0.1628
Absolute Sum of Squares	2.427e-021
Sy.x	1.050e-011
Data	
Number of X values	24
Number of Y replicates	1
Total number of values	24
Number of missing values	0

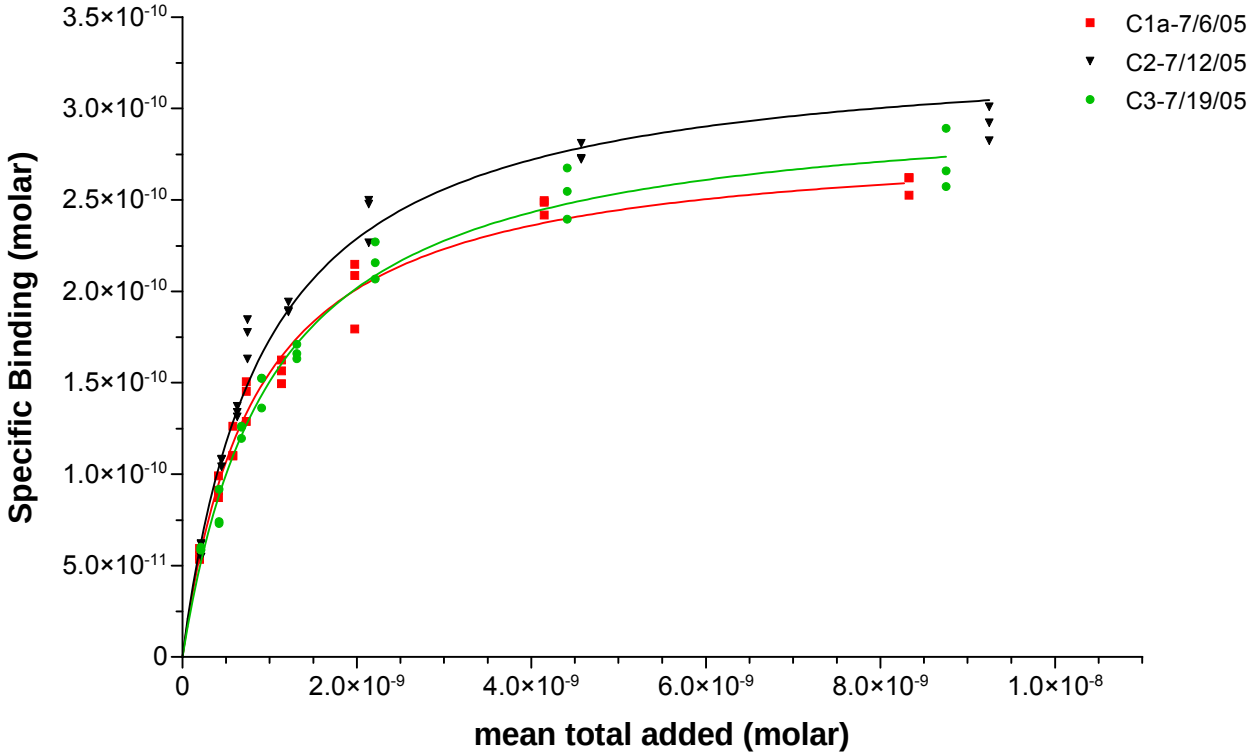

NSB Tubes
Lab C run 3
0.6 mg/tube
C3-7/19/05
Non-Specific Binding (DPM)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)


bound counts
Lab C run 3
0.6 mg/tube
Binding (DPM)
22000
21000
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)
NSB
SB
total bound
C3-7/19/05
C3-7/19/05
C3-7/19/05

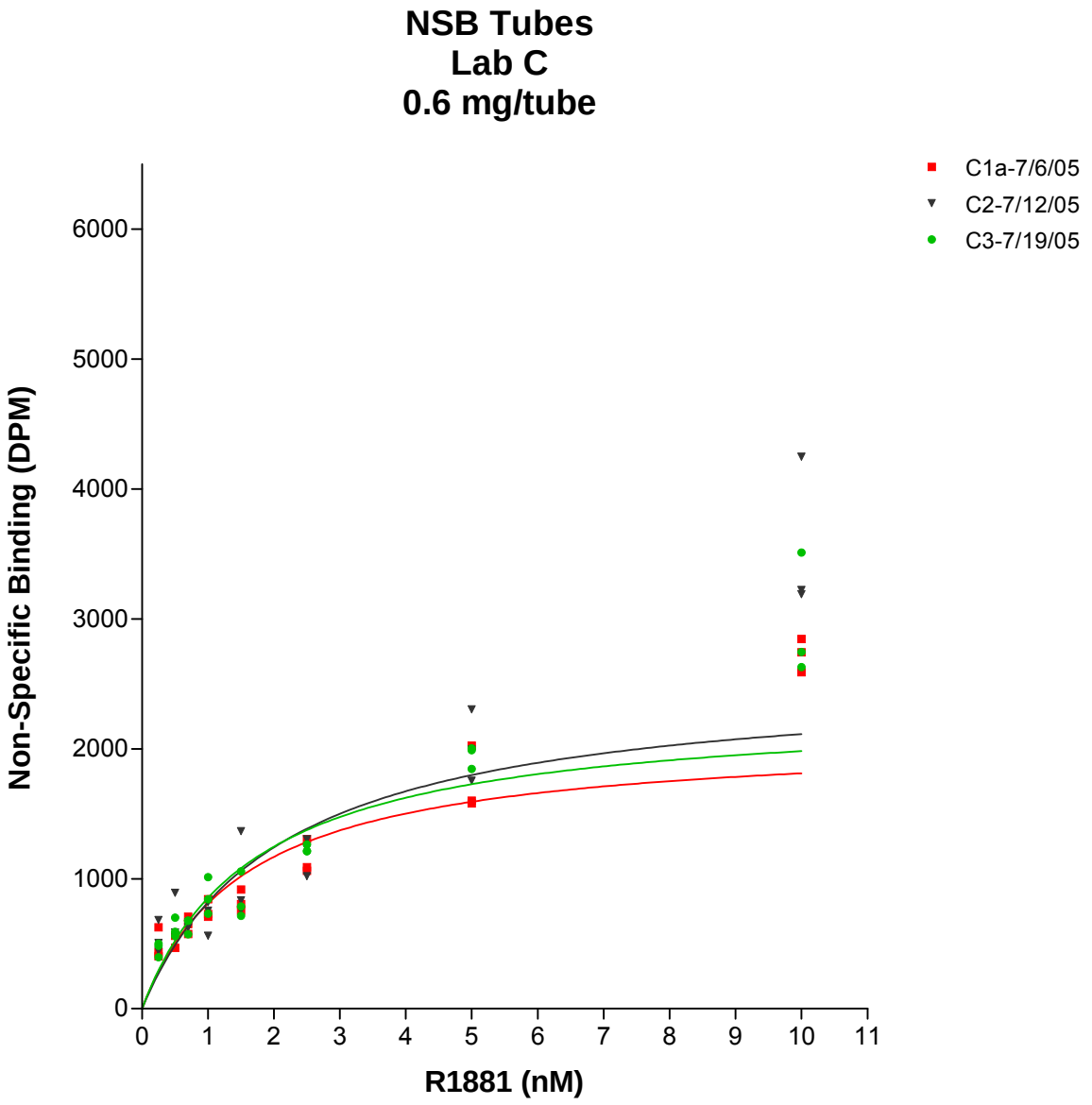

Hot Tubes
Lab C
0.6 mg/tube
Hot Counts (DPM)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
500
1000
1500
2000
2500
3000
3500
4000
R1881 (fmole)
C1a-7/6/05
C2-7/12/05
C3-7/19/05

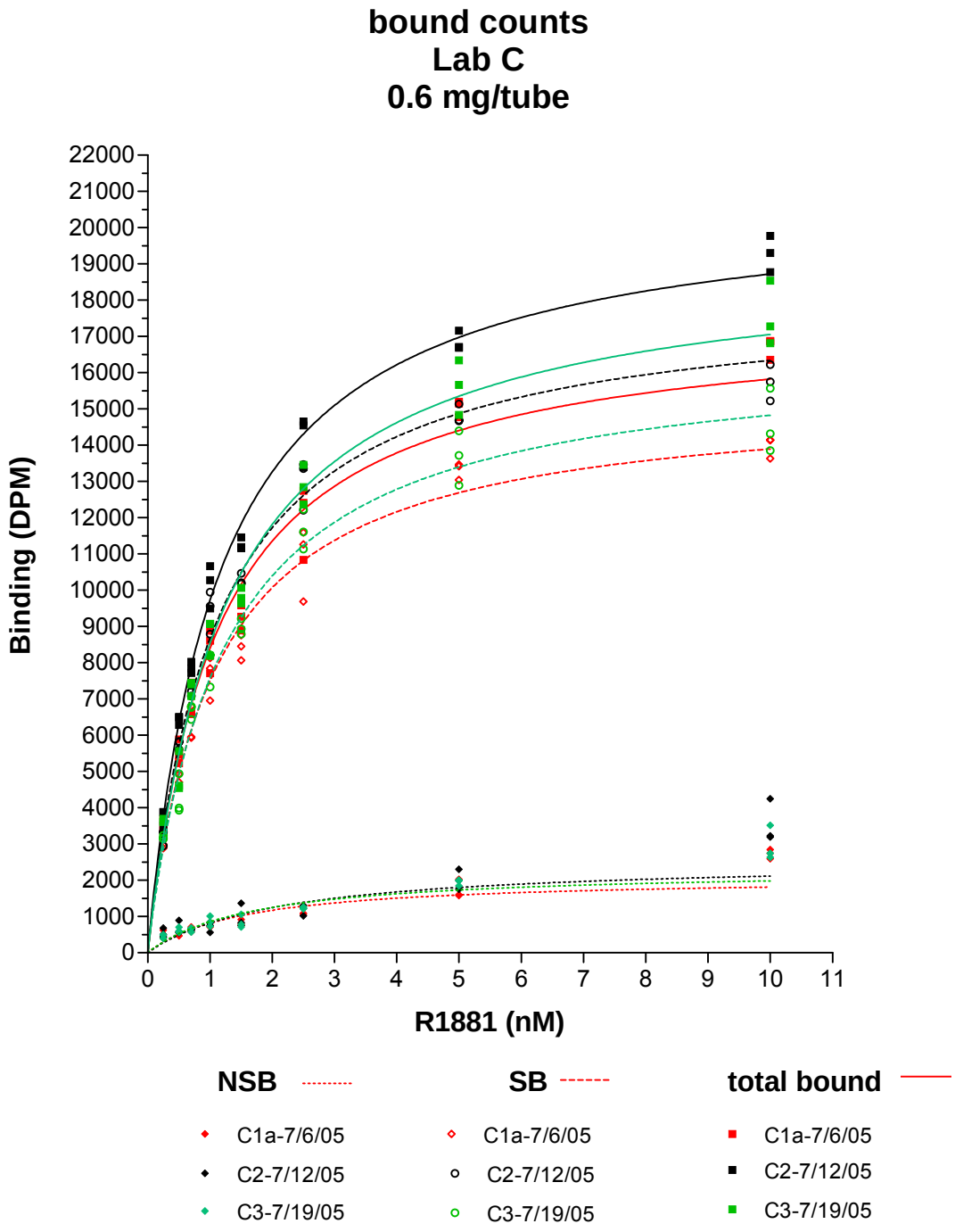

Scatchard Display
Lab C
0.6 mg/tube
C1a-7/6/05
C2-7/12/05
C3-7/19/05
Specific Bound/Free
5.0×10-1
4.0×10-1
3.0×10-1
2.0×10-1
1.0×10-1
0
0
5.0×10-11
1.0×10-10
1.5×10-10
2.0×10-10
2.5×10-10
3.0×10-10
3.5×10-10
Specific Bound (molar)

Lab C
0.6 mg/tube



Specific bound	C1a-7/6/05	C2-7/12/05	C3-7/19/05
BMAX	2.852e-010	3.351e-010	3.057e-010
KD	8.348e-010	9.300e-010	1.029e-009
Std. Error			
BMAX	8.160e-012	1.031e-011	1.203e-011
KD	4.740e-011	5.555e-011	7.795e-011
95% Confidence Intervals			
BMAX	2.683e-010 to 3.022e-010	3.138e-010 to 3.565e-010	2.808e-010 to 3.307e-010
KD	7.365e-010 to 9.331e-010	8.148e-010 to 1.045e-009	8.677e-010 to 1.191e-009
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9811	0.9747	0.9811
Weighted Sum of Squares ($1/Y^2$)	0.09067	0.1109	0.1628
Absolute Sum of Squares	2.109e-021	3.619e-021	2.427e-021
Sy.x	9.791e-012	1.283e-011	1.050e-011
Data			
Number of X values	24	24	24
Number of Y replicates	1	1	1
Total number of values	24	24	24
Number of missing values	0	0	0


NSB Tubes
Lab C
0.6 mg/tube
C1a-7/6/05
C2-7/12/05
C3-7/19/05
Non-Specific Binding (DPM)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)


bound counts
Lab C
0.6 mg/tube
Binding (DPM)
22000
21000
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
11
R1881 (nM)
NSB
SB
total bound
C1a-7/6/05
C2-7/12/05
C3-7/19/05
C1a-7/6/05
C2-7/12/05
C3-7/19/05
C1a-7/6/05
C2-7/12/05
C3-7/19/05

Appendix 4: Excel Files for Saturation Runs

Laboratory C

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:	C	
Run identification:	1a (Seq#6397a)	
Assay start date:	7/6/2005	
Tracer lot number:	3559-507	
Specific activity on day of assay:	81.01	**Ci/mmole**
Cytosol lot or vial number:	0725-06-01	
protein (cytosol) per tube:	600	**ug**
protein (cytosol) per tube:	0.6	**mg**
KD	8.35E-01	**nM**
Bmax	14.26	**fmole/100 ug**
total volume in tubes	300	**uL**
volume of ethanol counted:	2	**mL**
mulitply DPM in sample by :	3	

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

protcol 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)	Triamcelenone 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"	Ten Percent Rule	Saturation X values	Bound y values	NSB y values
1	1	H	10.0	7.5	0.25	—	—	—	50	300	—	1230.63	3691.884	TRUE		35.0%	0.25	3691.9	489.2
2	2	H	10.0	7.5	0.25	—	—	—	50	300	—	1124.17	3372.507	TRUE		32.0%	0.25	3372.5	489.2
3	3	H	10.0	7.5	0.25	—	—	—	50	300	—	1157.93	3473.781	TRUE		33.0%	0.25	3473.8	489.2
4	1	H	10.0	15	0.50	—	—	—	50	300	—	1820.06	5460.174	TRUE		24.5%	0.5	5460.2	532.2
5	2	H	10.0	15	0.50	—	—	—	50	300	—	1961.39	5884.161	TRUE		26.4%	0.5	5884.2	532.2
6	3	H	10.0	15	0.50	—	—	—	50	300	—	1745.06	5235.174	TRUE		23.5%	0.5	5235.2	532.2
7	1	H	10.0	21	0.70	—	—	—	50	300	—	2200.88	6602.649	TRUE		21.2%	0.7	6602.6	646.2
8	2	H	10.0	21	0.70	—	—	—	50	300	—	2485.01	7455.039	TRUE		23.9%	0.7	7455.0	646.2
9	3	H	10.0	21	0.70	—	—	—	50	300	—	2194.40	6583.185	TRUE		21.1%	0.7	6583.2	646.2
10	1	H	10.0	30	1.00	—	—	—	50	300	—	2962.86	8888.571	TRUE		22.5%	1	8888.6	760.9
11	2	H	10.0	30	1.00	—	—	—	50	300	—	2868.91	8606.715	TRUE		21.8%	1	8606.7	760.9
12	3	H	10.0	30	1.00	—	—	—	50	300	—	2571.67	7714.995	TRUE		19.5%	1	7715.0	760.9
13	1	H	10.0	45	1.50	—	—	—	50	300	—	2964.02	8892.072	TRUE		14.5%	1.5	8892.1	826.2
14	2	H	10.0	45	1.50	—	—	—	50	300	—	3196.11	9588.333	TRUE		15.7%	1.5	9588.3	826.2
15	3	H	10.0	45	1.50	—	—	—	50	300	—	3091.70	9275.097	TRUE		15.1%	1.5	9275.1	826.2
16	1	H	100.0	7.5	2.50	—	—	—	50	300	—	4248.69	12746.07	TRUE		12.0%	2.5	12746.1	1152.4
17	2	H	100.0	7.5	2.50	—	—	—	50	300	—	4137.53	12412.593	TRUE		11.7%	2.5	12412.6	1152.4
18	3	H	100.0	7.5	2.50	—	—	—	50	300	—	3612.60	10837.788	TRUE		10.2%	2.5	10837.8	1152.4
19	1	H	100.0	15	5.00	—	—	—	50	300	—	5051.51	15154.536	TRUE		6.8%	5	15154.5	1735.9
20	2	H	100.0	15	5.00	—	—	—	50	300	—	4925.65	14776.947	TRUE		6.6%	5	14776.9	1735.9
21	3	H	100.0	15	5.00	—	—	—	50	300	—	5067.23	15201.678	TRUE		6.8%	5	15201.7	1735.9
22	1	H	100.0	30	10.00	—	—	—	50	300	—	5624.73	16874.184	TRUE		3.8%	10	16874.2	2727.7
23	2	H	100.0	30	10.00	—	—	—	50	300	—	5453.39	16360.17	TRUE		3.6%	10	16360.2	2727.7
24	3	H	100.0	30	10.00	—	—	—	50	300	—	5621.30	16863.9	TRUE		3.8%	10	16863.9	2727.7

25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	145.16	435.4911	TRUE
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	209.31	627.9174	TRUE
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	134.69	404.0826	TRUE
28	1	HC	10.0	15	0.5	1.00	15	50	50	300	C7	188.52	565.5576	TRUE
29	2	HC	10.0	15	0.5	1.00	15	50	50	300	C7	156.60	469.7904	TRUE
30	3	HC	10.0	15	0.5	1.00	15	50	50	300	C7	187.09	561.279	TRUE
31	1	HC	10.0	21	0.7	1.00	21	70	50	300	C6	236.51	709.5177	TRUE
32	2	HC	10.0	21	0.7	1.00	21	70	50	300	C6	191.95	575.8404	TRUE
33	3	HC	10.0	21	0.7	1.00	21	70	50	300	C6	217.71	653.1249	TRUE
34	1	HC	10.0	30	1	1.00	30	100	50	300	C5	243.36	730.0836	TRUE
35	2	HC	10.0	30	1	1.00	30	100	50	300	C5	281.07	843.195	TRUE
36	3	HC	10.0	30	1	1.00	30	100	50	300	C5	236.51	709.5177	TRUE
37	1	HC	10.0	45	1.5	1.00	45	150	50	300	C4	251.27	753.8037	TRUE
38	2	HC	10.0	45	1.5	1.00	45	150	50	300	C4	306.15	918.4566	TRUE
39	3	HC	10.0	45	1.5	1.00	45	150	50	300	C4	268.73	806.2008	TRUE
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	363.33	1089.9837	TRUE
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	435.31	1305.924	TRUE
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	353.78	1061.3277	TRUE
43	1	HC	100.0	15	5	10.00	15	500	50	300	C2	534.07	1602.1965	TRUE
44	2	HC	100.0	15	5	10.00	15	500	50	300	C2	675.18	2025.54	TRUE
45	3	HC	100.0	15	5	10.00	15	500	50	300	C2	526.64	1579.9212	TRUE
46	1	HC	100.0	30	10	10.00	30	1000	50	300	C1	914.87	2744.6067	TRUE
47	2	HC	100.0	30	10	10.00	30	1000	50	300	C1	863.76	2591.2821	TRUE
48	3	HC	100.0	30	10	10.00	30	1000	50	300	C1	949.07	2847.2154	TRUE
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—	—	10604.9	10604.92	TRUE
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—	—	10625.1	10625.11	TRUE
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—	—	10392.8	10392.8	TRUE
52	1	Hot	10.0	15	0.06	—	—	—	—	—	—	22686.7	22686.73	TRUE
53	2	Hot	10.0	15	0.06	—	—	—	—	—	—	21874.1	21874.07	TRUE
54	3	Hot	10.0	15	0.06	—	—	—	—	—	—	22364.9	22364.88	TRUE
55	1	Hot	10.0	21	0.08	—	—	—	—	—	—	30545.8	30545.83	TRUE
56	2	Hot	10.0	21	0.08	—	—	—	—	—	—	29642.5	29642.5	TRUE
57	3	Hot	10.0	21	0.08	—	—	—	—	—	—	33196.9	33196.93	TRUE
58	1	Hot	10.0	30	0.10	—	—	—	—	—	—	34661.9	34661.91	TRUE
59	2	Hot	10.0	30	0.10	—	—	—	—	—	—	46474.5	46474.47	TRUE
60	3	Hot	10.0	30	0.10	—	—	—	—	—	—	37398.3	37398.3	TRUE
61	1	Hot	10.0	45	0.30	—	—	—	—	—	—	56797.1	56797.12	TRUE
62	2	Hot	10.0	45	0.30	—	—	—	—	—	—	60291.8	60291.79	TRUE
63	3	Hot	10.0	45	0.30	—	—	—	—	—	—	66638.9	66638.85	TRUE
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—	—	104035	104035.4	TRUE
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—	—	106439	106439.4	TRUE
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—	—	109057	109056.5	TRUE
67	1	Hot	100.0	15	1.00	—	—	—	—	—	—	223604	223604	TRUE
68	2	Hot	100.0	15	1.00	—	—	—	—	—	—	223891	223891.2	TRUE
69	3	Hot	100.0	15	1.00	—	—	—	—	—	—	223540	223540.3	TRUE
70	1	Hot	100.0	30	3.00	—	—	—	—	—	—	417015	417014.8	TRUE
71	2	Hot	100.0	30	3.00	—	—	—	—	—	—	432772	432771.6	TRUE
72	3	Hot	100.0	30	3.00	—	—	—	—	—	—	497774	497774.4	TRUE

		Total Binding -- Positions 1-24 radiolabeled R1881 plus cytosol (Panel A)												
		Tube Identification			Assay tube contents									
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. Initial	Hot R1881 Volume	Cold R1881 Conc. Initial	Cold R1881 volume	Triamcelenone Acetate	Cytosol	Hot Conc. Final	Cold Conc. Final	Total Volume
					(nM)	(ul)	(mM)	(ul)	(ul)	(ul)	(nM)	(nM)	(ul)
Seq#63	1	1	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	2	2	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	3	3	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	4	1	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	5	2	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	6	3	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	7	1	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	8	2	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	9	3	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	10	1	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	11	2	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	12	3	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	13	1	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	14	2	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	15	3	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	16	1	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	17	2	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	18	3	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	19	1	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	20	2	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	21	3	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	22	1	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	23	2	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	24	3	H	c8	100.0	30	—	—	—	300	10.00	—	300

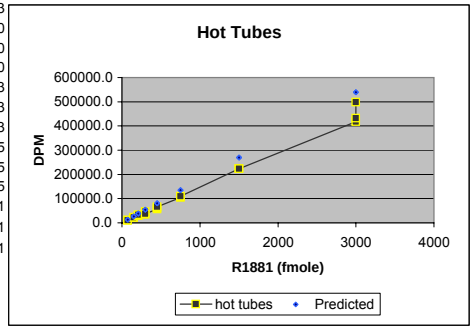
									Number of molecules					Ratio
Run	Position	Total Counts	Non Specific Binding (Mean of reps in pos. 25-48)	Specific Binding (Total - Non Specific)	Ratio of NSB/ total binding	Ratio Total binding/ Hot	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Total Binding molecules	Non Specific Binding molecules	Specific Binding molecules	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Specific Bound / Free
		(dpm)	(dpm)	(dpm)			(dpm)	(dpm)	(fmole)	(fmole)	(fmole)	(fmole)	(fmole)	
Seq#63	1	3691.9	489.2	3202.7	13.2%	35.0%	10540.9	6849.1	25	3	22	71	46	0.47
Seq#63	2	3372.5	489.2	2883.3	14.5%	32.0%	10540.9	7168.4	23	3	19	71	48	0.40
Seq#63	3	3473.8	489.2	2984.6	14.1%	33.0%	10540.9	7067.2	23	3	20	71	47	0.42
Seq#63	4	5460.2	532.2	4928.0	9.7%	24.5%	22308.6	16848.4	37	4	33	150	113	0.29
Seq#63	5	5884.2	532.2	5352.0	9.0%	26.4%	22308.6	16424.4	40	4	36	150	110	0.33
Seq#63	6	5235.2	532.2	4703.0	10.2%	23.5%	22308.6	17073.4	35	4	32	150	115	0.28
Seq#63	7	6602.6	646.2	5956.5	9.8%	21.2%	31128.4	24525.8	44	4	40	209	165	0.24
Seq#63	8	7455.0	646.2	6808.9	8.7%	23.9%	31128.4	23673.4	50	4	46	209	159	0.29
Seq#63	9	6583.2	646.2	5937.0	9.8%	21.1%	31128.4	24545.2	44	4	40	209	165	0.24
Seq#63	10	8888.6	760.9	8127.6	8.6%	22.5%	39511.6	30623.0	60	5	55	265	206	0.27
Seq#63	11	8606.7	760.9	7845.8	8.8%	21.8%	39511.6	30904.8	58	5	53	265	208	0.25
Seq#63	12	7715.0	760.9	6954.1	9.9%	19.5%	39511.6	31796.6	52	5	47	265	214	0.22
Seq#63	13	8892.1	826.2	8065.9	9.3%	14.5%	61242.6	52350.5	60	6	54	411	352	0.15
Seq#63	14	9588.3	826.2	8762.2	8.6%	15.7%	61242.6	51654.3	64	6	59	411	347	0.17
Seq#63	15	9275.1	826.2	8448.9	8.9%	15.1%	61242.6	51967.5	62	6	57	411	349	0.16
Seq#63	16	12746.1	1152.4	11593.7	9.0%	12.0%	106510.4	93764.4	86	8	78	715	630	0.12
Seq#63	17	12412.6	1152.4	11260.2	9.3%	11.7%	106510.4	94097.8	83	8	76	715	632	0.12
Seq#63	18	10837.8	1152.4	9685.4	10.6%	10.2%	106510.4	95672.6	73	8	65	715	643	0.10
Seq#63	19	15154.5	1735.9	13418.7	11.5%	6.8%	223678.5	208524.0	102	12	90	1502	1401	0.06
Seq#63	20	14776.9	1735.9	13041.1	11.7%	6.6%	223678.5	208901.6	99	12	88	1502	1403	0.06
Seq#63	21	15201.7	1735.9	13465.8	11.4%	6.8%	223678.5	208476.8	102	12	90	1502	1400	0.06
Seq#63	22	16874.2	2727.7	14146.5	16.2%	3.8%	449186.9	432312.7	113	18	95	3017	2904	0.03
Seq#63	23	16360.2	2727.7	13632.5	16.7%	3.6%	449186.9	432826.8	110	18	92	3017	2907	0.03
Seq#63	24	16863.9	2727.7	14136.2	16.2%	3.8%	449186.9	432323.0	113	18	95	3017	2904	0.03

		Non Specific Binding -- Positions 25-48 radiolabeled R1881 plus 100 X inert R1881 plus cytosol												
		Tube Identification			Assay tube contents								Scintillation Results	
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. R1881 Initial	Hot	Cold R1881 Conc. Initial	Cold	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)	(ul)	(ul)	(nM)	(nM)	(dpm)	(dpm)
Seq#63	25	1	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	435.5	489.2
Seq#63	26	2	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	627.9	489.2
Seq#63	27	3	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	404.1	489.2
Seq#63	28	1	HC	c2	10.0	15	1.00	15	50	300	0.5	50	565.6	532.2
Seq#63	29	2	HC	c2	10.0	15	1.00	15	50	300	0.5	50	469.8	532.2
Seq#63	30	3	HC	c2	10.0	15	1.00	15	50	300	0.5	50	561.3	532.2
Seq#63	31	1	HC	c3	10.0	21	1.00	21	50	300	0.7	70	709.5	646.2
Seq#63	32	2	HC	c3	10.0	21	1.00	21	50	300	0.7	70	575.8	646.2
Seq#63	33	3	HC	c3	10.0	21	1.00	21	50	300	0.7	70	653.1	646.2
Seq#63	34	1	HC	c4	10.0	30	1.00	30	50	300	1	100	730.1	760.9
Seq#63	35	2	HC	c4	10.0	30	1.00	30	50	300	1	100	843.2	760.9
Seq#63	36	3	HC	c4	10.0	30	1.00	30	50	300	1	100	709.5	760.9
Seq#63	37	1	HC	c5	10.0	45	1.00	45	50	300	1.5	150	753.8	826.2
Seq#63	38	2	HC	c5	10.0	45	1.00	45	50	300	1.5	150	918.5	826.2
Seq#63	39	3	HC	c5	10.0	45	1.00	45	50	300	1.5	150	806.2	826.2
Seq#63	40	1	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1090.0	1152.4
Seq#63	41	2	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1305.9	1152.4
Seq#63	42	3	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1061.3	1152.4
Seq#63	43	1	HC	c7	100.0	15	10.00	15	50	300	5	500	1602.2	1735.9
Seq#63	44	2	HC	c7	100.0	15	10.00	15	50	300	5	500	2025.5	1735.9
Seq#63	45	3	HC	c7	100.0	15	10.00	15	50	300	5	500	1579.9	1735.9
Seq#63	46	1	HC	c8	100.0	30	10.00	30	50	300	10	1000	2744.6	2727.7
Seq#63	47	2	HC	c8	100.0	30	10.00	30	50	300	10	1000	2591.3	2727.7
Seq#63	48	3	HC	c8	100.0	30	10.00	30	50	300	10	1000	2847.2	2727.7

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)	predicted dpm
Seq#63	49	1	Hot	c1	10	7.5	75	10604.9	71	10540.9	13488
Seq#63	50	2	Hot	c1	10	7.5	75	10625.1	71	10540.9	13488
Seq#63	51	3	Hot	c1	10	7.5	75	10392.8	70	10540.9	13488
Seq#63	52	1	Hot	c2	10	15	150	22686.7	152	22308.6	26977
Seq#63	53	2	Hot	c2	10	15	150	21874.1	147	22308.6	26977
Seq#63	54	3	Hot	c2	10	15	150	22364.9	150	22308.6	26977
Seq#63	55	1	Hot	c3	10	21	210	30545.8	205	31128.4	37767
Seq#63	56	2	Hot	c3	10	21	210	29642.5	199	31128.4	37767
Seq#63	57	3	Hot	c3	10	21	210	33196.9	223	31128.4	37767
Seq#63	58	1	Hot	c4	10	30	300	34661.9	233	39511.6	53953
Seq#63	59	2	Hot	c4	10	30	300	46474.5	312	39511.6	53953
Seq#63	60	3	Hot	c4	10	30	300	37398.3	251	39511.6	53953
Seq#63	61	1	Hot	c5	10	45	450	56797.1	381	61242.6	80930
Seq#63	62	2	Hot	c5	10	45	450	60291.8	405	61242.6	80930
Seq#63	63	3	Hot	c5	10	45	450	66638.9	448	61242.6	80930
Seq#63	64	1	Hot	c6	100	7.5	750	104035.4	699	106510.4	134883
Seq#63	65	2	Hot	c6	100	7.5	750	106439.4	715	106510.4	134883
Seq#63	66	3	Hot	c6	100	7.5	750	109056.5	732	106510.4	134883
Seq#63	67	1	Hot	c7	100	15	1500	223604.0	1502	223678.5	269765
Seq#63	68	2	Hot	c7	100	15	1500	223891.2	1504	223678.5	269765
Seq#63	69	3	Hot	c7	100	15	1500	223540.3	1501	223678.5	269765
Seq#63	70	1	Hot	c8	100	30	3000	417014.8	2801	449186.9	539531
Seq#63	71	2	Hot	c8	100	30	3000	432771.6	2907	449186.9	539531
Seq#63	72	3	Hot	c8	100	30	3000	497774.4	3343	449186.9	539531

Computation Check	
7/6/05	specific activity date
81.01	Ci/mMole 3H R1881
2.22E+12	DPM/Ci (definition)
1.7984E+14	DPM/mmole
1.7984E+11	DPM/nmole
179.8	DPM/fmole
0.005560	fmole/DPM



Linear regression results (LINEST function)		
(Regression line forced through 0,0)		
Slope	148.8884233 dpm/fmole	
1/slope	0.006716439 fmole/dpm	
	x	y
origin	0	0
end point	3343.3	497774.4
SLOPE function, used if missing HOT tubes		
Slope	150.5 dpm/fmole	
1/slope	0.006645 fmole/dpm	
	x	y
origin	0	0
end point	3307.6	497774.4

Prism input for bound/free		Prism input for specific bound	
specific bound/molar	bound/free	*average total added molar*	*specific bound/molar*
5.93612E-11	0.46761	1.95373E-10	5.93612E-11
5.34417E-11	0.40223	1.95373E-10	5.34417E-11
5.53188E-11	0.42232	1.95373E-10	5.53188E-11
9.1338E-11	0.29249	4.13481E-10	9.1338E-11
9.91965E-11	0.32585	4.13481E-10	9.91965E-11
8.71677E-11	0.27546	4.13481E-10	8.71677E-11
1.10401E-10	0.24287	5.76954E-10	1.10401E-10
1.262E-10	0.28762	5.76954E-10	1.262E-10
1.10041E-10	0.24188	5.76954E-10	1.10041E-10
1.50643E-10	0.26541	7.32332E-10	1.50643E-10
1.45419E-10	0.25387	7.32332E-10	1.45419E-10
1.28891E-10	0.21870	7.32332E-10	1.28891E-10
1.49499E-10	0.15408	1.13511E-09	1.49499E-10
1.62404E-10	0.16963	1.13511E-09	1.62404E-10
1.56598E-10	0.16258	1.13511E-09	1.56598E-10
2.14884E-10	0.12365	1.97413E-09	2.14884E-10
2.08703E-10	0.11966	1.97413E-09	2.08703E-10
1.79515E-10	0.10123	1.97413E-09	1.79515E-10
2.4871E-10	0.06435	4.1458E-09	2.4871E-10
2.41711E-10	0.06243	4.1458E-09	2.41711E-10
2.49584E-10	0.06459	4.1458E-09	2.49584E-10
2.622E-10	0.03272	8.32551E-09	2.622E-10
2.52673E-10	0.03150	8.32551E-09	2.52673E-10
2.62009E-10	0.03270	8.32551E-09	2.62009E-10

Laboratory C

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:	C	
Run identification:	2 (Seq#6398)	
Assay start date:	7/12/2005	
Tracer lot number:	3559-507	
Specific activity on day of assay:	80.94	**Ci/mmole**
Cytosol lot or vial number:	0725-06-01	
protein (cytosol) per tube:	600	**ug**
protein (cytosol) per tube:	0.6	**mg**
KD	9.30E-01	**nM**
Bmax	16.76	**fmole/100 ug**
total volume in tubes	300	**uL**
volume of ethanol counted:	2	**mL**
mulitply DPM in sample by :	3	

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

protcol 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)	Triamcelenone 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"	Ten Percent Rule	Saturation X values	Bound y values	NSB y values
1	1	H	10.0	7.5	0.25	—	—	—	50	300	—	1165.877	3497.631	TRUE		30.5%	0.25	3497.6	545.2
2	2	H	10.0	7.5	0.25	—	—	—	50	300	—	1289.587	3868.761	TRUE		33.7%	0.25	3868.8	545.2
3	3	H	10.0	7.5	0.25	—	—	—	50	300	—	1296.037	3888.111	TRUE		33.9%	0.25	3888.1	545.2
4	1	H	10.0	15	0.50	—	—	—	50	300	—	2163.940	6491.82	TRUE		26.7%	0.5	6491.8	678.0
5	2	H	10.0	15	0.50	—	—	—	50	300	—	2095.338	6286.014	TRUE		25.9%	0.5	6286.0	678.0
6	3	H	10.0	15	0.50	—	—	—	50	300	—	2169.673	6509.019	TRUE		26.8%	0.5	6509.0	678.0
7	1	H	10.0	21	0.70	—	—	—	50	300	—	2615.887	7847.661	TRUE		23.1%	0.7	7847.7	639.5
8	2	H	10.0	21	0.70	—	—	—	50	300	—	2574.131	7722.393	TRUE		22.8%	0.7	7722.4	639.5
9	3	H	10.0	21	0.70	—	—	—	50	300	—	2677.117	8031.351	TRUE		23.7%	0.7	8031.4	639.5
10	1	H	10.0	30	1.00	—	—	—	50	300	—	3426.051	10278.153	TRUE		25.5%	1	10278.2	712.3
11	2	H	10.0	30	1.00	—	—	—	50	300	—	3553.837	10661.511	TRUE		26.5%	1	10661.5	712.3
12	3	H	10.0	30	1.00	—	—	—	50	300	—	3166.577	9499.731	TRUE		23.6%	1	9499.7	712.3
13	1	H	10.0	45	1.50	—	—	—	50	300	—	3819.253	11457.759	TRUE		17.5%	1.5	11457.8	987.2
14	2	H	10.0	45	1.50	—	—	—	50	300	—	3720.222	11160.666	TRUE		17.0%	1.5	11160.7	987.2
15	3	H	10.0	45	1.50	—	—	—	50	300	—	3731.635	11194.905	TRUE		17.1%	1.5	11194.9	987.2
16	1	H	100.0	7.5	2.50	—	—	—	50	300	—	4884.901	14654.703	TRUE		12.7%	2.5	14654.7	1186.5
17	2	H	100.0	7.5	2.50	—	—	—	50	300	—	4847.792	14543.376	TRUE		12.6%	2.5	14543.4	1186.5
18	3	H	100.0	7.5	2.50	—	—	—	50	300	—	4465.078	13395.234	TRUE		11.6%	2.5	13395.2	1186.5
19	1	H	100.0	15	5.00	—	—	—	50	300	—	5571.971	16715.913	TRUE		6.8%	5	16715.9	2017.8
20	2	H	100.0	15	5.00	—	—	—	50	300	—	5563.030	16689.09	TRUE		6.8%	5	16689.1	2017.8
21	3	H	100.0	15	5.00	—	—	—	50	300	—	5719.325	17157.975	TRUE		7.0%	5	17158.0	2017.8
22	1	H	100.0	30	10.00	—	—	—	50	300	—	6259.112	18777.336	TRUE		3.8%	10	18777.3	3554.2
23	2	H	100.0	30	10.00	—	—	—	50	300	—	6592.478	19777.434	TRUE		4.0%	10	19777.4	3554.2
24	3	H	100.0	30	10.00	—	—	—	50	300	—	6434.390	19303.17	TRUE		3.9%	10	19303.2	3554.2

25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	168.7950	506.385	TRUE
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	148.5396	445.6188	TRUE
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	227.9133	683.7399	TRUE
28	1	HC	10.0	15	0.5	1.00	15	50	50	300	C7	185.0916	555.2748	TRUE
29	2	HC	10.0	15	0.5	1.00	15	50	50	300	C7	297.0792	891.2376	TRUE
30	3	HC	10.0	15	0.5	1.00	15	50	50	300	C7	195.8022	587.4066	TRUE
31	1	HC	10.0	21	0.7	1.00	21	70	50	300	C6	221.1099	663.3297	TRUE
32	2	HC	10.0	21	0.7	1.00	21	70	50	300	C6	209.3058	627.9174	TRUE
33	3	HC	10.0	21	0.7	1.00	21	70	50	300	C6	209.0849	627.2547	TRUE
34	1	HC	10.0	30	1	1.00	30	100	50	300	C5	251.7252	755.1756	TRUE
35	2	HC	10.0	30	1	1.00	30	100	50	300	C5	273.4479	820.3437	TRUE
36	3	HC	10.0	30	1	1.00	30	100	50	300	C5	187.0930	561.279	TRUE
37	1	HC	10.0	45	1.5	1.00	45	150	50	300	C4	455.8737	1367.6211	TRUE
38	2	HC	10.0	45	1.5	1.00	45	150	50	300	C4	253.6440	760.932	TRUE
39	3	HC	10.0	45	1.5	1.00	45	150	50	300	C4	277.6374	832.9122	TRUE
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	434.9912	1304.9736	TRUE
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	411.3146	1233.9438	TRUE
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	340.1691	1020.5073	TRUE
43	1	HC	100.0	15	5	10.00	15	500	50	300	C2	767.7874	2303.3622	TRUE
44	2	HC	100.0	15	5	10.00	15	500	50	300	C2	664.9587	1994.8761	TRUE
45	3	HC	100.0	15	5	10.00	15	500	50	300	C2	585.0909	1755.2727	TRUE
46	1	HC	100.0	30	10	10.00	30	1000	50	300	C1	1074.100	3222.3	TRUE
47	2	HC	100.0	30	10	10.00	30	1000	50	300	C1	1063.739	3191.217	TRUE
48	3	HC	100.0	30	10	10.00	30	1000	50	300	C1	1416.331	4248.993	TRUE
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—	—	11630.72	11630.72	TRUE
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—	—	11399.80	11399.8	TRUE
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—	—	11379.20	11379.2	TRUE
52	1	Hot	10.0	15	0.06	—	—	—	—	—	—	24009.79	24009.79	TRUE
53	2	Hot	10.0	15	0.06	—	—	—	—	—	—	24177.88	24177.88	TRUE
54	3	Hot	10.0	15	0.06	—	—	—	—	—	—	24677.04	24677.04	TRUE
55	1	Hot	10.0	21	0.08	—	—	—	—	—	—	34619.20	34619.2	TRUE
56	2	Hot	10.0	21	0.08	—	—	—	—	—	—	32971.57	32971.57	TRUE
57	3	Hot	10.0	21	0.08	—	—	—	—	—	—	34137.24	34137.24	TRUE
58	1	Hot	10.0	30	0.10	—	—	—	—	—	—	40644.12	40644.12	TRUE
59	2	Hot	10.0	30	0.10	—	—	—	—	—	—	40648.00	40648	TRUE
60	3	Hot	10.0	30	0.10	—	—	—	—	—	—	39487.02	39487.02	TRUE
61	1	Hot	10.0	45	0.30	—	—	—	—	—	—	66911.00	66911	TRUE
62	2	Hot	10.0	45	0.30	—	—	—	—	—	—	73444.51	73444.51	TRUE
63	3	Hot	10.0	45	0.30	—	—	—	—	—	—	56465.69	56465.69	TRUE
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—	—	114049.8	114049.8	TRUE
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—	—	116908.8	116908.8	TRUE
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—	—	114148.7	114148.7	TRUE
67	1	Hot	100.0	15	1.00	—	—	—	—	—	—	244327.3	244327.3	TRUE
68	2	Hot	100.0	15	1.00	—	—	—	—	—	—	247764.6	247764.6	TRUE
69	3	Hot	100.0	15	1.00	—	—	—	—	—	—	246793.9	246793.9	TRUE
70	1	Hot	100.0	30	3.00	—	—	—	—	—	—	477002.8	477002.8	TRUE
71	2	Hot	100.0	30	3.00	—	—	—	—	—	—	507456.5	507456.5	TRUE
72	3	Hot	100.0	30	3.00	—	—	—	—	—	—	510790.4	510790.4	TRUE

		Total Binding -- Positions 1-24 radiolabeled R1881 plus cytosol (Panel A)												
		Tube Identification			Assay tube contents									
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. Initial	Hot R1881 Volume	Cold R1881 Conc. Initial	Cold R1881 volume	Triamcelenone Acetate	Cytosol	Hot Conc. Final	Cold Conc. Final	Total Volume
					(nM)	(ul)	(mM)	(ul)	(ul)	(ul)	(nM)	(nM)	(ul)
Seq#63	1	1	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	2	2	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	3	3	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	4	1	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	5	2	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	6	3	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	7	1	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	8	2	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	9	3	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	10	1	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	11	2	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	12	3	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	13	1	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	14	2	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	15	3	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	16	1	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	17	2	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	18	3	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	19	1	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	20	2	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	21	3	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	22	1	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	23	2	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	24	3	H	c8	100.0	30	—	—	—	300	10.00	—	300

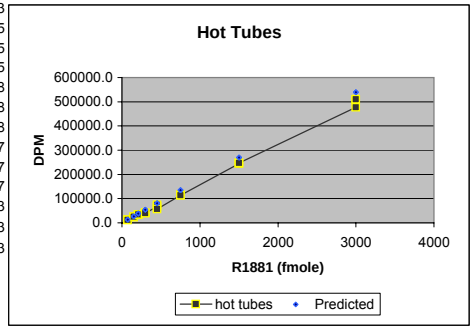
									Number of molecules					Ratio
Run	Position	Total Counts	Non Specific Binding (Mean of reps in pos. 25-48)	Specific Binding (Total - Non Specific)	Ratio of NSB/ total binding	Ratio Total binding/ Hot	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Total Binding molecules	Non Specific Binding molecules	Specific Binding molecules	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Specific Bound / Free
		(dpm)	(dpm)	(dpm)			(dpm)	(dpm)	(fmole)	(fmole)	(fmole)	(fmole)	(fmole)	
Seq#63	1	3497.6	545.2	2952.4	15.6%	30.5%	11469.9	7972.3	21	3	18	70	48	0.37
Seq#63	2	3868.8	545.2	3323.5	14.1%	33.7%	11469.9	7601.1	24	3	20	70	46	0.44
Seq#63	3	3888.1	545.2	3342.9	14.0%	33.9%	11469.9	7581.8	24	3	20	70	46	0.44
Seq#63	4	6491.8	678.0	5813.8	10.4%	26.7%	24288.2	17796.4	39	4	35	148	108	0.33
Seq#63	5	6286.0	678.0	5608.0	10.8%	25.9%	24288.2	18002.2	38	4	34	148	109	0.31
Seq#63	6	6509.0	678.0	5831.0	10.4%	26.8%	24288.2	17779.2	40	4	35	148	108	0.33
Seq#63	7	7847.7	639.5	7208.2	8.1%	23.1%	33909.3	26061.7	48	4	44	206	158	0.28
Seq#63	8	7722.4	639.5	7082.9	8.3%	22.8%	33909.3	26186.9	47	4	43	206	159	0.27
Seq#63	9	8031.4	639.5	7391.9	8.0%	23.7%	33909.3	25878.0	49	4	45	206	157	0.29
Seq#63	10	10278.2	712.3	9565.9	6.9%	25.5%	40259.7	29981.6	62	4	58	245	182	0.32
Seq#63	11	10661.5	712.3	9949.2	6.7%	26.5%	40259.7	29598.2	65	4	60	245	180	0.34
Seq#63	12	9499.7	712.3	8787.5	7.5%	23.6%	40259.7	30760.0	58	4	53	245	187	0.29
Seq#63	13	11457.8	987.2	10470.6	8.6%	17.5%	65607.1	54149.3	70	6	64	399	329	0.19
Seq#63	14	11160.7	987.2	10173.5	8.8%	17.0%	65607.1	54446.4	68	6	62	399	331	0.19
Seq#63	15	11194.9	987.2	10207.7	8.8%	17.1%	65607.1	54412.2	68	6	62	399	331	0.19
Seq#63	16	14654.7	1186.5	13468.2	8.1%	12.7%	115035.8	100381.1	89	7	82	699	610	0.13
Seq#63	17	14543.4	1186.5	13356.9	8.2%	12.6%	115035.8	100492.4	88	7	81	699	611	0.13
Seq#63	18	13395.2	1186.5	12208.8	8.9%	11.6%	115035.8	101640.5	81	7	74	699	618	0.12
Seq#63	19	16715.9	2017.8	14698.1	12.1%	6.8%	246295.3	229579.4	102	12	89	1496	1395	0.06
Seq#63	20	16689.1	2017.8	14671.3	12.1%	6.8%	246295.3	229606.2	101	12	89	1496	1395	0.06
Seq#63	21	17158.0	2017.8	15140.1	11.8%	7.0%	246295.3	229137.3	104	12	92	1496	1392	0.07
Seq#63	22	18777.3	3554.2	15223.2	18.9%	3.8%	498416.6	479639.2	114	22	92	3028	2914	0.03
Seq#63	23	19777.4	3554.2	16223.3	18.0%	4.0%	498416.6	478639.1	120	22	99	3028	2908	0.03
Seq#63	24	19303.2	3554.2	15749.0	18.4%	3.9%	498416.6	479113.4	117	22	96	3028	2911	0.03

		Non Specific Binding -- Positions 25-48 radiolabeled R1881 plus 100 X inert R1881 plus cytosol												
		Tube Identification			Assay tube contents								Scintillation Results	
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. R1881 Initial	Hot	Cold R1881 Conc. Initial	Cold	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)	(ul)	(ul)	(nM)	(nM)	(dpm)	(dpm)
Seq#63	25	1	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	506.4	545.2
Seq#63	26	2	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	445.6	545.2
Seq#63	27	3	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	683.7	545.2
Seq#63	28	1	HC	c2	10.0	15	1.00	15	50	300	0.5	50	555.3	678.0
Seq#63	29	2	HC	c2	10.0	15	1.00	15	50	300	0.5	50	891.2	678.0
Seq#63	30	3	HC	c2	10.0	15	1.00	15	50	300	0.5	50	587.4	678.0
Seq#63	31	1	HC	c3	10.0	21	1.00	21	50	300	0.7	70	663.3	639.5
Seq#63	32	2	HC	c3	10.0	21	1.00	21	50	300	0.7	70	627.9	639.5
Seq#63	33	3	HC	c3	10.0	21	1.00	21	50	300	0.7	70	627.3	639.5
Seq#63	34	1	HC	c4	10.0	30	1.00	30	50	300	1	100	755.2	712.3
Seq#63	35	2	HC	c4	10.0	30	1.00	30	50	300	1	100	820.3	712.3
Seq#63	36	3	HC	c4	10.0	30	1.00	30	50	300	1	100	561.3	712.3
Seq#63	37	1	HC	c5	10.0	45	1.00	45	50	300	1.5	150	1367.6	987.2
Seq#63	38	2	HC	c5	10.0	45	1.00	45	50	300	1.5	150	760.9	987.2
Seq#63	39	3	HC	c5	10.0	45	1.00	45	50	300	1.5	150	832.9	987.2
Seq#63	40	1	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1305.0	1186.5
Seq#63	41	2	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1233.9	1186.5
Seq#63	42	3	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1020.5	1186.5
Seq#63	43	1	HC	c7	100.0	15	10.00	15	50	300	5	500	2303.4	2017.8
Seq#63	44	2	HC	c7	100.0	15	10.00	15	50	300	5	500	1994.9	2017.8
Seq#63	45	3	HC	c7	100.0	15	10.00	15	50	300	5	500	1755.3	2017.8
Seq#63	46	1	HC	c8	100.0	30	10.00	30	50	300	10	1000	3222.3	3554.2
Seq#63	47	2	HC	c8	100.0	30	10.00	30	50	300	10	1000	3191.2	3554.2
Seq#63	48	3	HC	c8	100.0	30	10.00	30	50	300	10	1000	4249.0	3554.2

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)	predicted dpm
Seq#63	49	1	Hot	c1	10	7.5	75	11630.7	71	11469.9	13476
Seq#63	50	2	Hot	c1	10	7.5	75	11399.8	69	11469.9	13476
Seq#63	51	3	Hot	c1	10	7.5	75	11379.2	69	11469.9	13476
Seq#63	52	1	Hot	c2	10	15	150	24009.8	146	24288.2	26952
Seq#63	53	2	Hot	c2	10	15	150	24177.9	147	24288.2	26952
Seq#63	54	3	Hot	c2	10	15	150	24677.0	150	24288.2	26952
Seq#63	55	1	Hot	c3	10	21	210	34619.2	210	33909.3	37732
Seq#63	56	2	Hot	c3	10	21	210	32971.6	200	33909.3	37732
Seq#63	57	3	Hot	c3	10	21	210	34137.2	207	33909.3	37732
Seq#63	58	1	Hot	c4	10	30	300	40644.1	247	40259.7	53903
Seq#63	59	2	Hot	c4	10	30	300	40648.0	247	40259.7	53903
Seq#63	60	3	Hot	c4	10	30	300	39487.0	240	40259.7	53903
Seq#63	61	1	Hot	c5	10	45	450	66911.0	407	65607.1	80855
Seq#63	62	2	Hot	c5	10	45	450	73444.5	446	65607.1	80855
Seq#63	63	3	Hot	c5	10	45	450	56465.7	343	65607.1	80855
Seq#63	64	1	Hot	c6	100	7.5	750	114049.8	693	115035.8	134758
Seq#63	65	2	Hot	c6	100	7.5	750	116908.8	710	115035.8	134758
Seq#63	66	3	Hot	c6	100	7.5	750	114148.7	694	115035.8	134758
Seq#63	67	1	Hot	c7	100	15	1500	244327.3	1485	246295.3	269517
Seq#63	68	2	Hot	c7	100	15	1500	247764.6	1505	246295.3	269517
Seq#63	69	3	Hot	c7	100	15	1500	246793.9	1499	246295.3	269517
Seq#63	70	1	Hot	c8	100	30	3000	477002.8	2898	498416.6	539033
Seq#63	71	2	Hot	c8	100	30	3000	507456.5	3083	498416.6	539033
Seq#63	72	3	Hot	c8	100	30	3000	510790.4	3103	498416.6	539033

Computation Check	
7/12/05	specific activity date
80.94	Ci/mMole 3H R1881
2.22E+12	DPM/Ci (definition)
1.7968E+14	DPM/mmole
1.7968E+11	DPM/nmole
179.7	DPM/fmole
0.005566	fmole/DPM



Linear regression results (LINEST function)		
(Regression line forced through 0,0)		
Slope	164.5855241 dpm/fmole	
1/slope	0.006075868 fmole/dpm	
	x	y
origin	0	0
end point	3103.5	510790.4
SLOPE function, used if missing HOT tubes		
Slope	167.3 dpm/fmole	
1/slope	0.005976 fmole/dpm	
	x	y
origin	0	0
end point	3052.7	510790.4

Prism input for bound/free		Prism input for specific bound	
specific bound/molar	bound/free	*average total added molar*	*specific bound/molar*
5.47718E-11	0.37033	2.12787E-10	5.47718E-11
6.16569E-11	0.43724	2.12787E-10	6.16569E-11
6.20159E-11	0.44091	2.12787E-10	6.20159E-11
1.07857E-10	0.32669	4.50589E-10	1.07857E-10
1.04039E-10	0.31152	4.50589E-10	1.04039E-10
1.08176E-10	0.32797	4.50589E-10	1.08176E-10
1.33724E-10	0.27658	6.29077E-10	1.33724E-10
1.314E-10	0.27047	6.29077E-10	1.314E-10
1.37132E-10	0.28564	6.29077E-10	1.37132E-10
1.77464E-10	0.31906	7.46887E-10	1.77464E-10
1.84576E-10	0.33614	7.46887E-10	1.84576E-10
1.63023E-10	0.28568	7.46887E-10	1.63023E-10
1.94248E-10	0.19337	1.21712E-09	1.94248E-10
1.88736E-10	0.18685	1.21712E-09	1.88736E-10
1.89371E-10	0.18760	1.21712E-09	1.89371E-10
2.49859E-10	0.13417	2.13411E-09	2.49859E-10
2.47794E-10	0.13291	2.13411E-09	2.47794E-10
2.26494E-10	0.12012	2.13411E-09	2.26494E-10
2.72675E-10	0.06402	4.5692E-09	2.72675E-10
2.72177E-10	0.06390	4.5692E-09	2.72177E-10
2.80876E-10	0.06607	4.5692E-09	2.80876E-10
2.82416E-10	0.03174	9.24649E-09	2.82416E-10
3.0097E-10	0.03389	9.24649E-09	3.0097E-10
2.92171E-10	0.03287	9.24649E-09	2.92171E-10

Laboratory C

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:	C	
Run identification:	3 (Seq#6399)	
Assay start date:	7/19/2005	
Tracer lot number:	3559-507	
Specific activity on day of assay:	80.85	**Ci/mmole**
Cytosol lot or vial number:	0725-06-01	
protein (cytosol) per tube:	600	**ug**
protein (cytosol) per tube:	0.6	**mg**
KD	1.03E+00	**nM**
Bmax	15.29	**fmole/100 ug**
total volume in tubes	300	**uL**
volume of ethanol counted:	2	**mL**
mulitply DPM in sample by :	3	

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

protcol 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)	Triamcelenone 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"	Ten Percent Rule	Saturation X values	Bound y values	NSB y values
1	1	H	10.0	7.5	0.25	—	—	—	50	300	—	1203.09	3609.267	TRUE		31.5%	0.25	3609.3	460.6
2	2	H	10.0	7.5	0.25	—	—	—	50	300	—	1207.59	3622.782	TRUE		31.6%	0.25	3622.8	460.6
3	3	H	10.0	7.5	0.25	—	—	—	50	300	—	1232.88	3698.646	TRUE		32.3%	0.25	3698.6	460.6
4	1	H	10.0	15	0.50	—	—	—	50	300	—	1853.91	5561.736	TRUE		24.6%	0.5	5561.7	617.7
5	2	H	10.0	15	0.50	—	—	—	50	300	—	1516.17	4548.498	TRUE		20.2%	0.5	4548.5	617.7
6	3	H	10.0	15	0.50	—	—	—	50	300	—	1536.70	4610.085	TRUE		20.4%	0.5	4610.1	617.7
7	1	H	10.0	21	0.70	—	—	—	50	300	—	2469.62	7408.845	TRUE		20.4%	0.7	7408.8	643.2
8	2	H	10.0	21	0.70	—	—	—	50	300	—	2360.76	7082.283	TRUE		19.5%	0.7	7082.3	643.2
9	3	H	10.0	21	0.70	—	—	—	50	300	—	2481.18	7443.531	TRUE		20.5%	0.7	7443.5	643.2
10	1	H	10.0	30	1.00	—	—	—	50	300	—	2731.54	8194.632	TRUE		16.8%	1	8194.6	862.2
11	2	H	10.0	30	1.00	—	—	—	50	300	—	3025.42	9076.272	TRUE		18.6%	1	9076.3	862.2
12	3	H	10.0	30	1.00	—	—	—	50	300	—	3024.04	9072.129	TRUE		18.6%	1	9072.1	862.2
13	1	H	10.0	45	1.50	—	—	—	50	300	—	3355.63	10066.881	TRUE		14.3%	1.5	10066.9	853.2
14	2	H	10.0	45	1.50	—	—	—	50	300	—	3214.58	9643.743	TRUE		13.7%	1.5	9643.7	853.2
15	3	H	10.0	45	1.50	—	—	—	50	300	—	3263.08	9789.237	TRUE		13.9%	1.5	9789.2	853.2
16	1	H	100.0	7.5	2.50	—	—	—	50	300	—	4121.95	12365.856	TRUE		10.4%	2.5	12365.9	1230.5
17	2	H	100.0	7.5	2.50	—	—	—	50	300	—	4281.10	12843.3	TRUE		10.8%	2.5	12843.3	1230.5
18	3	H	100.0	7.5	2.50	—	—	—	50	300	—	4486.35	13459.062	TRUE		11.3%	2.5	13459.1	1230.5
19	1	H	100.0	15	5.00	—	—	—	50	300	—	5221.60	15664.788	TRUE		6.6%	5	15664.8	1947.0
20	2	H	100.0	15	5.00	—	—	—	50	300	—	5448.70	16346.106	TRUE		6.9%	5	16346.1	1947.0
21	3	H	100.0	15	5.00	—	—	—	50	300	—	4946.06	14838.18	TRUE		6.2%	5	14838.2	1947.0
22	1	H	100.0	30	10.00	—	—	—	50	300	—	5605.99	16817.961	TRUE		3.6%	10	16818.0	2963.0
23	2	H	100.0	30	10.00	—	—	—	50	300	—	6178.67	18536.001	TRUE		3.9%	10	18536.0	2963.0
24	3	H	100.0	30	10.00	—	—	—	50	300	—	5760.89	17282.67	TRUE		3.7%	10	17282.7	2963.0

25	1	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	161.29	483.8727	TRUE
26	2	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	167.04	501.1098	TRUE
27	3	HC	10.0	7.5	0.25	1.00	7.5	25	50	300	C8	132.24	396.7119	TRUE
28	1	HC	10.0	15	0.5	1.00	15	50	50	300	C7	186.50	559.4991	TRUE
29	2	HC	10.0	15	0.5	1.00	15	50	50	300	C7	197.30	591.8943	TRUE
30	3	HC	10.0	15	0.5	1.00	15	50	50	300	C7	233.91	701.7378	TRUE
31	1	HC	10.0	21	0.7	1.00	21	70	50	300	C6	226.20	678.5862	TRUE
32	2	HC	10.0	21	0.7	1.00	21	70	50	300	C6	190.78	572.328	TRUE
33	3	HC	10.0	21	0.7	1.00	21	70	50	300	C6	226.20	678.5862	TRUE
34	1	HC	10.0	30	1	1.00	30	100	50	300	C5	281.07	843.195	TRUE
35	2	HC	10.0	30	1	1.00	30	100	50	300	C5	243.60	730.7853	TRUE
36	3	HC	10.0	30	1	1.00	30	100	50	300	C5	337.55	1012.6596	TRUE
37	1	HC	10.0	45	1.5	1.00	45	150	50	300	C4	352.28	1056.8319	TRUE
38	2	HC	10.0	45	1.5	1.00	45	150	50	300	C4	262.48	787.4433	TRUE
39	3	HC	10.0	45	1.5	1.00	45	150	50	300	C4	238.43	715.29	TRUE
40	1	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	421.60	1264.7925	TRUE
41	2	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	404.46	1213.3782	TRUE
42	3	HC	100.0	7.5	2.5	10.00	7.5	250	50	300	C3	404.46	1213.3782	TRUE
43	1	HC	100.0	15	5	10.00	15	500	50	300	C2	668.15	2004.4395	TRUE
44	2	HC	100.0	15	5	10.00	15	500	50	300	C2	615.71	1847.1186	TRUE
45	3	HC	100.0	15	5	10.00	15	500	50	300	C2	663.11	1989.3306	TRUE
46	1	HC	100.0	30	10	10.00	30	1000	50	300	C1	1170.80	3512.412	TRUE
47	2	HC	100.0	30	10	10.00	30	1000	50	300	C1	915.23	2745.6906	TRUE
48	3	HC	100.0	30	10	10.00	30	1000	50	300	C1	876.94	2630.8269	TRUE
49	1	Hot	10.0	7.5	0.03	—	—	—	—	—	—	11931.1	11931.13	TRUE
50	2	Hot	10.0	7.5	0.03	—	—	—	—	—	—	11558.7	11558.65	TRUE
51	3	Hot	10.0	7.5	0.03	—	—	—	—	—	—	10856.5	10856.46	TRUE
52	1	Hot	10.0	15	0.06	—	—	—	—	—	—	22559.9	22559.89	TRUE
53	2	Hot	10.0	15	0.06	—	—	—	—	—	—	23065.7	23065.7	TRUE
54	3	Hot	10.0	15	0.06	—	—	—	—	—	—	22082.8	22082.78	TRUE
55	1	Hot	10.0	21	0.08	—	—	—	—	—	—	36102.0	36102	TRUE
56	2	Hot	10.0	21	0.08	—	—	—	—	—	—	36710.8	36710.8	TRUE
57	3	Hot	10.0	21	0.08	—	—	—	—	—	—	36305.3	36305.27	TRUE
58	1	Hot	10.0	30	0.10	—	—	—	—	—	—	47380.8	47380.76	TRUE
59	2	Hot	10.0	30	0.10	—	—	—	—	—	—	48911.0	48911.01	TRUE
60	3	Hot	10.0	30	0.10	—	—	—	—	—	—	50099.1	50099.08	TRUE
61	1	Hot	10.0	45	0.30	—	—	—	—	—	—	69637.4	69637.37	TRUE
62	2	Hot	10.0	45	0.30	—	—	—	—	—	—	70906.3	70906.25	TRUE
63	3	Hot	10.0	45	0.30	—	—	—	—	—	—	71123.2	71123.15	TRUE
64	1	Hot	100.0	7.5	0.60	—	—	—	—	—	—	116614	116613.9	TRUE
65	2	Hot	100.0	7.5	0.60	—	—	—	—	—	—	118916	118915.7	TRUE
66	3	Hot	100.0	7.5	0.60	—	—	—	—	—	—	121175	121175	TRUE
67	1	Hot	100.0	15	1.00	—	—	—	—	—	—	237521	237520.6	TRUE
68	2	Hot	100.0	15	1.00	—	—	—	—	—	—	238638	238638.1	TRUE
69	3	Hot	100.0	15	1.00	—	—	—	—	—	—	236236	236235.9	TRUE
70	1	Hot	100.0	30	3.00	—	—	—	—	—	—	482447	482447.1	TRUE
71	2	Hot	100.0	30	3.00	—	—	—	—	—	—	463932	463931.6	TRUE
72	3	Hot	100.0	30	3.00	—	—	—	—	—	—	466933	466932.6	TRUE

		Total Binding -- Positions 1-24 radiolabeled R1881 plus cytosol (Panel A)												
		Tube Identification			Assay tube contents									
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. Initial	Hot R1881 Volume	Cold R1881 Conc. Initial	Cold R1881 volume	Triamcelenone Acetate	Cytosol	Hot Conc. Final	Cold Conc. Final	Total Volume
					(nM)	(ul)	(mM)	(ul)	(ul)	(ul)	(nM)	(nM)	(ul)
Seq#63	1	1	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	2	2	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	3	3	H	c1	10.0	7.5	—	—	—	300	0.25	—	300
Seq#63	4	1	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	5	2	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	6	3	H	c2	10.0	15	—	—	—	300	0.50	—	300
Seq#63	7	1	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	8	2	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	9	3	H	c3	10.0	21	—	—	—	300	0.70	—	300
Seq#63	10	1	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	11	2	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	12	3	H	c4	10.0	30	—	—	—	300	1.00	—	300
Seq#63	13	1	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	14	2	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	15	3	H	c5	10.0	45	—	—	—	300	1.50	—	300
Seq#63	16	1	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	17	2	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	18	3	H	c6	100.0	7.5	—	—	—	300	2.50	—	300
Seq#63	19	1	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	20	2	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	21	3	H	c7	100.0	15	—	—	—	300	5.00	—	300
Seq#63	22	1	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	23	2	H	c8	100.0	30	—	—	—	300	10.00	—	300
Seq#63	24	3	H	c8	100.0	30	—	—	—	300	10.00	—	300

										Number of molecules					Ratio
Run	Position	Total Counts	Non Specific Binding (Mean of reps in pos. 25-48)	Specific Binding (Total - Non Specific)	Ratio of NSB/ total binding	Ratio Total binding/ Hot	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Total Binding molecules	Non Specific Binding molecules	Specific Binding molecules	Total Added (Mean of reps in pos. 49-72)	Free (total added - bound)	Specific Bound / Free
		(dpm)	(dpm)	(dpm)			(dpm)	(dpm)	(fmole)	(fmole)	(fmole)	(fmole)	(fmole)	
Seq#63	1	3609.3	460.6	3148.7	12.8%	31.5%	11448.7	7839.5	23	3	20	73	50	0.40
Seq#63	2	3622.8	460.6	3162.2	12.7%	31.6%	11448.7	7826.0	23	3	20	73	50	0.40
Seq#63	3	3698.6	460.6	3238.1	12.5%	32.3%	11448.7	7750.1	23	3	21	73	49	0.42
Seq#63	4	5561.7	617.7	4944.0	11.1%	24.6%	22569.5	17007.7	35	4	31	143	108	0.29
Seq#63	5	4548.5	617.7	3930.8	13.6%	20.2%	22569.5	18021.0	29	4	25	143	114	0.22
Seq#63	6	4610.1	617.7	3992.4	13.4%	20.4%	22569.5	17959.4	29	4	25	143	114	0.22
Seq#63	7	7408.8	643.2	6765.7	8.7%	20.4%	36372.7	28963.8	47	4	43	231	184	0.23
Seq#63	8	7082.3	643.2	6439.1	9.1%	19.5%	36372.7	29290.4	45	4	41	231	186	0.22
Seq#63	9	7443.5	643.2	6800.4	8.6%	20.5%	36372.7	28929.2	47	4	43	231	184	0.24
Seq#63	10	8194.6	862.2	7332.4	10.5%	16.8%	48797.0	40602.3	52	5	47	310	258	0.18
Seq#63	11	9076.3	862.2	8214.1	9.5%	18.6%	48797.0	39720.7	58	5	52	310	252	0.21
Seq#63	12	9072.1	862.2	8209.9	9.5%	18.6%	48797.0	39724.8	58	5	52	310	252	0.21
Seq#63	13	10066.9	853.2	9213.7	8.5%	14.3%	70555.6	60488.7	64	5	59	448	384	0.15
Seq#63	14	9643.7	853.2	8790.6	8.8%	13.7%	70555.6	60911.8	61	5	56	448	387	0.14
Seq#63	15	9789.2	853.2	8936.0	8.7%	13.9%	70555.6	60766.4	62	5	57	448	386	0.15
Seq#63	16	12365.9	1230.5	11135.3	10.0%	10.4%	118901.5	106535.7	79	8	71	755	677	0.10
Seq#63	17	12843.3	1230.5	11612.8	9.6%	10.8%	118901.5	106058.2	82	8	74	755	674	0.11
Seq#63	18	13459.1	1230.5	12228.5	9.1%	11.3%	118901.5	105442.5	85	8	78	755	670	0.12
Seq#63	19	15664.8	1947.0	13717.8	12.4%	6.6%	237464.9	221800.1	100	12	87	1508	1409	0.06
Seq#63	20	16346.1	1947.0	14399.1	11.9%	6.9%	237464.9	221118.8	104	12	91	1508	1405	0.07
Seq#63	21	14838.2	1947.0	12891.2	13.1%	6.2%	237464.9	222626.7	94	12	82	1508	1414	0.06
Seq#63	22	16818.0	2963.0	13855.0	17.6%	3.6%	471103.8	454285.8	107	19	88	2993	2886	0.03
Seq#63	23	18536.0	2963.0	15573.0	16.0%	3.9%	471103.8	452567.8	118	19	99	2993	2875	0.03
Seq#63	24	17282.7	2963.0	14319.7	17.1%	3.7%	471103.8	453821.1	110	19	91	2993	2883	0.03

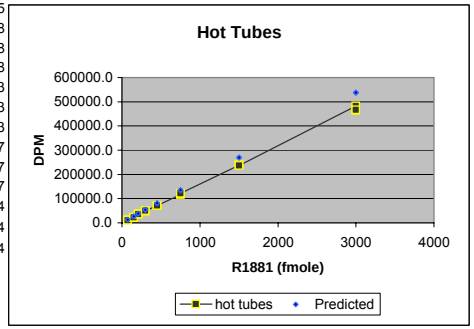
		Non Specific Binding -- Positions 25-48 radiolabeled R1881 plus 100 X inert R1881 plus cytosol												
		Tube Identification			Assay tube contents								Scintillation Results	
Run	Position	Rep	Tube Type Code	Conc. Code	Hot Conc. R1881 Initial	Hot	Cold R1881 Conc. Initial	Cold	Triamcelenone Acetate	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)	(ul)	(ul)	(nM)	(nM)	(dpm)	(dpm)
Seq#63	25	1	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	483.9	460.6
Seq#63	26	2	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	501.1	460.6
Seq#63	27	3	HC	c1	10.0	7.5	1.00	7.5	50	300	0.25	25	396.7	460.6
Seq#63	28	1	HC	c2	10.0	15	1.00	15	50	300	0.5	50	559.5	617.7
Seq#63	29	2	HC	c2	10.0	15	1.00	15	50	300	0.5	50	591.9	617.7
Seq#63	30	3	HC	c2	10.0	15	1.00	15	50	300	0.5	50	701.7	617.7
Seq#63	31	1	HC	c3	10.0	21	1.00	21	50	300	0.7	70	678.6	643.2
Seq#63	32	2	HC	c3	10.0	21	1.00	21	50	300	0.7	70	572.3	643.2
Seq#63	33	3	HC	c3	10.0	21	1.00	21	50	300	0.7	70	678.6	643.2
Seq#63	34	1	HC	c4	10.0	30	1.00	30	50	300	1	100	843.2	862.2
Seq#63	35	2	HC	c4	10.0	30	1.00	30	50	300	1	100	730.8	862.2
Seq#63	36	3	HC	c4	10.0	30	1.00	30	50	300	1	100	1012.7	862.2
Seq#63	37	1	HC	c5	10.0	45	1.00	45	50	300	1.5	150	1056.8	853.2
Seq#63	38	2	HC	c5	10.0	45	1.00	45	50	300	1.5	150	787.4	853.2
Seq#63	39	3	HC	c5	10.0	45	1.00	45	50	300	1.5	150	715.3	853.2
Seq#63	40	1	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1264.8	1230.5
Seq#63	41	2	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1213.4	1230.5
Seq#63	42	3	HC	c6	100.0	7.5	10.00	7.5	50	300	2.5	250	1213.4	1230.5
Seq#63	43	1	HC	c7	100.0	15	10.00	15	50	300	5	500	2004.4	1947.0
Seq#63	44	2	HC	c7	100.0	15	10.00	15	50	300	5	500	1847.1	1947.0
Seq#63	45	3	HC	c7	100.0	15	10.00	15	50	300	5	500	1989.3	1947.0
Seq#63	46	1	HC	c8	100.0	30	10.00	30	50	300	10	1000	3512.4	2963.0
Seq#63	47	2	HC	c8	100.0	30	10.00	30	50	300	10	1000	2745.7	2963.0
Seq#63	48	3	HC	c8	100.0	30	10.00	30	50	300	10	1000	2630.8	2963.0

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)	predicted dpm
Seq#63	49	1	Hot	c1	10	7.5	75	11931.1	76	11448.7	13461
Seq#63	50	2	Hot	c1	10	7.5	75	11558.7	73	11448.7	13461
Seq#63	51	3	Hot	c1	10	7.5	75	10856.5	69	11448.7	13461
Seq#63	52	1	Hot	c2	10	15	150	22559.9	143	22569.5	26923
Seq#63	53	2	Hot	c2	10	15	150	23065.7	147	22569.5	26923
Seq#63	54	3	Hot	c2	10	15	150	22082.8	140	22569.5	26923
Seq#63	55	1	Hot	c3	10	21	210	36102.0	229	36372.7	37692
Seq#63	56	2	Hot	c3	10	21	210	36710.8	233	36372.7	37692
Seq#63	57	3	Hot	c3	10	21	210	36305.3	231	36372.7	37692
Seq#63	58	1	Hot	c4	10	30	300	47380.8	301	48797.0	53845
Seq#63	59	2	Hot	c4	10	30	300	48911.0	311	48797.0	53845
Seq#63	60	3	Hot	c4	10	30	300	50099.1	318	48797.0	53845
Seq#63	61	1	Hot	c5	10	45	450	69637.4	442	70555.6	80768
Seq#63	62	2	Hot	c5	10	45	450	70906.3	450	70555.6	80768
Seq#63	63	3	Hot	c5	10	45	450	71123.2	452	70555.6	80768
Seq#63	64	1	Hot	c6	100	7.5	750	116613.9	741	118901.5	134613
Seq#63	65	2	Hot	c6	100	7.5	750	118915.7	755	118901.5	134613
Seq#63	66	3	Hot	c6	100	7.5	750	121175.0	770	118901.5	134613
Seq#63	67	1	Hot	c7	100	15	1500	237520.6	1509	237464.9	269227
Seq#63	68	2	Hot	c7	100	15	1500	238638.1	1516	237464.9	269227
Seq#63	69	3	Hot	c7	100	15	1500	236235.9	1501	237464.9	269227
Seq#63	70	1	Hot	c8	100	30	3000	482447.1	3065	471103.8	538454
Seq#63	71	2	Hot	c8	100	30	3000	463931.6	2947	471103.8	538454
Seq#63	72	3	Hot	c8	100	30	3000	466932.6	2966	471103.8	538454

Computation Check

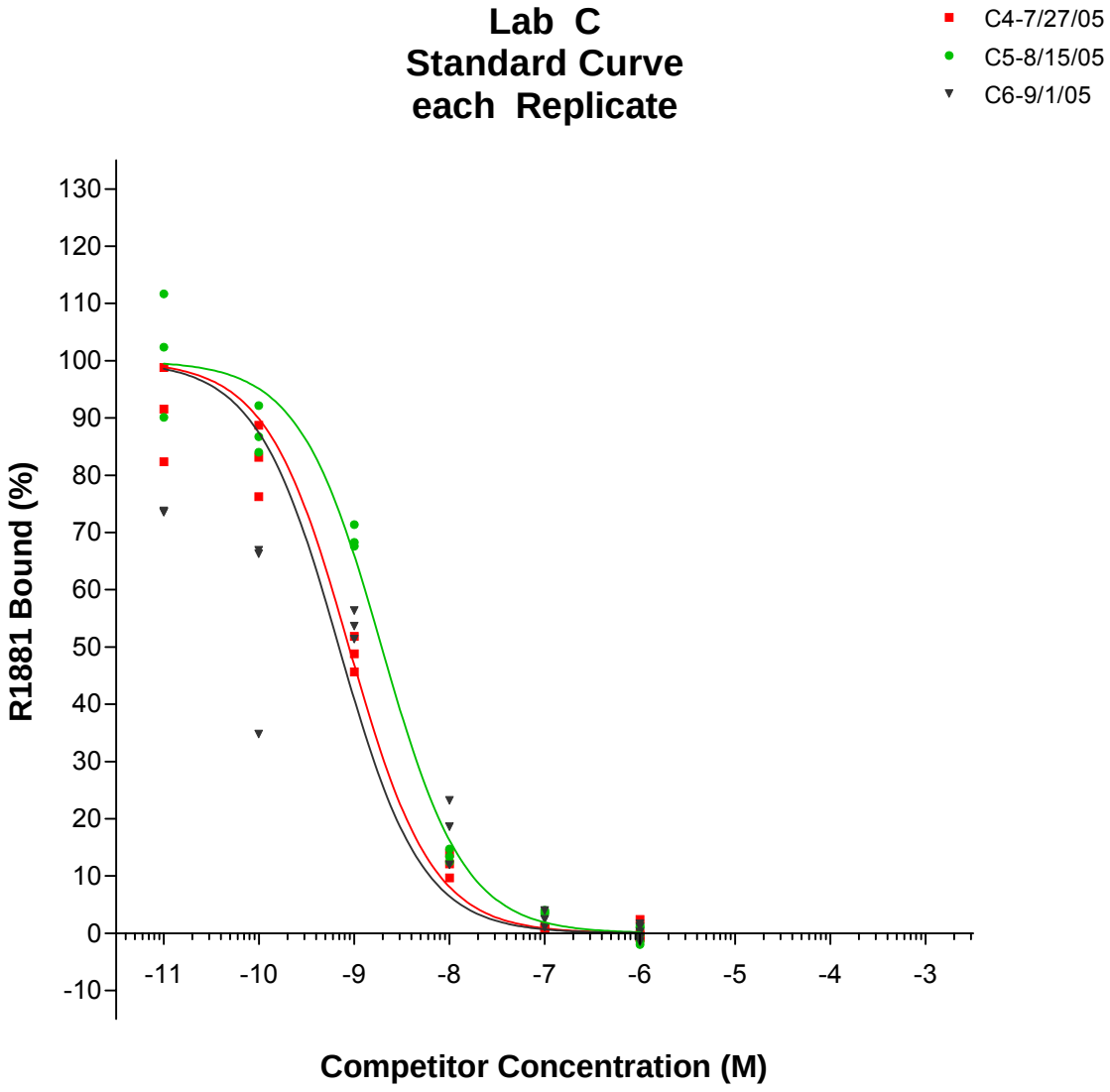
7/19/05	specific activity date
80.85	Ci/mMole 3H R1881
2.22E+12	DPM/Ci (definition)
1.7948E+14	DPM/mmole
1.7948E+11	DPM/nmole
179.5	DPM/fmole
0.005572	fmole/DPM

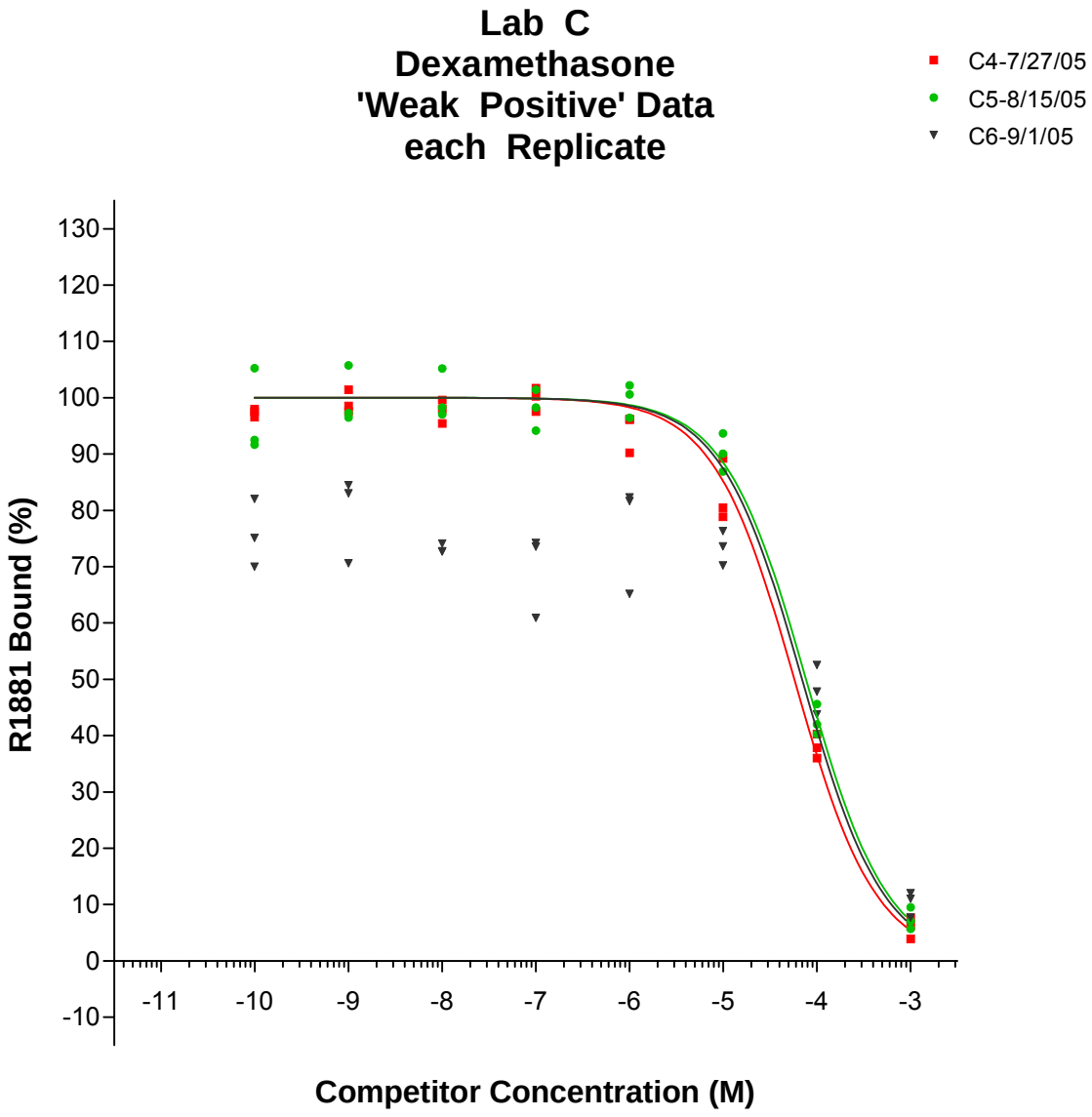


Linear regression results (LINEST function)		
(Regression line forced through 0,0)		
Slope	157.4214314 dpm/fmole	
1/slope	0.006352375 fmole/dpm	
	x	y
origin	0	0
end point	3064.7	482447.1
SLOPE function, used if missing HOT tubes		
Slope	156.9 dpm/fmole	
1/slope	0.006372 fmole/dpm	
	x	y
origin	0	0
end point	3074.1	482447.1

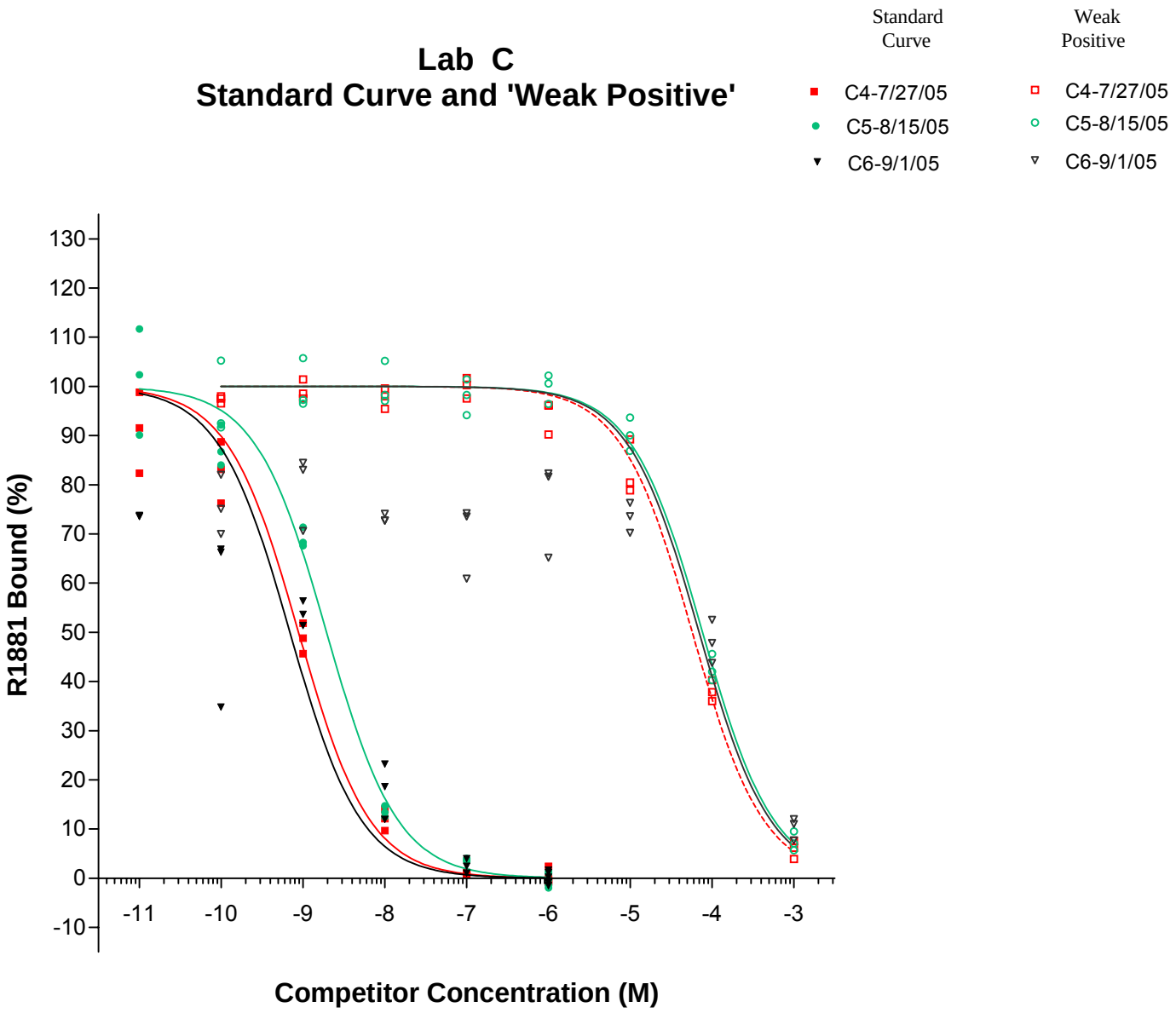
Prism input for bound/free		Prism input for specific bound	
specific bound/molar	bound/free	*average total added molar*	*specific bound/molar*
5.84767E-11	0.40165	2.12623E-10	5.84767E-11
5.87277E-11	0.40407	2.12623E-10	5.87277E-11
6.01367E-11	0.41781	2.12623E-10	6.01367E-11
9.18189E-11	0.29069	4.19153E-10	9.18189E-11
7.30014E-11	0.21812	4.19153E-10	7.30014E-11
7.41452E-11	0.22230	4.19153E-10	7.41452E-11
1.2565E-10	0.23359	6.75503E-10	1.2565E-10
1.19585E-10	0.21984	6.75503E-10	1.19585E-10
1.26294E-10	0.23507	6.75503E-10	1.26294E-10
1.36175E-10	0.18059	9.06242E-10	1.36175E-10
1.52549E-10	0.20680	9.06242E-10	1.52549E-10
1.52472E-10	0.20667	9.06242E-10	1.52472E-10
1.71114E-10	0.15232	1.31034E-09	1.71114E-10
1.63256E-10	0.14432	1.31034E-09	1.63256E-10
1.65958E-10	0.14706	1.31034E-09	1.65958E-10
2.06802E-10	0.10452	2.2082E-09	2.06802E-10
2.15669E-10	0.10949	2.2082E-09	2.15669E-10
2.27105E-10	0.11597	2.2082E-09	2.27105E-10
2.54763E-10	0.06185	4.41013E-09	2.54763E-10
2.67416E-10	0.06512	4.41013E-09	2.67416E-10
2.39412E-10	0.05791	4.41013E-09	2.39412E-10
2.57311E-10	0.03050	8.7492E-09	2.57311E-10
2.89217E-10	0.03441	8.7492E-09	2.89217E-10
2.65941E-10	0.03155	8.7492E-09	2.65941E-10

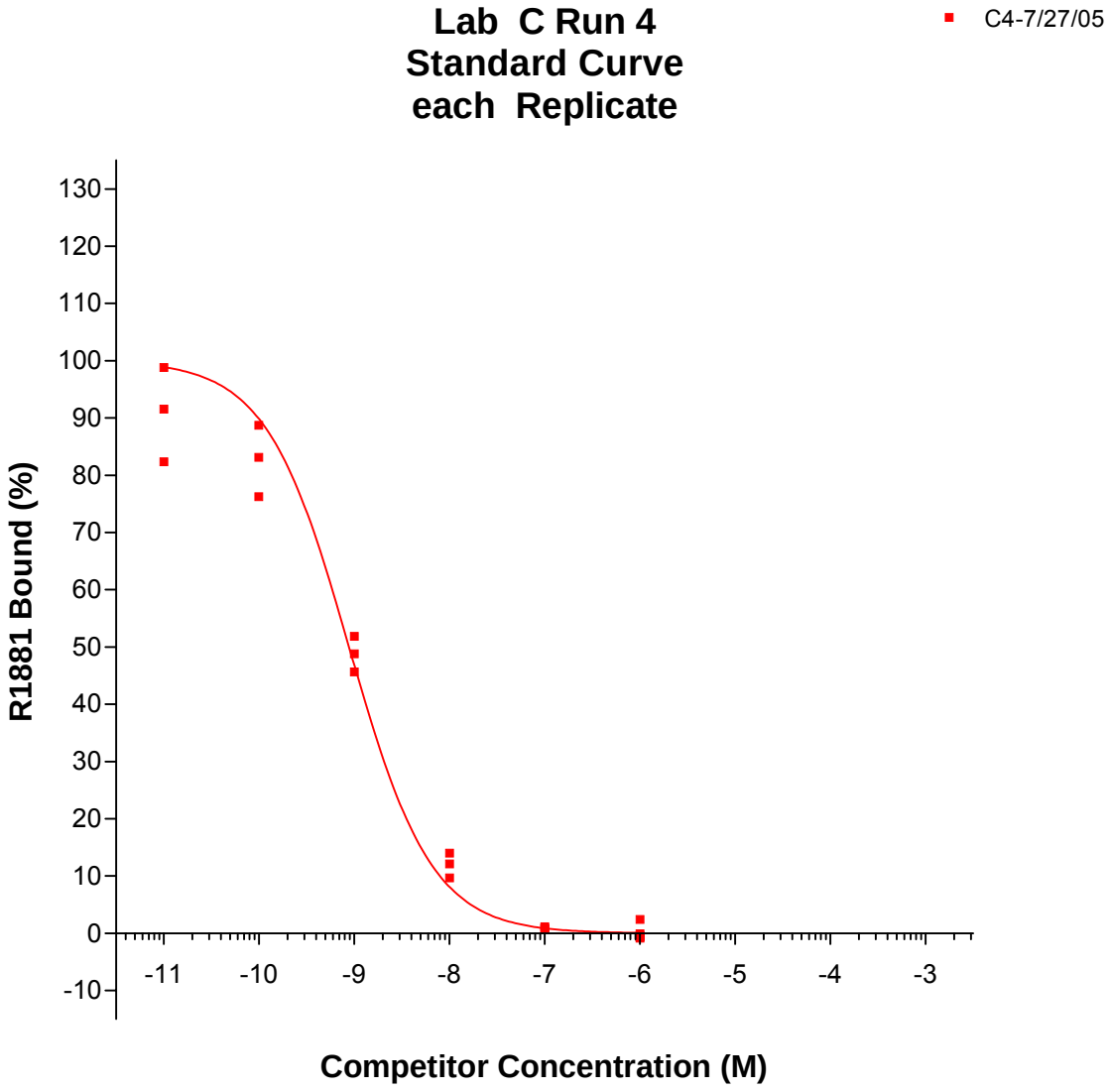
Appendix 5: Prism Files for Competitive Binding Experiments

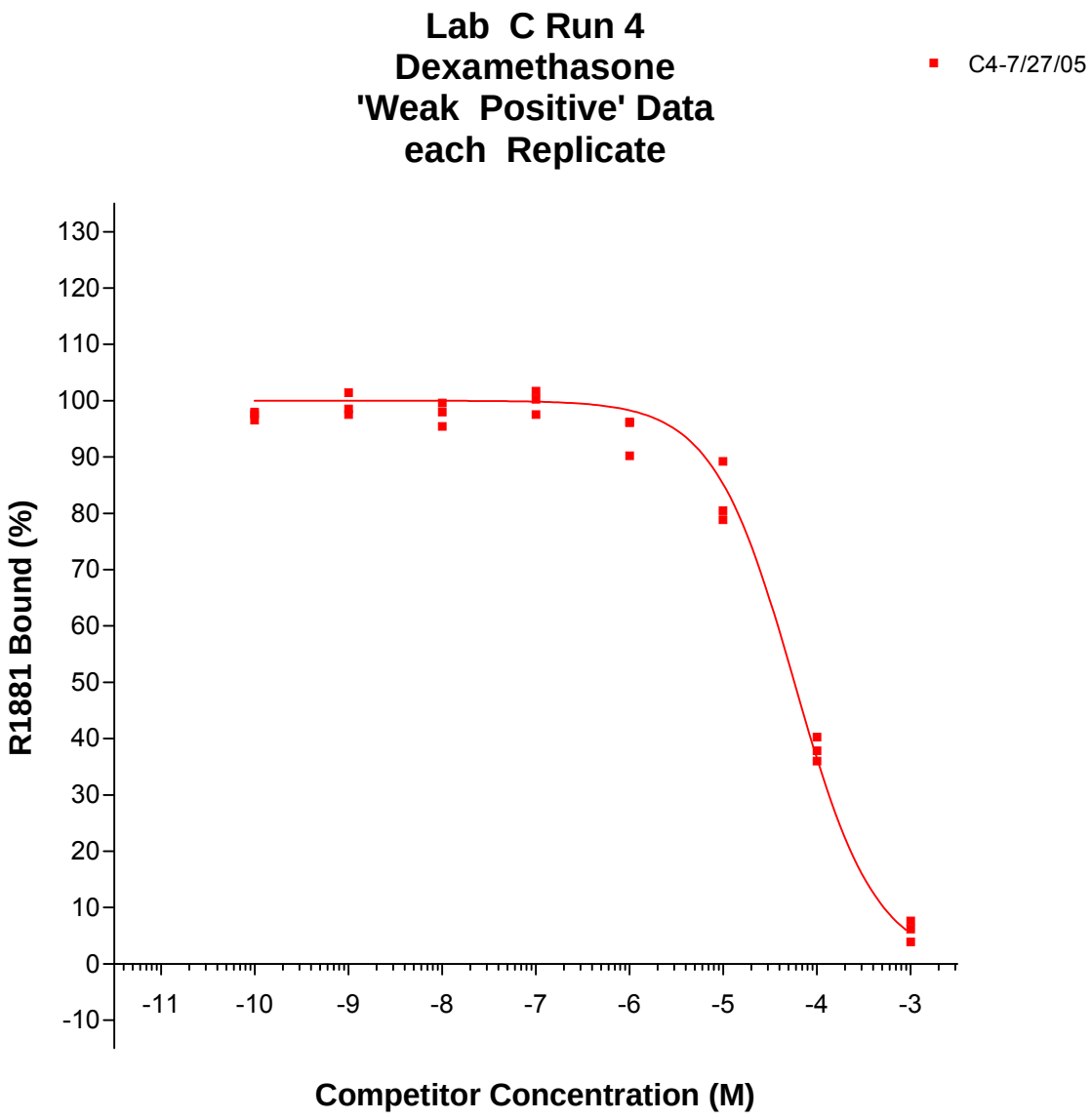

Lab C
Standard Curve
each Replicate
C4-7/27/05
C5-8/15/05
C6-9/1/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)


Lab C
Dexamethasone
'Weak Positive' Data
each Replicate
C4-7/27/05
C5-8/15/05
C6-9/1/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)

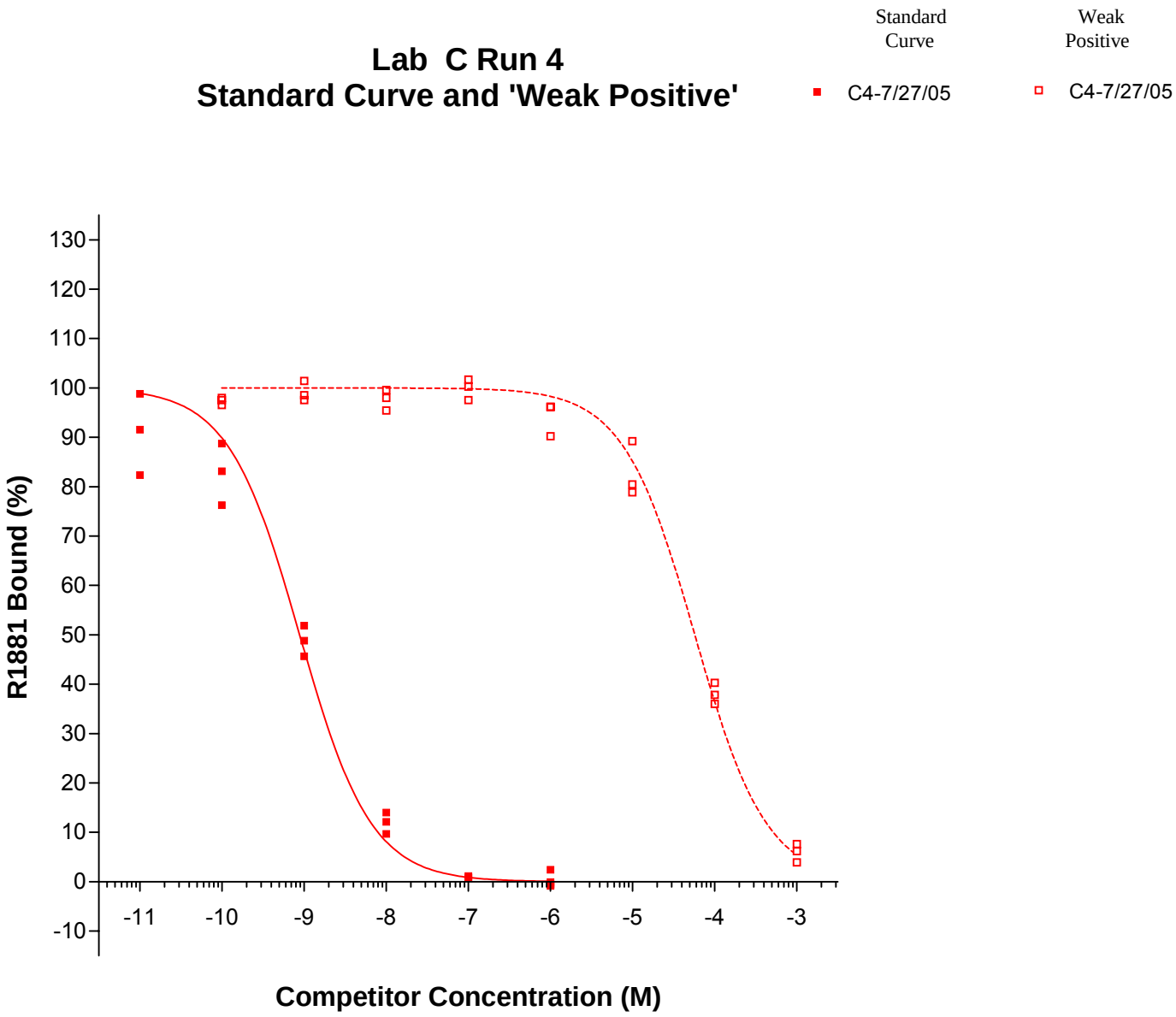
Lab C
Standard Curve and 'Weak Positive'

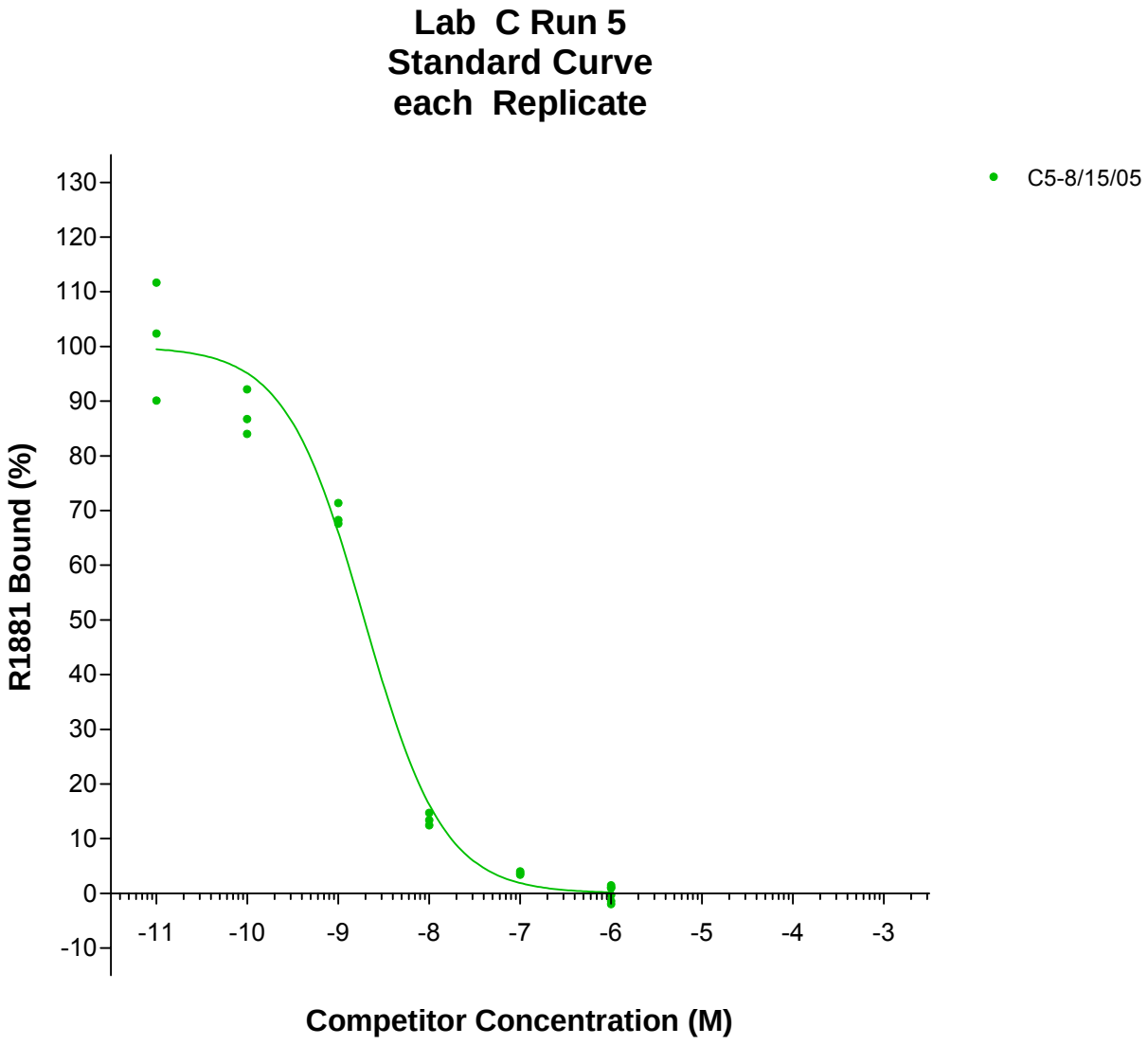


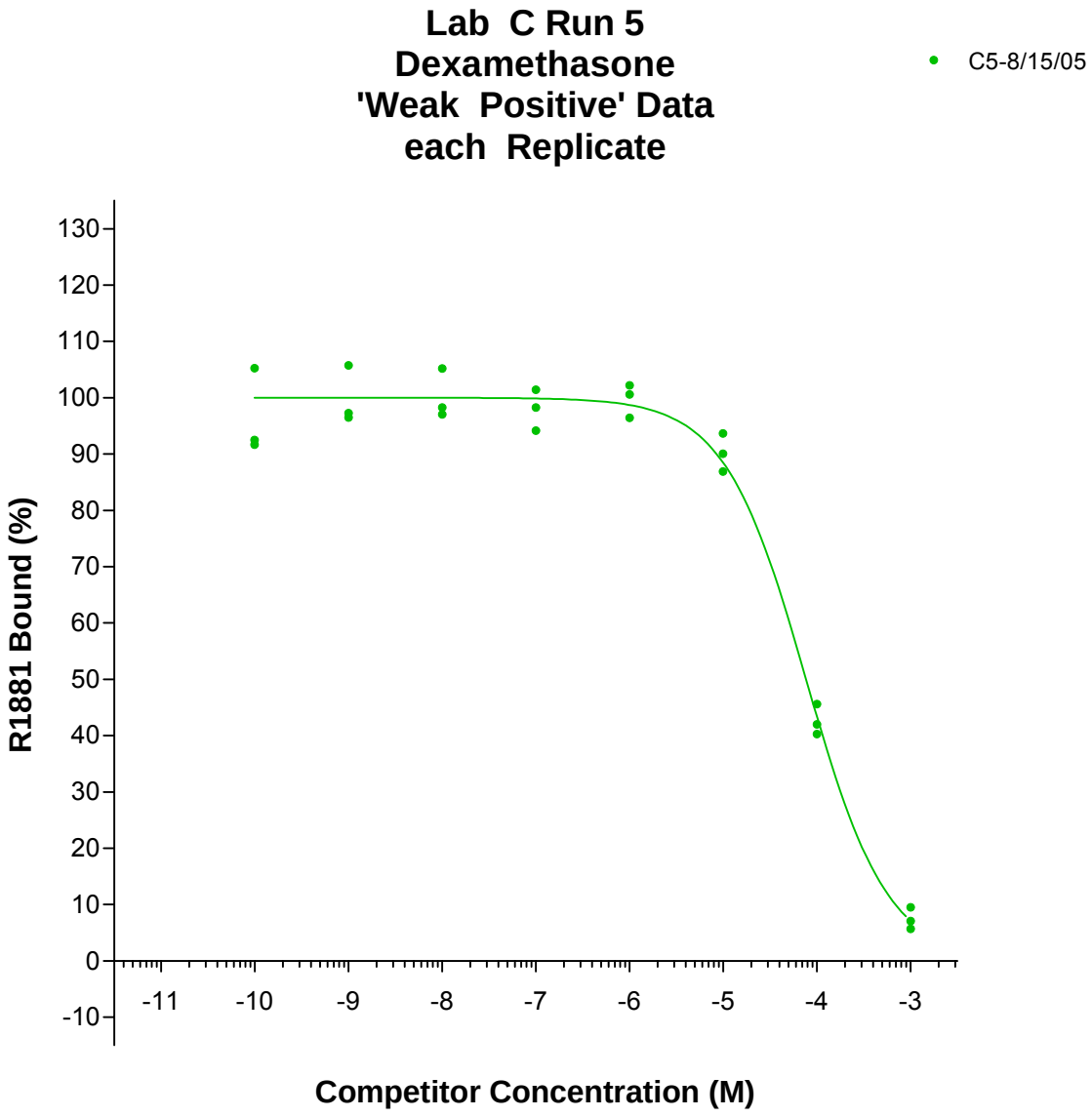

Lab C Run 4
Standard Curve
each Replicate
C4-7/27/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)


Lab C Run 4
Dexamethasone
'Weak Positive' Data
each Replicate
C4-7/27/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)

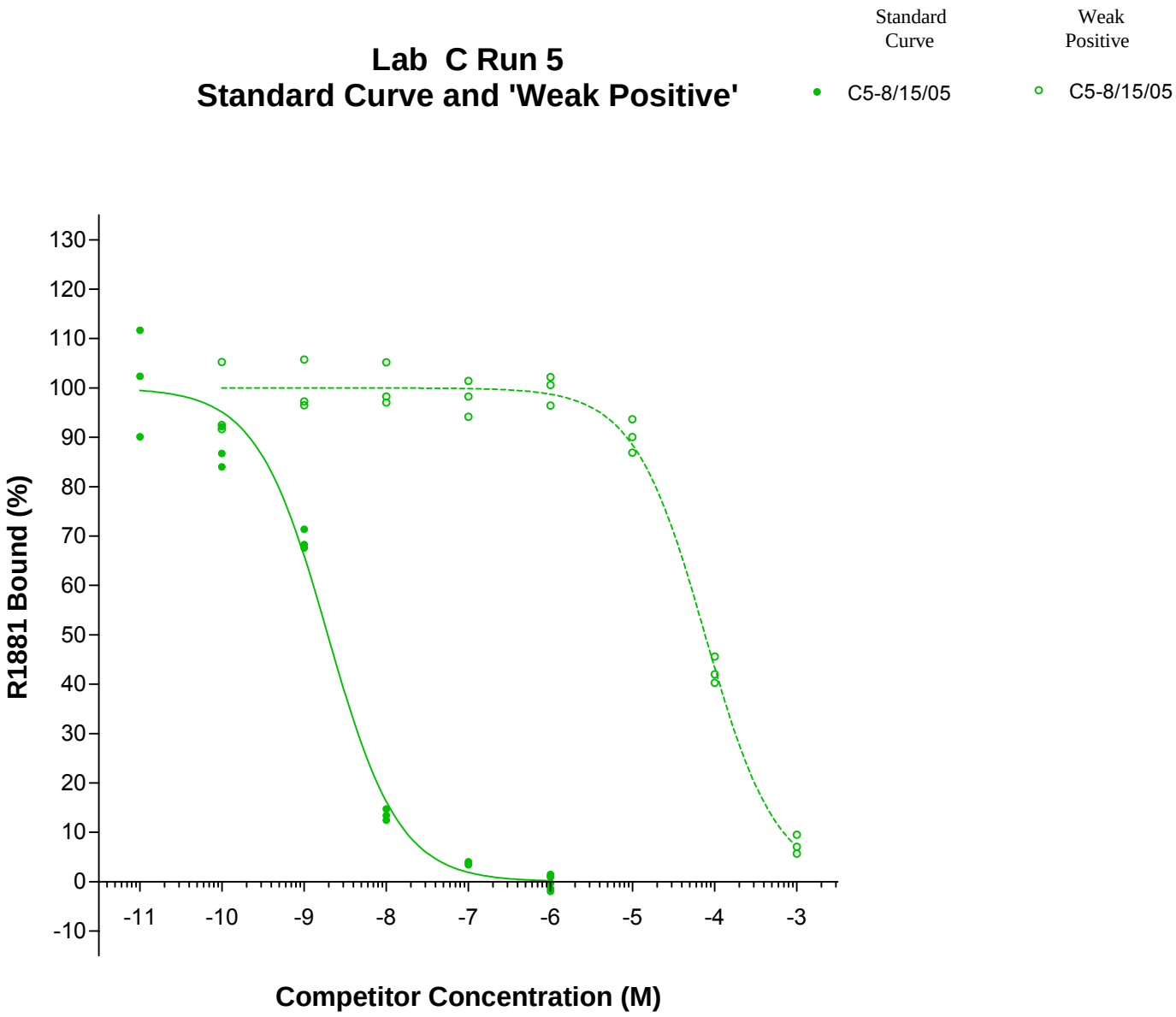
Lab C Run 4
Standard Curve and 'Weak Positive'

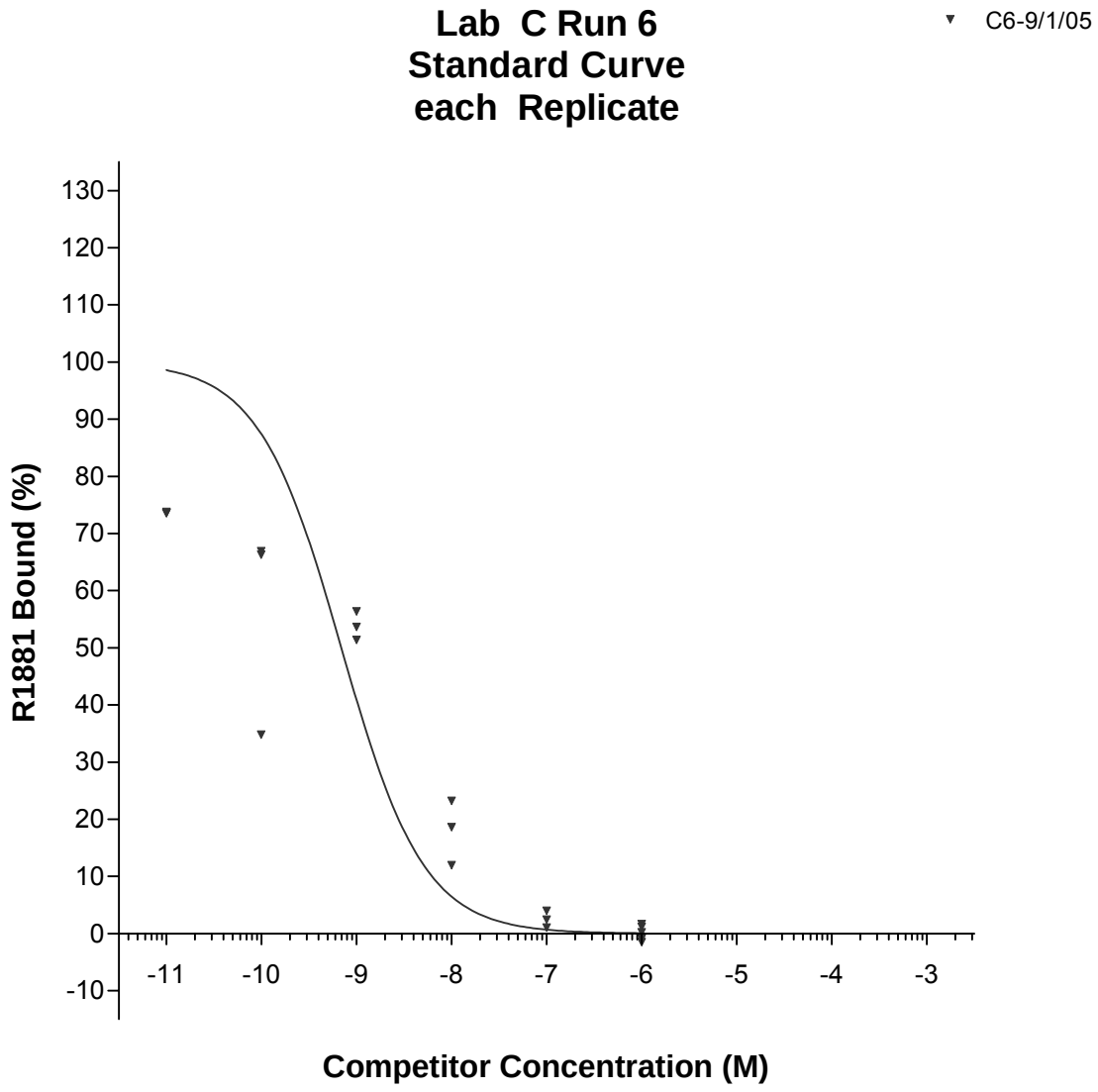


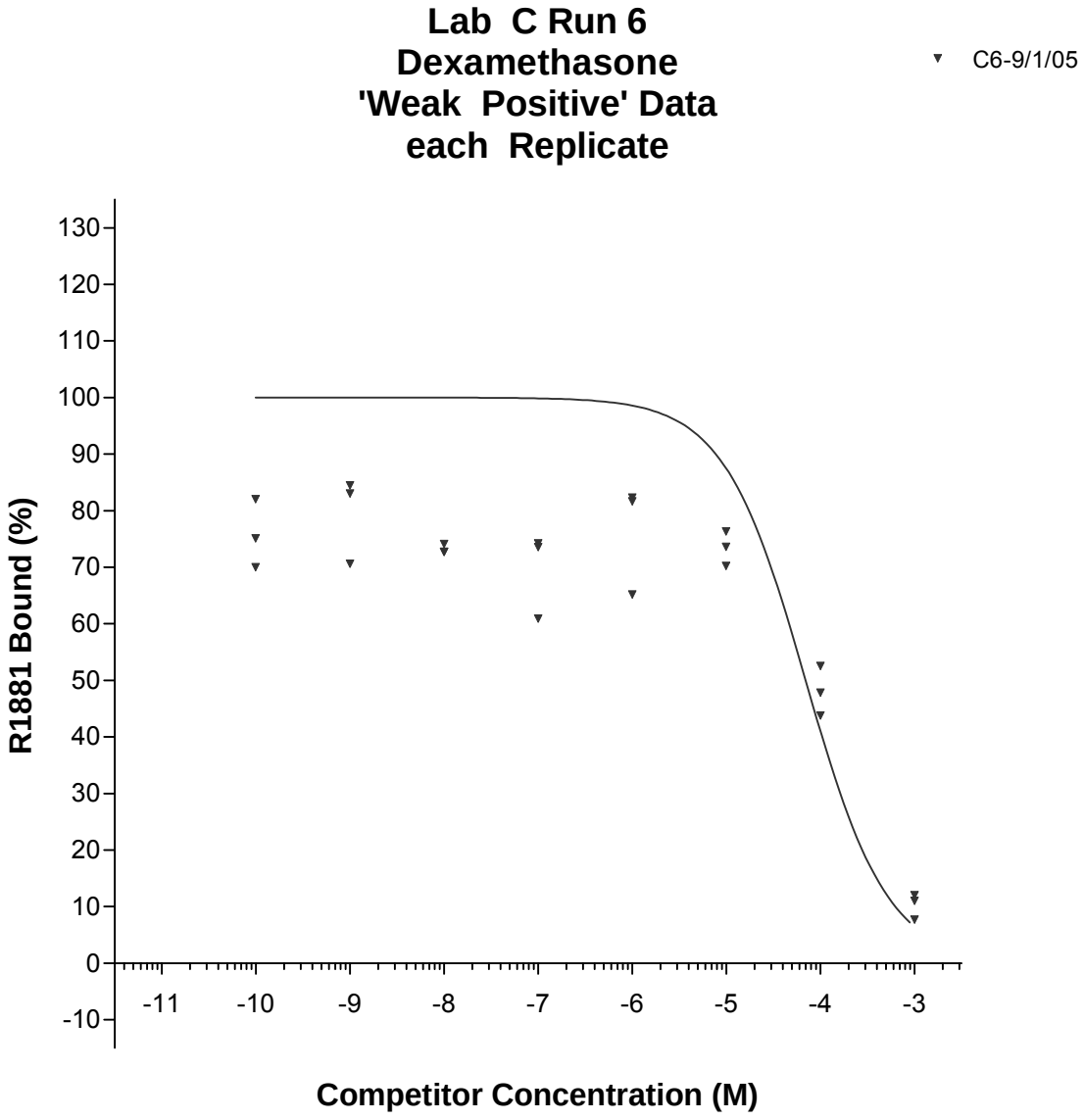

Lab C Run 5
Standard Curve
each Replicate
C5-8/15/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)


Lab C Run 5
Dexamethasone
'Weak Positive' Data
each Replicate
C5-8/15/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)

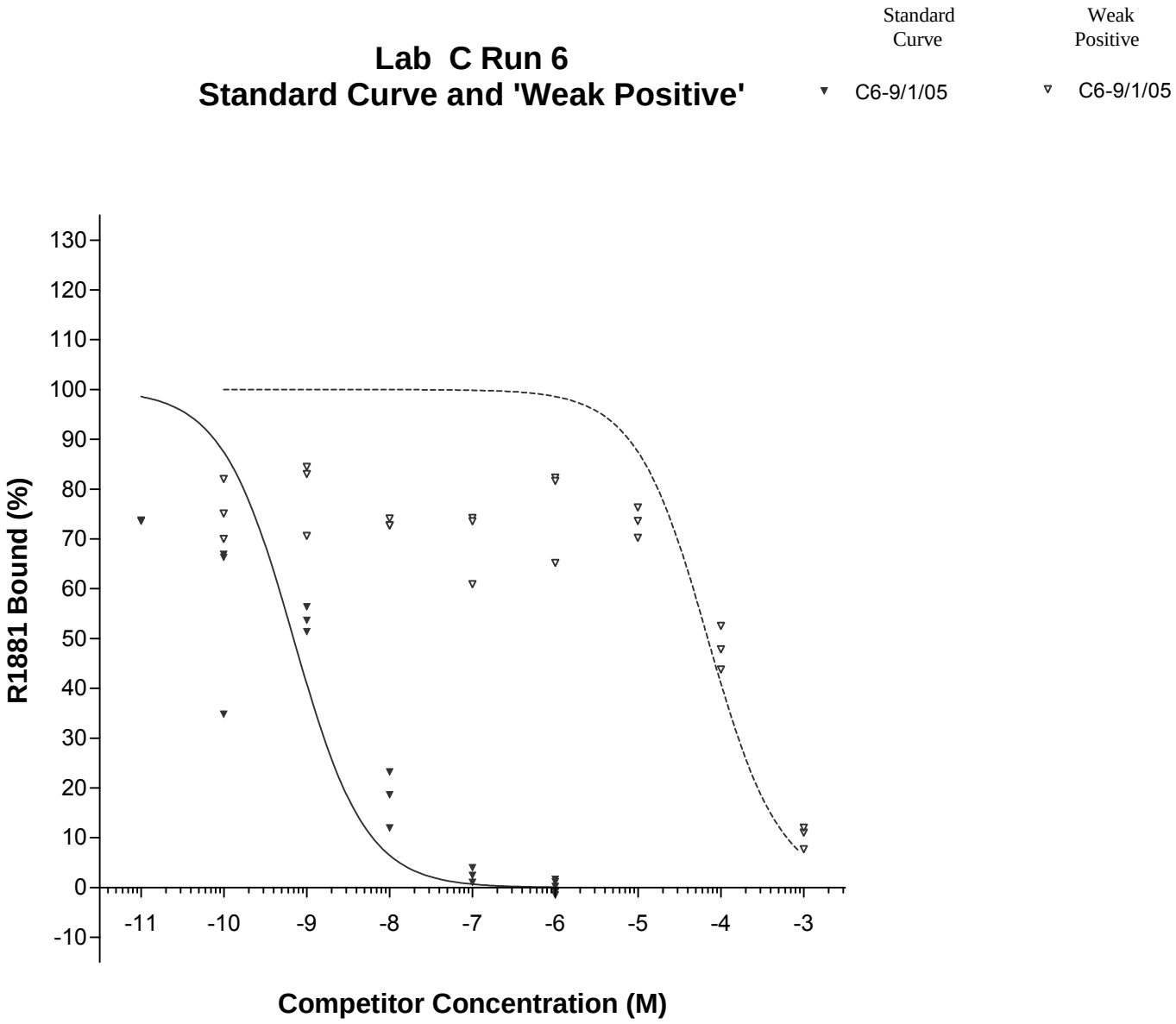
Lab C Run 5
Standard Curve and 'Weak Positive'




Lab C Run 6
Standard Curve
each Replicate
C6-9/1/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)


Lab C Run 6
Dexamethasone
'Weak Positive' Data
each Replicate
C6-9/1/05
R1881 Bound (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
-4
-3
Competitor Concentration (M)

Lab C Run 6
Standard Curve and 'Weak Positive'



Appendix 6: Excel Files for Competitive Binding Experiments

IC50 - RBA

run	1 (Seq#643	5 (Seq#6578)	6 (Seq#6604)
St Crv IC50	8.86E-10	1.95E-09	6.95E-10
WP IC50	5.77E-05	7.69E-05	6.98E-05
RBA	0.0015%	0.0025%	0.0010%

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1				**Competitive Assay of a known Weak Positive**											*Provide information in all blue cells in this column*			
2				**57 Assay Tubes**									**Laboratory Code:**		**C**			
3				Please return by eMail to n.a.Holter@pnl.gov									**Run identification:**		1 (Seq#6430)			
4													**Assay start date:**		7/27/2005			
5				Provide information in all blue cells									**Tracer lot number:**		3559-507			
6				in columns O, AE and AI									**Specific activity on day of assay:**		80.75	**Ci/mmole**		
7				If the DPM value for a tube was judged unreliable,									**Cytosol vial or lot identification:**		0725-06-01			
8				Include the DPM value in column O									**Protein (cytosol):**		600	**micro gram per tube**		
9				Provide a reason in column R														
10				The value in column Q will automatically change to FALSE									**Standard Curve IC50:**		8.86E-10	**M**		
11													**Weak Positive, Max Concentration:**		**3.00E-02**	**M**		
12													**Weak Positive IC50:**		5.77E-05	**M**		
13				Columns T and U contain values to be analyzed									**RBA:**		1.54E-05			
14				by nonlinear regression software														
15				They are also presented in table form in columns														
16				AC thorugh AJ														
17													**volume of ethanol counted:**		2	**mL**		protcol calls for counting decanted EtOH s
18																		reflects 100ul of reaction mixture processed

	S	T	U	V	W
	Summary values				
		n	**Mean**	**SD**	
	EtOH	6	10046.6	538.17	Total Binding, solvent control, tubes
	Hot	6	50112.1	844.00	Total hot R1881 added to each tube
	NSB	6	603.9	114.67	Nonspecific Binding
	Specific EtOH	6	9442.7	538.17	

Assay Characterization Values			
EtOH / Hot	0.20	less than 0.1?	
NSB / EtOH	0.06	around 0.25 ?	

upernate

Column O, Rows 10 through 13 will contain output parameters from the nonlinear regression software.

total volume in tubes **3.1E+00** uL

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 20.05% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

Position	Replicate	Competitor	Competitor Code	Concentration Code	Labels on vials in set 1-1-A	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Final Volume (ul)	Competitor Final Concentration (M)	Aliquot (uL)	DPM as sampled	corrected DPM for 2.0 mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
1	1	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3507.541	10873.3771	TRUE	
2	2	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3364.255	10429.1905	TRUE	
3	3	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3283.645	10179.2995	TRUE	
4	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	182.2976	546.8928	TRUE	
5	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	183.6904	551.0712	TRUE	
6	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	277.6359	832.9077	TRUE	
7	1	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	229.5600	711.636	TRUE	
8	2	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	221.1088	685.43728	TRUE	
9	3	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	219.4323	680.24013	TRUE	
10	1	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	489.5029	1517.45899	TRUE	
11	2	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	564.6778	1750.50118	TRUE	
12	3	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	622.5062	1929.76922	TRUE	
13	1	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1585.875	4916.2125	TRUE	
14	2	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1775.617	5504.4127	TRUE	
15	3	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1681.811	5213.6141	TRUE	
16	1	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2518.408	7807.0648	TRUE	
17	2	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2728.156	8457.2836	TRUE	
18	3	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2898.241	8984.5471	TRUE	
19	1	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	2983.283	9248.1773	TRUE	
20	2	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	2704.096	8382.6976	TRUE	
21	3	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	3204.827	9934.9637	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
24	1	1		—	108.8	10873.38	10269.4	-1430.7	108.8	21.69809
25	2	2		—	104.1	10429.19	9825.3	-986.5	104.1	20.8117
26	3	3		—	101.4	10179.3	9575.4	-736.6	101.4	20.31304
27	4	1		-6.0	-0.6	546.8928	-57.0	8895.8	-0.6	1.091338
28	5	2		-6.0	-0.6	551.0712	-52.9	8891.6	-0.6	1.099676
29	6	3		-6.0	0.0	832.9077	229.0	8609.8	2.4	1.662088
30	7	1	cold R1881	-7.0	1.1	711.636	107.7	8731.1	1.1	1.420087
31	8	2	cold R1881	-7.0	0.9	685.4373	81.5	8757.2	0.9	1.367807
32	9	3	cold R1881	-7.0	0.8	680.2401	76.3	8762.4	0.8	1.357436
33	10	1	cold R1881	-8.0	9.7	1517.459	913.5	7925.2	9.7	3.028126
34	11	2	cold R1881	-8.0	12.1	1750.501	1146.6	7692.2	12.1	3.493168
35	12	3	cold R1881	-8.0	14.0	1929.769	1325.8	7512.9	14.0	3.850901
36	13	1	cold R1881	-9.0	45.7	4916.213	4312.3	4526.5	45.7	9.810422
37	14	2	cold R1881	-9.0	51.9	5504.413	4900.5	3938.3	51.9	10.98419
38	15	3	cold R1881	-9.0	48.8	5213.614	4609.7	4229.1	48.8	10.40389
39	16	1	cold R1881	-10.0	76.3	7807.065	7203.1	1635.6	76.3	15.57919
40	17	2	cold R1881	-10.0	83.2	8457.284	7853.4	985.4	83.2	16.87671
41	18	3	cold R1881	-10.0	88.8	8984.547	8380.6	458.1	88.8	17.92888
42	19	1	cold R1881	-11.0	91.5	9248.177	8644.2	194.5	91.5	18.45496
43	20	2	cold R1881	-11.0	82.4	8382.698	7778.8	1060.0	82.4	16.72788
44	21	3	cold R1881	-11.0	98.8	9934.964	9331.0	-492.3	98.8	19.82546

Column O, Rows 10 through 13 will contain output parameters from the nonlinear regression software. ***total volume in tubes*** **3.1E+00** uL

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 20.05% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

Position	Replicate	Competitor	Competitor Code	Concentration Code	Labels on vials in set 1-1-A	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Final Volume (ul)	Competitor Final Concentration (M)	Aliquot (uL)	DPM as sampled	corrected DPM for 2.0 mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
22	1	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	314.2866	974.28846	TRUE	
23	2	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	383.3606	1188.41786	TRUE	
24	3	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	428.2586	1327.60166	TRUE	
25	1	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1421.907	4407.9117	TRUE	
26	2	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1347.070	4175.917	TRUE	
27	3	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1292.970	4008.207	TRUE	
28	1	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2913.401	9031.5431	TRUE	
29	2	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2596.594	8049.4414	TRUE	
30	3	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2646.706	8204.7886	TRUE	
31	1	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	3125.597	9689.3507	TRUE	
32	2	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	2943.785	9125.7335	TRUE	
33	3	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	3122.753	9680.5343	TRUE	
34	1	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	3292.837	10207.7947	TRUE	
35	2	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	3167.042	9817.8302	TRUE	
36	3	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	3249.386	10073.0966	TRUE	
37	1	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	3180.098	9858.3038	TRUE	
38	2	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	3102.343	9617.2633	TRUE	
39	3	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	3228.820	10009.342	TRUE	
40	1	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	3196.977	9910.6287	TRUE	
41	2	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	3284.467	10181.8477	TRUE	
42	3	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	3166.594	9816.4414	TRUE	
43	1	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	3180.581	9859.8011	TRUE	
44	2	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	3166.975	9817.6225	TRUE	
45	3	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	3136.359	9722.7129	TRUE	
46	1	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3091.975	9585.1225	TRUE	
47	2	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3098.570	9605.567	TRUE	
48	3	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	3099.076	9607.1356	TRUE	
49	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	189.5081	568.5243	TRUE	
50	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	175.5468	526.6404	TRUE	
51	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	199.1791	597.5373	TRUE	
52	1	none	Hot		—	—	—	30	—	—	—	—		50953.06	50953.06	TRUE	
53	2	none	Hot		—	—	—	30	—	—	—	—		49907.45	49907.45	TRUE	
54	3	none	Hot		—	—	—	30	—	—	—	—		49900.36	49900.36	TRUE	
55	1	none	Hot		—	—	—	30	—	—	—	—		48668.04	48668.04	TRUE	
56	2	none	Hot		—	—	—	30	—	—	—	—		50336.91	50336.91	TRUE	
57	3	none	Hot		—	—	—	30	—	—	—	—		50907.04	50907.04	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
45	22	1	Weak Positive	-3.0	3.9	974.2885	370.4	8468.4	3.9	1.944216
46	23	2	Weak Positive	-3.0	6.2	1188.418	584.5	8254.3	6.2	2.371517
47	24	3	Weak Positive	-3.0	7.7	1327.602	723.7	8115.1	7.7	2.649261
48	25	1	Weak Positive	-4.0	40.3	4407.912	3804.0	5034.8	40.3	8.796095
49	26	2	Weak Positive	-4.0	37.8	4175.917	3572.0	5266.8	37.8	8.333144
50	27	3	Weak Positive	-4.0	36.1	4008.207	3404.3	5434.5	36.1	7.998474
51	28	1	Weak Positive	-5.0	89.3	9031.543	8427.6	411.1	89.3	18.02266
52	29	2	Weak Positive	-5.0	78.8	8049.441	7445.5	1393.2	78.8	16.06286
53	30	3	Weak Positive	-5.0	80.5	8204.789	7600.9	1237.9	80.5	16.37286
54	31	1	Weak Positive	-6.0	96.2	9689.351	9085.4	-246.7	96.2	19.33533
55	32	2	Weak Positive	-6.0	90.2	9125.734	8521.8	317.0	90.2	18.21062
56	33	3	Weak Positive	-6.0	96.1	9680.534	9076.6	-237.8	96.1	19.31774
57	34	1	Weak Positive	-7.0	101.7	10207.79	9603.9	-765.1	101.7	20.3699
58	35	2	Weak Positive	-7.0	97.6	9817.83	9213.9	-375.1	97.6	19.59172
59	36	3	Weak Positive	-7.0	100.3	10073.1	9469.2	-630.4	100.3	20.10111
60	37	1	Weak Positive	-8.0	98.0	9858.304	9254.4	-415.6	98.0	19.67248
61	38	2	Weak Positive	-8.0	95.5	9617.263	9013.3	-174.6	95.5	19.19148
62	39	3	Weak Positive	-8.0	99.6	10009.34	9405.4	-566.7	99.6	19.97389
63	40	1	Weak Positive	-9.0	98.6	9910.629	9306.7	-467.9	98.6	19.7769
64	41	2	Weak Positive	-9.0	101.4	10181.85	9577.9	-739.2	101.4	20.31812
65	42	3	Weak Positive	-9.0	97.6	9816.441	9212.5	-373.8	97.6	19.58895
66	43	1	Weak Positive	-10.0	98.0	9859.801	9255.9	-417.1	98.0	19.67547
67	44	2	Weak Positive	-10.0	97.6	9817.623	9213.7	-374.9	97.6	19.5913
68	45	3	Weak Positive	-10.0	96.6	9722.713	9118.8	-280.0	96.6	19.40191
69	46	1		—	95.1	9585.123	8981.2	-142.4	95.1	19.12734
70	47	2		—	95.3	9605.567	9001.6	-162.9	95.3	19.16814
71	48	3		—	95.3	9607.136	9003.2	-164.4	95.3	19.17127
72	49	1		-6.0	-0.4	568.5243	-35.4	8874.2	-0.4	1.134504
73	50	2		-6.0	-0.8	526.6404	-77.3	8916.0	-0.8	1.050924
74	51	3		-6.0	-0.1	597.5373	-6.4	8845.1	-0.1	1.1924
75	52	1				50953.06	50349.1			
76	53	2				49907.45	49303.5			
77	54	3				49900.36	49296.4			
78	55	1				48668.04	48064.1			
79	56	2				50336.91	49733.0			
80	57	3				50907.04	50303.1			

	A	B	AC	AD	AE	AF	AG	AH	AI
19									
20									
21			Prism data						
22			***standard curve***				***weak positive***		
23	***Position***	***Replicate***	***concentration (log)***	Y1-SC	Y2-SC	Y3-SC	***concentration (log)***	y1-PC	y2-PC
33	10	1	Standard Curve		C4-7/27/05		Positive Control		C4-7/27/05
34	11	2	One site competition				One site competition		
35	12	3	Best-fit values				Best-fit values		
36	13	1	BOTTOM		0		BOTTOM		0
37	14	2	TOP		100		TOP		100
38	15	3	LOGEC50		-9.053		LOGEC50		-4.239
39	16	1	EC50		8.86E-10		EC50		5.77E-05
40	17	2	Std. Error				Std. Error		
41	18	3	LOGEC50		0.05167		LOGEC50		0.03014
42	19	1	95% Confidence Intervals				95% Confidence Intervals		
43	20	2	LOGEC50		-9.160 to -8.945		LOGEC50		-4.301 to -4.
44	21	3	*EC50*		6.914e-010 to 1.136e-00		EC50		4.997e-005 t
45	22	1	Goodness of Fit				Goodness of Fit		
46	23	2	Degrees of Freedor		20		Degrees of Freedor		23
47	24	3	R^2		0.978		R^2		0.9909
48	25	1	Absolute Sum of Squa		646.6		Absolute Sum of Sc		241.4
49	26	2	Sy.x		5.686		Sy.x		3.239
50	27	3	Constraints				Constraints		
51	28	1	BOTTOM		BOTTOM = 0.0		BOTTOM		BOTTOM =
52	29	2	TOP		TOP = 100.0		TOP		TOP = 100.0
53	30	3	Data				Data		
54	31	1	Number of X values		7		Number of X values		8
55	32	2	Number of Y replica		3		Number of Y replica		3
56	33	3	Total number of val		21		Total number of val		24
57	34	1	Number of missing		0		Number of missing		0

Competitive Assay of a known Weak Positive

57 Assay Tubes

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

Provide information in all blue cells
in columns O, AE and AI

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

Columns T and U contain values to be analyzed
by nonlinear regression software
They are also presented in table form in columns
AC thorugh AJ

Provide information in all blue cells in this column

Laboratory Code:	**C**	
Run identification:	5 (Seq#6578)	
Assay start date:	8/15/2005	
Tracer lot number:	3559-507	
Specific activity on day of assay:	80.51	**Ci/mmole**
Cytosol vial or lot identification:	0725-06-01	
Protein (cytosol):	600	**micro gram per tube**
Standard Curve IC50:	1.95E-09	**M**
Weak Positive, Max Concentration:	**3.00E-02**	**M**
Weak Positive IC50:	7.69E-05	**M**
RBA:	2.53E-05	

volume of ethanol counted:	2	**mL**

protcol calls for counting decanted EtOH s
reflects 100ul of reaction mixture processed

	S	T	U	V	W
	Summary values				
		n	**Mean**	**SD**	
	EtOH	6	8242.7	104.97	Total Binding, solvent control, tubes
	Hot	6	48373.7	960.16	Total hot R1881 added to each tube
	NSB	6	648.0	109.14	Nonspecific Binding
	Specific EtOH	6	7594.8	104.97	

Assay Characterization Values			
EtOH / Hot	0.17	less than 0.1?	
NSB / EtOH	0.08	around 0.25 ?	

upernate

Column O, Rows 10 through 13 will contain output parameters from the nonlinear regression software.

total volume in tubes **3.1E+00** uL

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 17.04% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

Position	Replicate	Competitor	Competitor Code	Concentration Code	Labels on vials in set 1-1-A	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Final Volume (ul)	Competitor Final Concentration (M)	Aliquot (uL)	DPM as sampled	corrected DPM for 2.0 mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
1	1	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	2646.450	8203.995	TRUE	
2	2	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	2605.513	8077.0903	TRUE	
3	3	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	2688.905	8335.6055	TRUE	
4	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	254.0335	762.1005	TRUE	
5	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	180.0681	540.2043	TRUE	
6	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	240.8467	722.5401	TRUE	
7	1	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	300.2336	930.72416	TRUE	
8	2	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	307.7528	954.03368	TRUE	
9	3	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	293.7017	910.47527	TRUE	
10	1	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	514.6861	1595.52691	TRUE	
11	2	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	537.7809	1667.12079	TRUE	
12	3	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	570.5240	1768.6244	TRUE	
13	1	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1882.530	5835.843	TRUE	
14	2	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1866.161	5785.0991	TRUE	
15	3	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1958.240	6070.544	TRUE	
16	1	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2267.436	7029.0516	TRUE	
17	2	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2466.931	7647.4861	TRUE	
18	3	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	2333.942	7235.2202	TRUE	
19	1	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	2945.865	9132.1815	TRUE	
20	2	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	2717.599	8424.5569	TRUE	
21	3	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	2417.060	7492.886	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
24	1	1		—	99.5	8203.995	7556.0	-609.2	99.5	16.95962
25	2	2		—	97.8	8077.09	7429.1	-482.3	97.8	16.69727
26	3	3		—	101.2	8335.606	7687.6	-740.8	101.2	17.23169
27	4	1		-6.0	1.5	762.1005	114.1	6832.7	1.5	1.575444
28	5	2		-6.0	-1.4	540.2043	-107.8	7054.6	-1.4	1.116731
29	6	3		-6.0	0.0	722.5401	74.6	6872.2	1.0	1.493663
30	7	1	cold R1881	-7.0	3.7	930.7242	282.8	6664.0	3.7	1.924029
31	8	2	cold R1881	-7.0	4.0	954.0337	306.1	6640.7	4.0	1.972215
32	9	3	cold R1881	-7.0	3.5	910.4753	262.5	6684.3	3.5	1.88217
33	10	1	cold R1881	-8.0	12.5	1595.527	947.6	5999.2	12.5	3.298335
34	11	2	cold R1881	-8.0	13.4	1667.121	1019.1	5927.6	13.4	3.446337
35	12	3	cold R1881	-8.0	14.8	1768.624	1120.7	5826.1	14.8	3.656169
36	13	1	cold R1881	-9.0	68.3	5835.843	5187.9	1758.9	68.3	12.06408
37	14	2	cold R1881	-9.0	67.6	5785.099	5137.1	1809.7	67.6	11.95918
38	15	3	cold R1881	-9.0	71.4	6070.544	5422.6	1524.2	71.4	12.54926
39	16	1	cold R1881	-10.0	84.0	7029.052	6381.1	565.7	84.0	14.53073
40	17	2	cold R1881	-10.0	92.2	7647.486	6999.5	-52.7	92.2	15.80918
41	18	3	cold R1881	-10.0	86.7	7235.22	6587.2	359.6	86.7	14.95693
42	19	1	cold R1881	-11.0	111.7	9132.182	8484.2	-1537.4	111.7	18.8784
43	20	2	cold R1881	-11.0	102.4	8424.557	7776.6	-829.8	102.4	17.41557
44	21	3	cold R1881	-11.0	90.1	7492.886	6844.9	101.9	90.1	15.48958

Column O, Rows 10 through 13 will contain output parameters

total volume in tubes **3.1E+00** uL

from the nonlinear regression software.

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 17.04% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
23	*Position*	*Replicate*	*Competitor*	*Competitor Code*	*Concentration Code*	*Labels on vials in set 1-1-A*	*Competitor Initial Concentration (M)*	*Cytosol (uL)*	*Tracer (Hot R1881) Volume (uL)*	*Competitor Volume (uL)*	*triamcelenone Volume (uL)*	*Final Volume (ul)*	*Competitor Final Concentration (M)*	*Aliquot (uL)*	*DPM as sampled*	*corrected DPM for 2.0 mL*	*Use this value?*	*Notes to explain why "Use this value" is set to "FALSE"*
45	22	1	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	441.9878	1370.16218	TRUE	
46	23	2	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	383.3569	1188.40639	TRUE	
47	24	3	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	349.0939	1082.19109	TRUE	
48	25	1	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1196.035	3707.7085	TRUE	
49	26	2	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1326.695	4112.7545	TRUE	
50	27	3	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1238.216	3838.4696	TRUE	
51	28	1	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2338.467	7249.2477	TRUE	
52	29	2	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2416.200	7490.22	TRUE	
53	30	3	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	2504.597	7764.2507	TRUE	
54	31	1	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	2572.436	7974.5516	TRUE	
55	32	2	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	2712.958	8410.1698	TRUE	
56	33	3	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	2673.491	8287.8221	TRUE	
57	34	1	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	2616.542	8111.2802	TRUE	
58	35	2	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	2694.140	8351.834	TRUE	
59	36	3	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	2516.030	7799.693	TRUE	
60	37	1	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	2586.385	8017.7935	TRUE	
61	38	2	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	2785.985	8636.5535	TRUE	
62	39	3	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	2616.537	8111.2647	TRUE	
63	40	1	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	2799.592	8678.7352	TRUE	
64	41	2	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	2572.984	7976.2504	TRUE	
65	42	3	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	2592.089	8035.4759	TRUE	
66	43	1	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	2788.493	8644.3283	TRUE	
67	44	2	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	2455.725	7612.7475	TRUE	
68	45	3	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	2475.827	7675.0637	TRUE	
69	46	1	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	2676.841	8298.2071	TRUE	
70	47	2	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	2642.104	8190.5224	TRUE	
71	48	3	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	2693.885	8351.0435	TRUE	
72	49	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	166.9731	500.9193	TRUE	
73	50	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	244.5675	733.7025	TRUE	
74	51	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	209.4575	628.3725	TRUE	
75	52	1	none	Hot		**—**	—	—	30	—	—	—	—		49618.01	49618.01	TRUE	
76	53	2	none	Hot		**—**	—	—	30	—	—	—	—		48974.80	48974.80	TRUE	
77	54	3	none	Hot		**—**	—	—	30	—	—	—	—		47847.54	47847.54	TRUE	
78	55	1	none	Hot		**—**	—	—	30	—	—	—	—		48257.46	48257.46	TRUE	
79	56	2	none	Hot		**—**	—	—	30	—	—	—	—		48690.37	48690.37	TRUE	
80	57	3	none	Hot		**—**	—	—	30	—	—	—	—		46854.07	46854.07	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
45	22	1	Weak Positive	-3.0	9.5	1370.162	722.2	6224.6	9.5	2.832452
46	23	2	Weak Positive	-3.0	7.1	1188.406	540.4	6406.4	7.1	2.45672
47	24	3	Weak Positive	-3.0	5.7	1082.191	434.2	6512.6	5.7	2.237147
48	25	1	Weak Positive	-4.0	40.3	3707.709	3059.7	3887.1	40.3	7.664718
49	26	2	Weak Positive	-4.0	45.6	4112.755	3464.8	3482.0	45.6	8.502045
50	27	3	Weak Positive	-4.0	42.0	3838.47	3190.5	3756.3	42.0	7.935033
51	28	1	Weak Positive	-5.0	86.9	7249.248	6601.3	345.5	86.9	14.98593
52	29	2	Weak Positive	-5.0	90.1	7490.22	6842.2	104.6	90.1	15.48407
53	30	3	Weak Positive	-5.0	93.7	7764.251	7116.3	-169.5	93.7	16.05056
54	31	1	Weak Positive	-6.0	96.5	7974.552	7326.6	-379.8	96.5	16.4853
55	32	2	Weak Positive	-6.0	102.2	8410.17	7762.2	-815.4	102.2	17.38583
56	33	3	Weak Positive	-6.0	100.6	8287.822	7639.8	-693.1	100.6	17.13291
57	34	1	Weak Positive	-7.0	98.3	8111.28	7463.3	-516.5	98.3	16.76795
58	35	2	Weak Positive	-7.0	101.4	8351.834	7703.9	-757.1	101.4	17.26523
59	36	3	Weak Positive	-7.0	94.2	7799.693	7151.7	-204.9	94.2	16.12383
60	37	1	Weak Positive	-8.0	97.0	8017.794	7369.8	-423.0	97.0	16.57469
61	38	2	Weak Positive	-8.0	105.2	8636.554	7988.6	-1041.8	105.2	17.85382
62	39	3	Weak Positive	-8.0	98.3	8111.265	7463.3	-516.5	98.3	16.76792
63	40	1	Weak Positive	-9.0	105.7	8678.735	8030.8	-1084.0	105.7	17.94102
64	41	2	Weak Positive	-9.0	96.5	7976.25	7328.3	-381.5	96.5	16.48881
65	42	3	Weak Positive	-9.0	97.3	8035.476	7387.5	-440.7	97.3	16.61125
66	43	1	Weak Positive	-10.0	105.3	8644.328	7996.4	-1049.6	105.3	17.86989
67	44	2	Weak Positive	-10.0	91.7	7612.748	6964.8	-18.0	91.7	15.73737
68	45	3	Weak Positive	-10.0	92.5	7675.064	7027.1	-80.3	92.5	15.86619
69	46	1		—	100.7	8298.207	7650.2	-703.4	100.7	17.15437
70	47	2		—	99.3	8190.522	7542.5	-595.8	99.3	16.93176
71	48	3		—	101.4	8351.044	7703.1	-756.3	101.4	17.2636
72	49	1		-6.0	-1.9	500.9193	-147.1	7093.9	-1.9	1.03552
73	50	2		-6.0	1.1	733.7025	85.7	6861.1	1.1	1.516738
74	51	3		-6.0	-0.3	628.3725	-19.6	6966.4	-0.3	1.298996
75	52	1				49618.01	48970.0			
76	53	2				48974.8	48326.8			
77	54	3				47847.54	47199.6			
78	55	1				48257.46	47609.5			
79	56	2				48690.37	48042.4			
80	57	3				46854.07	46206.1			

	A	B	AC	AD	AE	AF	AG	AH	AI
19									
20									
21			Prism data						
22			***standard curve***				***weak positive***		
23	***Position***	***Replicate***	***concentration (log)***	Y1-SC	Y2-SC	Y3-SC	***concentration (log)***	y1-PC	y2-PC
33	10	1	Standard Curve		C5-8/15/05		Positive Control		C5-8/15/05
34	11	2	One site competition				One site competition		
35	12	3	Best-fit values				Best-fit values		
36	13	1	BOTTOM		0		BOTTOM		0
37	14	2	TOP		100		TOP		100
38	15	3	LOGEC50		-8.71		LOGEC50		-4.114
39	16	1	EC50		1.95E-09		EC50		7.69E-05
40	17	2	Std. Error				Std. Error		
41	18	3	LOGEC50		0.04828		LOGEC50		0.03609
42	19	1	95% Confidence Intervals				95% Confidence Intervals		
43	20	2	LOGEC50		-8.811 to -8.610		LOGEC50		-4.189 to -4.03
44	21	3	*EC50*		1.545e-009 to 2.457e-00		EC50		6.479e-005 to
45	22	1	Goodness of Fit				Goodness of Fit		
46	23	2	Degrees of Freedor		20		Degrees of Freedor		23
47	24	3	R^2		0.9855		R^2		0.9862
48	25	1	Absolute Sum of Squa		529.4		Absolute Sum of Sc		359
49	26	2	Sy.x		5.145		Sy.x		3.951
50	27	3	Constraints				Constraints		
51	28	1	BOTTOM		BOTTOM = 0.0		BOTTOM		BOTTOM = 0.
52	29	2	TOP		TOP = 100.0		TOP		TOP = 100.0
53	30	3	Data				Data		
54	31	1	Number of X values		7		Number of X values		8
55	32	2	Number of Y replica		3		Number of Y replica		3
56	33	3	Total number of val		21		Total number of val		24
57	34	1	Number of missing		0		Number of missing		0

Competitive Assay of a known Weak Positive

57 Assay Tubes

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

Provide information in all blue cells
in columns O, AE and AI

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

Columns T and U contain values to be analyzed
by nonlinear regression software
They are also presented in table form in columns
AC thorugh AJ

Provide information in all blue cells in this column

Laboratory Code:	**C**	
Run identification:	6 (Seq#6604)	
Assay start date:	9/1/2005	
Tracer lot number:	3559-507	
Specific activity on day of assay:	80.30	**Ci/mmole**
Cytosol vial or lot identification:	0725-06-01	
Protein (cytosol):	600	**micro gram per tube**
Standard Curve IC50:	6.95E-10	**M**
Weak Positive, Max Concentration:	**3.00E-02**	**M**
Weak Positive IC50:	6.98E-05	**M**
RBA:	9.95E-06	

volume of ethanol counted: 2 **mL**

protcol calls for counting decanted EtOH s
reflects 100ul of reaction mixture processed

	S	T	U	V	W
2		**Summary values**			
3		**n**	**Mean**	**SD**	
4	EtOH	6	5555.8	1042.42	Total Binding, solvent control, tubes
5	Hot	6	48810.9	1082.81	Total hot R1881 added to each tube
6	NSB	6	606.5	60.69	Nonspecific Binding
7	Specific EtOH	6	4949.3	1042.42	

	S	T	U
11	**Assay Characterization Values**		
12	EtOH / Hot	0.11	less than 0.1?
13	NSB / EtOH	0.11	around 0.25 ?

17 upernate

Column O, Rows 10 through 13 will contain output parameters

total volume in tubes **3.1E+00** uL

from the nonlinear regression software.

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 11.38% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

Position	Replicate	Competitor	Competitor Code	Concentration Code	Labels on vials in set 1-1-A	Competitor Initial Concentration (M)	Cytosol (uL)	Tracer (Hot R1881) Volume (uL)	Competitor Volume (uL)	triamcelenone Volume (uL)	Final Volume (ul)	Competitor Final Concentration (M)	Aliquot (uL)	DPM as sampled	corrected DPM for 2.0 mL	Use this value?	Notes to explain why "Use this value" is set to "FALSE"
1	1	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	1234.285	3826.2835	TRUE	
2	2	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	1665.668	5163.5708	TRUE	
3	3	ethanol	EtOH	0	—	—	300	30	10	50	310	—	100	1718.832	5328.3792	TRUE	
4	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	221.0511	663.1533	TRUE	
5	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	229.4034	688.2102	TRUE	
6	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	191.3576	574.0728	TRUE	
7	1	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	259.3256	803.90936	TRUE	
8	2	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	212.7799	659.61769	TRUE	
9	3	Inert R1881	S	1	**C-1-S1**	3.00E-06	300	30	10	50	310	9.7E-08	100	234.2482	726.16942	TRUE	
10	1	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	566.1874	1755.18094	TRUE	
11	2	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	492.8788	1527.92428	TRUE	
12	3	Inert R1881	S	2	**C-1-S2**	3.00E-07	300	30	10	50	310	9.7E-09	100	386.3280	1197.6168	TRUE	
13	1	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1095.522	3396.1182	TRUE	
14	2	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1016.175	3150.1425	TRUE	
15	3	Inert R1881	S	3	**C-1-S3**	3.00E-08	300	30	10	50	310	9.7E-10	100	1052.467	3262.6477	TRUE	
16	1	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	1253.723	3886.5413	TRUE	
17	2	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	1263.627	3917.2437	TRUE	
18	3	Inert R1881	S	4	**C-1-S4**	3.00E-09	300	30	10	50	310	9.7E-11	100	751.383	2329.28761	TRUE	
19	1	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	1370.248	4247.7688	TRUE	
20	2	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	1373.103	4256.6193	TRUE	
21	3	Inert R1881	S	5	**C-1-S5**	3.00E-10	300	30	10	50	310	9.7E-12	100	1370.882	4249.7342	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
24	1	1		—	65.1	3826.284	3219.8	1123.0	65.1	7.838997
25	2	2		—	92.1	5163.571	4557.1	-214.3	92.1	10.57873
26	3	3		—	95.4	5328.379	4721.9	-379.1	95.4	10.91638
27	4	1		-6.0	1.1	663.1533	56.7	4286.1	1.1	1.358618
28	5	2		-6.0	1.7	688.2102	81.8	4261.1	1.7	1.409952
29	6	3		-6.0	0.0	574.0728	-32.4	4375.2	-0.7	1.176116
30	7	1	cold R1881	-7.0	4.0	803.9094	197.4	4145.4	4.0	1.646988
31	8	2	cold R1881	-7.0	1.1	659.6177	53.2	4289.7	1.1	1.351374
32	9	3	cold R1881	-7.0	2.4	726.1694	119.7	4223.1	2.4	1.48772
33	10	1	cold R1881	-8.0	23.2	1755.181	1148.7	3194.1	23.2	3.59588
34	11	2	cold R1881	-8.0	18.6	1527.924	921.5	3421.4	18.6	3.130294
35	12	3	cold R1881	-8.0	11.9	1197.617	591.2	3751.7	11.9	2.453586
36	13	1	cold R1881	-9.0	56.4	3396.118	2789.7	1553.2	56.4	6.957707
37	14	2	cold R1881	-9.0	51.4	3150.143	2543.7	1799.2	51.4	6.453771
38	15	3	cold R1881	-9.0	53.7	3262.648	2656.2	1686.7	53.7	6.684263
39	16	1	cold R1881	-10.0	66.3	3886.541	3280.1	1062.8	66.3	7.962448
40	17	2	cold R1881	-10.0	66.9	3917.244	3310.8	1032.1	66.9	8.025349
41	18	3	cold R1881	-10.0	34.8	2329.288	1722.8	2620.0	34.8	4.772066
42	19	1	cold R1881	-11.0	73.6	4247.769	3641.3	701.5	73.6	8.702503
43	20	2	cold R1881	-11.0	73.8	4256.619	3650.2	692.7	73.8	8.720636
44	21	3	cold R1881	-11.0	73.6	4249.734	3643.3	699.6	73.6	8.70653

Column O, Rows 10 through 13 will contain output parameters

total volume in tubes **3.1E+00** uL

from the nonlinear regression software.

and the maximum concentration for the weak positive

Competitive Assay Tube Layout - One Test Chemical (Weak Positive)

Check the 10% rule: 11.38% If the ratio of EtOH / Hot is > 10% then there are problems with the assay

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
23	*Position*	*Replicate*	*Competitor*	*Competitor Code*	*Concentration Code*	*Labels on vials in set 1-1-A*	*Competitor Initial Concentration (M)*	*Cytosol (uL)*	*Tracer (Hot R1881) Volume (uL)*	*Competitor Volume (uL)*	*triamcelenone Volume (uL)*	*Final Volume (ul)*	*Competitor Final Concentration (M)*	*Aliquot (uL)*	*DPM as sampled*	*corrected DPM for 2.0 mL*	*Use this value?*	*Notes to explain why "Use this value" is set to "FALSE"*
45	22	1	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	371.3490	1151.1819	TRUE	
46	23	2	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	318.2727	986.64537	TRUE	
47	24	3	Weak Positive	P	1	**C-1-P1**	3.00E-02	300	30	10	50	310	9.7E-04	100	387.7928	1202.15768	TRUE	
48	25	1	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	959.277	2973.75901	TRUE	
49	26	2	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	1034.114	3205.7534	TRUE	
50	27	3	Weak Positive	P	2	**C-1-P2**	3.00E-03	300	30	10	50	310	9.7E-05	100	894.346	2772.47105	TRUE	
51	28	1	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	1316.583	4081.4073	TRUE	
52	29	2	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	1413.801	4382.7831	TRUE	
53	30	3	Weak Positive	P	3	**C-1-P3**	3.00E-04	300	30	10	50	310	9.7E-06	100	1370.882	4249.7342	TRUE	
54	31	1	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	1236.238	3832.3378	TRUE	
55	32	2	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	1509.416	4679.1896	TRUE	
56	33	3	Weak Positive	P	4	**C-1-P4**	3.00E-05	300	30	10	50	310	9.7E-07	100	1498.900	4646.59	TRUE	
57	34	1	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	1380.299	4278.9269	TRUE	
58	35	2	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	1369.778	4246.3118	TRUE	
59	36	3	Weak Positive	P	5	**C-1-P5**	3.00E-06	300	30	10	50	310	9.7E-08	100	1168.061	3620.9891	TRUE	
60	37	1	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	1379.102	4275.2162	TRUE	
61	38	2	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	1356.847	4206.2257	TRUE	
62	39	3	Weak Positive	P	6	**C-1-P6**	3.00E-07	300	30	10	50	310	9.7E-09	100	1356.847	4206.2257	TRUE	
63	40	1	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	1544.460	4787.826	TRUE	
64	41	2	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	1323.353	4102.3943	TRUE	
65	42	3	Weak Positive	P	7	**C-1-P7**	3.00E-08	300	30	10	50	310	9.7E-10	100	1521.009	4715.1279	TRUE	
66	43	1	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	1394.693	4323.5483	TRUE	
67	44	2	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	1313.225	4070.9975	TRUE	
68	45	3	Weak Positive	P	8	**C-1-P8**	3.00E-09	300	30	10	50	310	9.7E-11	100	1505.651	4667.5181	TRUE	
69	46	1	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	1885.107	5843.8317	TRUE	
70	47	2	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	2085.237	6464.2347	TRUE	
71	48	3	ethanol	EtOH	0	**—**	—	300	30	10	50	310	—	100	2163.952	6708.2512	TRUE	
72	49	1	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	177.5627	532.6881	TRUE	
73	50	2	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	205.9299	617.7897	TRUE	
74	51	3	Inert R1881	NSB		**C-1-S0**	1.00E-05	300	30	30	50	300	1.0E-06	100	187.6144	562.8432	TRUE	
75	52	1	none	Hot		**—**	—	—	30	—	—	—	—		47555.39	47555.39	TRUE	
76	53	2	none	Hot		**—**	—	—	30	—	—	—	—		49187.17	49187.17	TRUE	
77	54	3	none	Hot		**—**	—	—	30	—	—	—	—		49835.21	49835.21	TRUE	
78	55	1	none	Hot		**—**	—	—	30	—	—	—	—		47386.04	47386.04	TRUE	
79	56	2	none	Hot		**—**	—	—	30	—	—	—	—		49099.86	49099.86	TRUE	
80	57	3	none	Hot		**—**	—	—	30	—	—	—	—		49801.63	49801.63	TRUE	

	A	B	S	T	U	V	W	X	Y	AA
19										
20				*Values for analysis by nonlinear regression*						
21				Standard Curve						
22				Weak Positive						
23	*Position*	*Replicate*		*concentration (log)*	*percent bound*	*Usable DPM values*	*Specific Binding (Total - mean NSB)*	*Free DPM (mean total add - total bound)*	*Percent Binding (specific bound / mean specific EtOH)*	*Ratio Total binding/ Hot*
45	22	1	Weak Positive	-3.0	11.0	1151.182	544.7	3798.1	11.0	2.358453
46	23	2	Weak Positive	-3.0	7.7	986.6454	380.2	3962.7	7.7	2.021363
47	24	3	Weak Positive	-3.0	12.0	1202.158	595.7	3747.1	12.0	2.462889
48	25	1	Weak Positive	-4.0	47.8	2973.759	2367.3	1975.5	47.8	6.09241
49	26	2	Weak Positive	-4.0	52.5	3205.753	2599.3	1743.5	52.5	6.567702
50	27	3	Weak Positive	-4.0	43.8	2772.471	2166.0	2176.8	43.8	5.680026
51	28	1	Weak Positive	-5.0	70.2	4081.407	3474.9	867.9	70.2	8.361675
52	29	2	Weak Positive	-5.0	76.3	4382.783	3776.3	566.5	76.3	8.97911
53	30	3	Weak Positive	-5.0	73.6	4249.734	3643.3	699.6	73.6	8.70653
54	31	1	Weak Positive	-6.0	65.2	3832.338	3225.9	1117.0	65.2	7.8514
55	32	2	Weak Positive	-6.0	82.3	4679.19	4072.7	270.1	82.3	9.586365
56	33	3	Weak Positive	-6.0	81.6	4646.59	4040.1	302.7	81.6	9.519578
57	34	1	Weak Positive	-7.0	74.2	4278.927	3672.5	670.4	74.2	8.766338
58	35	2	Weak Positive	-7.0	73.5	4246.312	3639.9	703.0	73.5	8.699518
59	36	3	Weak Positive	-7.0	60.9	3620.989	3014.5	1328.3	60.9	7.418405
60	37	1	Weak Positive	-8.0	74.1	4275.216	3668.8	674.1	74.1	8.758736
61	38	2	Weak Positive	-8.0	72.7	4206.226	3599.8	743.1	72.7	8.617393
62	39	3	Weak Positive	-8.0	72.7	4206.226	3599.8	743.1	72.7	8.617393
63	40	1	Weak Positive	-9.0	84.5	4787.826	4181.4	161.5	84.5	9.808931
64	41	2	Weak Positive	-9.0	70.6	4102.394	3495.9	846.9	70.6	8.404671
65	42	3	Weak Positive	-9.0	83.0	4715.128	4108.7	234.2	83.0	9.659993
66	43	1	Weak Positive	-10.0	75.1	4323.548	3717.1	625.8	75.1	8.857755
67	44	2	Weak Positive	-10.0	70.0	4070.998	3464.5	878.3	70.0	8.340348
68	45	3	Weak Positive	-10.0	82.1	4667.518	4061.1	281.8	82.1	9.562454
69	46	1		—	105.8	5843.832	5237.4	-894.5	105.8	11.97239
70	47	2		—	118.4	6464.235	5857.8	-1514.9	118.4	13.24343
71	48	3		—	123.3	6708.251	6101.8	-1759.0	123.3	13.74335
72	49	1		-6.0	-1.5	532.6881	-73.8	4416.6	-1.5	1.091331
73	50	2		-6.0	0.2	617.7897	11.3	4331.5	0.2	1.26568
74	51	3		-6.0	-0.9	562.8432	-43.6	4386.5	-0.9	1.15311
75	52	1				47555.39	46948.9			
76	53	2				49187.17	48580.7			
77	54	3				49835.21	49228.8			
78	55	1				47386.04	46779.6			
79	56	2				49099.86	48493.4			
80	57	3				49801.63	49195.2			

	A	B	AC	AD	AE	AF	AG	AH	AI
19									
20									
21			Prism data						
22			*standard curve*				*weak positive*		
23	*Position*	*Replicate*	*concentration (log)*	Y1-SC	Y2-SC	Y3-SC	*concentration (log)*	y1-PC	y2-PC
33	10	1	Standard Curve		C6-9/1/05		Positive Control		C6-9/1/05
34	11	2	One site competition				One site competition		
35	12	3	Best-fit values				Best-fit values		
36	13	1	BOTTOM		0		BOTTOM		0
37	14	2	TOP		100		TOP		100
38	15	3	LOGEC50		-9.158		LOGEC50		-4.156
39	16	1	EC50		6.95E-10		EC50		6.98E-05
40	17	2	Std. Error				Std. Error		
41	18	3	LOGEC50		0.1654		LOGEC50		0.1988
42	19	1	95% Confidence Intervals				95% Confidence Intervals		
43	20	2	LOGEC50		-9.503 to -8.813		LOGEC50		-4.567 to -3
44	21	3	*EC50*		3.141e-010 to 1.538e-0(		EC50		2.709e-005
45	22	1	Goodness of Fit				Goodness of Fit		
46	23	2	Degrees of Freedor		20		Degrees of Freedor		23
47	24	3	R^2		0.6456		R^2		0.1146
48	25	1	Absolute Sum of Squa		6484		Absolute Sum of Sc		10773
49	26	2	Sy.x		18.01		Sy.x		21.64
50	27	3	Constraints				Constraints		
51	28	1	BOTTOM		BOTTOM = 0.0		BOTTOM		BOTTOM =
52	29	2	TOP		TOP = 100.0		TOP		TOP = 100.
53	30	3	Data				Data		
54	31	1	Number of X values		7		Number of X values		8
55	32	2	Number of Y replica		3		Number of Y replica		3
56	33	3	Total number of val		21		Total number of val		24
57	34	1	Number of missing		0		Number of missing		0

Appendix 7: Copies of Biological Methods

COLORIMETRIC QUANTIFICATION OF TOTAL PROTEIN USING BIORAD® PROTEIN ASSAY KIT II IN MICROPLATE FORMAT

Written By: _____ Date: 09Feb04
Becky Challmes, B.Sc.
Principal Research Scientist, Technology Development

Approved By: _____ Date: 10FEB2004
Neil S. Jensen, Ph.D.
Director, Technology Development

Approved By: _____ Date: 10 feb 2004
Paul Silber, Ph.D.
President & CEO

I. Introduction

This document describes the procedure for colorimetric quantification of total protein using the BioRad® Protein Assay Kit.

II. Safety Considerations

A. General laboratory safety procedures apply.
B. BSA Protein Standard contains sodium azide. DO NOT DISPOSE IN THE SANITARY SEWER. Sodium azide is a health hazard.
C. Dye reagent must be adjusted to neutral pH before disposal in sanitary sewer. Preferably, dispose of dye reagent in chemical waste.
D. Refer to MSDS for further information concerning safety and handling of reagents.

III. Materials and Equipment

A. Wallac Victor^2 Plate Reader with 600 nm filter.
B. Clear 96-well microtiter plate
C. Vortex
D. -20°C freezer
E. 4°C refrigerator
F. Whatman 1 filter paper
G. Funnel
H. Clean bottle
I. Assorted pipets and tips
J. Microplate shaker
K. Plastic tubes or vials to dilute protein standard curve and samples.

IV. Reagents and Chemicals

A. BioRad® Protein Assay Kit II (dye reagent concentrate and BSA stock) (Catalog # 500-0002)

B. OR
1) BioRad Dye Concentrate (Catalog #500-0006)
2) And Protein Assay Standard II (BSA Stock) (Catalog #500-0007)

C. Deionized water

V. Preparation of Reagents and Solutions

A. 1X BioRad Dye Reagent:
1) 40 mL Deionized water
2) 10 mL Dye Reagent Concentrate
3) Gravity or vacuum filter through a Whatman 1 filter into a clean bottle to remove particulates.
4) The diluted dye reagent is stable for 14 days at 4°C.

B. BSA Protein Standard Stock Solution
1) Determine the total amount of BSA protein in the stock bottle.
(a) Multiply the final mg/mL concentration given on the bottle by the total volume of deionized water stated in the directions on the bottle.
(b) Divide the total mg by 1.4 mg/mL to obtain the volume of deionized water needed.
2) Add the volume of deionized water determined in steps B1 to 1 bottle of lyophilized protein standard to achieve 1.4 mg/mL stock.
3) Aliquot this into labeled vials at 0.4 mL per aliquot.
4) Store at -20°C for up to 1 year.

C. BSA Standard Curve
1) Dilute the BSA standard into the same buffer or vehicle as the protein samples.

Standard #	Concentration	µL of BSA Protein Standard Stock	µL of buffer or vehicle
6	1.0 mg/mL	100	40
5	0.9 mg/mL	90	65
4	0.7 mg/mL	70	70
3	0.5 mg/mL	50	90
2	0.3 mg/mL	30	110
1	0.1 mg/mL	10	130

VI. Protein Assay Procedure

A. Alkaline (basic) pH conditions and detergents other than 0.1% SDS and 0.1% Triton X-100 interfere with this assay. For a complete list of reagents that are compatible with this assay see table 1 on page 2 of the *Bio-Rad Protein Assay Instruction Manual.*

B. Add 10 µL of protein standard in triplicate to separate wells of a 96-well plate.

C. Add 10 µL of each protein sample in triplicate to separate wells of the 96-well plate.

D. Add 200 µL of diluted dye reagent to each well.

E. Mix on a microplate shaker for 30 ±5 seconds at 50-60 rpm.

F. Incubate at room temperature for 5 to 60 minutes.

G. Measure absorbance (optical density, O.D.) with the Wallac Victor^2 Plate Reader at 600 nm.

H. Only if all sample plates are run within 55 minutes one standard curve can be used for all sample plates.

VII. Data Processing

A. The standard curve will be plotted in respect to the theoretical concentration in a computer program that can plot and determine a trendline for power regression, e.g. Excel.

1. In Excel, plot the corrected average of the calibration samples on the y-axis with respect to its theoretical concentrations on the x-axis in a scattered plot format.
2. Determine the standard curve equation and coefficient of determination (r^2) by selecting power regression type in the 'Add Trendline' function from the 'Chart' drop menu, and checking the box for 'Displace equation of chart' and 'Display R-squared value on chart' under the 'Options' tab.

B. The trendline will be determined by power regression which will yield the equation $y=bx^a$.

C. The curve will be accepted if the coefficient of determination (r^2) is greater than 0.9599.

1) The concentration of the experimental samples will be determined by solving the trendline equation with the y-value as the calculated absorbance for the experimental sample in the equation $x=(y/b)^{1/a}$.

2) Multiply the calculated sample concentration by applicable dilution factors to obtain the protein concentration of the undiluted protein suspension.

VIII. Reference:

A. http://www.biorad.com/LifeScience/pdf/Bulletin_9004.pdf
(*Instruction Manual, BioRad Protein Assay*, a copy is kept by the Technology Development Department)

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ANDROGEN RECEPTOR COMPETITIVE BINDING ASSAY USING [^{3}H]-R1881

Written By: _____ Date: 06 JUN 05
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Aruna Koganti, Ph. D.
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Approved By: _____ Date: 06 JUN 2005
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President & CEO

OFFICIAL QA ISSUE

I. Introduction

A. This document describes a radioligand binding assay for the evaluation of the ability of test compounds to inhibit binding of [^{3}H]-R1881 to the Androgen receptor.

II. Safety Considerations

A. General laboratory safety and radiation safety procedures apply.

III. Materials and Equipment

A. pH meter
B. Timer
C. 20 ml scintillation vials
D. assorted **amber** vials
E. 12 × 75 mm siliconized borosilicate glass test tubes
F. 4°C refrigerator
G. Tabletop centrifuge
H. LS6500 T/A Liquid Scintillation Counter
I. Assorted pipettes and tips
J. Shaker
K. Stir/hot plate
L. Assorted graduated cylinders
M. Parafilm®
N. Vacuum concentrator

IV. Reagents and Chemicals (ACS reagent grade)

A. dithiothreitol (DTT), (FW 154.3)
B. hydrochloric acid (HCl) (FW 36.46), 1 N
C. sodium hydroxide (NaOH) (FW 40.0), 1 N
D. absolute ethanol (FW 46.07)
E. [^{3}H]-R1881 (PerkinElmer, purity >97%)
F. deionized water (dH_2O)
G. ethylenediaminetetraacetic acid disodium salt (EDTA) (FW 372.2)
H. glycerol (FW 92.09, 99%+)
I. hydroxyapatite, hydrated (HAP) (BioRad catalog # 130-0151)
J. phenylmethylsulfonyl fluoride (PMSF) (FW 174.2)
K. R1881 (non-radioactive methyltrienolone) (PerkinElmer, FW 284.4, 5 mg/bottle)
L. Formula 989 scintillation cocktail
M. 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris Base) (FW 121.1)
N. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (FW 157.6)
O. Sodium molybdate dehydrate (FW 241.95, Sigma S-6646)
P. Triamcinolone acetonide (FW 434.5, >99%)
Q. Dexamethasone (FW 392.5)

V. Preparation of Reagents and Solutions

A. 200 mM EDTA Stock Solution
1. Add 7.444 g EDTA to 50 ml dH_2O.
2. Bring pH to 7.4 with 1N NaOH.
3. When EDTA has dissolved, Q.S. to 95 ml with dH_2O.
4. Adjust pH to 7.4 with 1N NaOH.
5. Q.S. to 100 ml.
6. Sterile filter the solution.
7. Store up to 180 days at 4°C.

B. 100 mM PMSF Stock Solution
1. Dissolve 1.742 g of PMSF in 100 ml of ethanol.
2. Store up to 180 days at 4°C.

C. 1 M Sodium Molybdate Stock Solution
1. Dissolve 2.419 g sodium molybdate to 8 mL of dH_2O
2. Q.S. to 10 mL with dH_2O.
3. Sterile filter the solution.
4. Store up to 180 days at 4°C.

D. 1 M Tris Stock Buffer
1. Dissolve 147.24 g of Tris-HCl and 8 g of Tris base to 800 ml of cold (~4°C) dH_2O.
2. Adjust pH to 7.4 ± 0.1 at 4 (±3) °C using 1N HCl or 1N NaOH
3. Q.S. to 1 L.
4. Sterile filter the buffer.
5. Store up to 180 days at 4°C.

E. Low Salt TEDG + PMSF Buffer
1. 871.5 ml dH_2O.
2. 10 ml 1M Tris Stock Buffer
3. 1 mL sodium molybdate stock solution
4. 100 ml glycerol
5. 7.5 ml 200 mM EDTA Stock Solution
6. Store at 4°C until addition of DTT and PMSF.
7. Dissolve 154 mg DTT in the buffer immediately before use.
8. 10 ml 100 mM PMSF Stock Solution
9. Check pH and adjust to 7.4 ± 0.1 (at 4 ± 3°C).
10. Prepare fresh daily.

F. 50 mM Tris Buffer
1. 950 mL of dH_2O
2. 50 mL of 1 M Tris Stock Buffer
3. Check pH and adjust to 7.4 ± 0.1 at 4 ±3 °C.
4. Sterile filter the buffer.
5. Store up to 30 days at 4°C.

G. HAP Slurry
1. Begin preparation on the day the assay incubation starts.
2. 100 mL HAP
   (a) HAP comes hydrated at 2.5 ml/g (0.4 g/ml)
   (b) Mix by gently rolling the bottle on the countertop, shaking will destroy the HAP crystals.
   (c) Pour 100 ml into a graduated cylinder.
3. Allow it to settle for at least 2 hours at 4°C.
4. Aspirate the supernatant and discard.
5. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
6. Allow the HAP to settle for at least 2 hours at 4°C.
7. Aspirate the supernatant and discard.
8. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
9. Allow the HAP to settle overnight at 4°C.
10. Aspirate the supernatant and discard.
11. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
12. Allow the HAP to settle for at least 2 hours at 4°C.
13. Measure the volume of HAP remaining in the graduated cylinder.
14. Divide that volume by 0.6; this will give you the total volume of HAP slurry to be made.
15. Q.S. to the total volume calculated in step V.G.14
16. Store at 4°C until ready for use in the extraction.

H. Labelling of assay tubes
1. Each tube will be labelled with a unique identifying code consisting of a position code, a "tube type code", a replicate code and a concentration code.
2. The position code represents the number in the counting sequence of the final sample in the scintillation counter.
3. The replicate code identifies the tube as being the first, second or third tube of each triplicate.
4. The concentration code refers to the number of different concentrations of the competitor that are used.
5. The tube type code identifies the type of sample:
   (a) EtOH = tubes containing cytosol, buffer, triamcinolone, EtOH (vehicle) and [^{3}H]-R1881
   (b) NSB = tubes containing cytosol, buffer, triamcinolone, R1881 at a final concentration of 1 µM and [^{3}H]-R1881.
   (c) S = tubes containing cytosol, buffer, triamcinolone, R1881 at 5 different concentrations, and [^{3}H]-R1881. Each concentration is assigned a number (S1, S2, S3, S4 and S5).
   (d) WP = tubes containing cytosol, buffer, triamcinolone, dexamethasone at 8 different concentrations, and [^{3}H]-

R1881. Each concentration is assigned a number (WP1, WP2, WP3, WP4, WP5, WP6, WP7, AND WP8).

(e) Hot = sample of [^{3}H]-R1881 in scintillation vial containing only the amount of [^{3}H]-R1881 used in the incubation tubes.

6. Label two sets of tubes, one for the incubation (incubation tubes) and one for the harvest (HAP tubes) except for the "Hot" samples in scintillation vials.

VI. Androgen Receptor Competition Binding Assay Start Solutions

A. [^{3}H]-R1881 Stock solutions will be provided by the sponsor

1. 0.1 µM [^{3}H]-R1881 Stock Solution (**amber** vial)
   (a) Dispense 1 µL per specific activity unit into a 20 mL **amber** vial and bringing the volume to 10 mL with absolute ethanol.
   (b) **Example:** if the specific activity is 86 Ci/mmol, dispense 86 µL of the original bottle into a 20 mL **amber** vial and add 9.914 mL of absolute ethanol.
   (c) Store at -20°C.

2. 10 nM [^{3}H]-R1881 Stock Solution (**amber** vial)
   (a) 1 mL of 0.1 µM [^{3}H]-R1881 Stock Solution
   (b) 9 mL absolute ethanol
   (c) Store at -20°C in an **amber** vial.

B. 60 µM triamcinolone acetonide

1. First prepare 6 mM triamcinolone acetonide
   (a) 2.61 mg
   (b) dissolved in 1 mL absolute ethanol
2. 60 µM triamcinolone acetonide
   (a) 0.1 mL of 6 mM triamcinolone acetonide
   (b) 9.9 mL absolute ethanol

C. 1X Cytosol

1. Thaw on ice while the assay tube contents are in the vacuum concentrator.
2. Dilute to 3.3 mg of protein per mL in low salt TEDG + PMSF buffer.
   (a) Keep on ice until addition to incubation tubes.

D. 30X stock drug solutions provided by the sponsor

1. R1881 concentrations
   (a) 10 µM (NSB)
   (b) 3 µM
   (c) 300 nM
   (d) 30 nM

(e) 3 nM
(f) 0.3 nM

2. weak positive (dexamethasone)
(a) 30 mM
(b) 3 mM
(c) 300 µM
(d) 30 µM
(e) 3 µM
(f) 300 nM
(g) 30 nM
(h) 3 nM

VII. Androgen receptor binding assay incubation

A. Place labelled incubation tubes on ice.
B. Add 50 µL of 60 µM triamcinolone to each assay tube.
C. Add 30 µL of 10 µM R1881 to each tube labeled "NSB".
D. Add 30 µL of 10 nM [^{3}H]R1881 to each tube, including the scintillation vials labeled "Hot".
E. Add 14 mL of Formula 989 to "hot" tubes (#52-57) and cap the tubes.
F. Place the incubation tubes (#1-51) in the vacuum concentrator and dry according to the instrument instructions.
G. When the tube contents are dry, transfer the tubes to a rack on ice.
H. Add the following components to each incubation tube:

Tube #	Tube type	Replicate	Competitor	Competitor Initial concentration	Volume of initial concentration
1	EtOH	1	Ethanol	100%	10 µL
2	EtOH	2	Ethanol	100%	10 µL
3	EtOH	3	Ethanol	100%	10 µL
4	NSB	1	R1881	10 µM	***N/A***
5	NSB	2	R1881	10 µM	***N/A***
6	NSB	3	R1881	10 µM	***N/A***
7	S1	1	R1881	3 µM	10 µL
8	S1	2	R1881	3 µM	10 µL
9	S1	3	R1881	3 µM	10 µL
10	S2	1	R1881	300 nM	10 µL
11	S2	2	R1881	300 nM	10 µL
12	S2	3	R1881	300 nM	10 µL
13	S3	1	R1881	30 nM	10 µL
14	S3	2	R1881	30 nM	10 µL
15	S3	3	R1881	30 nM	10 µL
16	S4	1	R1881	3 nM	10 µL

Tube #	Tube type	Replicate	Competitor	Competitor Initial concentration	Volume of initial concentration
17	S4	2	R1881	3 nM	10 µL
18	S4	3	R1881	3 nM	10 µL
19	S5	1	R1881	0.3 nM	10 µL
20	S5	2	R1881	0.3 nM	10 µL
21	S5	3	R1881	0.3 nM	10 µL
22	WP1	1	Weak positive	30 mM	10 µL
23	WP1	2	Weak positive	30 mM	10 µL
24	WP1	3	Weak positive	30 mM	10 µL
25	WP2	1	Weak positive	3 mM	10 µL
26	WP2	2	Weak positive	3 mM	10 µL
27	WP2	3	Weak positive	3 mM	10 µL
28	WP3	1	Weak positive	300 µM	10 µL
29	WP3	2	Weak positive	300 µM	10 µL
30	WP3	3	Weak positive	300 µM	10 µL
31	WP4	1	Weak positive	30 µM	10 µL
32	WP4	2	Weak positive	30 µM	10 µL
33	WP4	3	Weak positive	30 µM	10 µL
34	WP5	1	Weak positive	3 µM	10 µL
35	WP5	2	Weak positive	3 µM	10 µL
36	WP5	3	Weak positive	3 µM	10 µL
37	WP6	1	Weak positive	300 nM	10 µL
38	WP6	2	Weak positive	300 nM	10 µL
39	WP6	3	Weak positive	300 nM	10 µL
40	WP7	1	Weak positive	30 nM	10 µL
41	WP7	2	Weak positive	30 nM	10 µL
42	WP7	3	Weak positive	30 nM	10 µL
43	WP8	1	Weak positive	3 nM	10 µL
44	WP8	2	Weak positive	3 nM	10 µL
45	WP8	3	Weak positive	3 nM	10 µL
46	EtOH	1	ethanol	100%	10 µL
47	EtOH	2	ethanol	100%	10 µL
48	EtOH	3	ethanol	100%	10 µL
49	NSB	1	R1881	10 µM	***N/A***
50	NSB	2	R1881	10 µM	***N/A***
51	NSB	3	R1881	10 µM	***N/A***

I. Add 300 µL of 1X Cytosol to all incubation tubes (#1-51).

J. Incubate for 20 ± 1 hour in the refrigerator on an orbital shaker at approximately 50 rpm.

VIII. Completion of the androgen receptor binding assay

A. Separation of [^{3}H]-R1881 bound to receptor from free (unbound) [^{3}H]-R1881
   1. Add 500 µL of HAP to empty, labeled tubes in an ice-water bath in centrifuge rotor buckets (one HAP tube for each incubation tube). NOTE: stir the HAP continuously while adding it to the tubes.
   2. Remove the incubation tubes from the refrigerator and place in an ice-water bath.
   3. Transfer 100 µL from each incubation tube to its corresponding HAP tube.
   4. Transfer the HAP tubes to centrifuge rotor buckets on ice.
   5. Vortex the tubes (entire rotor bucket) for 15 ± 5 seconds in 5 minutes intervals for a total of 20 minutes with the tubes remaining on ice between vortexing. Tubes should be vortexed a total of 5 times. (NOTE: cover the tubes with Parafilm when performing any vortexing step to prevent splashing of radioactive material.)
   6. Add 2 ml of 50 mM Tris buffer to each tube.
   7. Quickly vortex the tubes.
   8. Centrifuge the tubes for 2-3 minutes at 600 × *g* at 4°C.
   9. Decant or carefully aspirate the supernatant from the tubes.
   10. Add 2 ml of 50 mM Tris buffer to each tube.
   11. Quickly vortex the tubes.
   12. Centrifuge the tubes for 2-3 minutes at 600 × *g* at 4°C.
   13. Decant or carefully aspirate the supernatant from the tubes.
   14. Repeat steps 7 through 10 two more times.

B. Extraction and quantifying [^{3}H]-R1881 bound to receptor
   1. Add 2 ml of absolute ethanol to each HAP tube.
   2. Allow the tubes to sit at room temparature, vortexing at 5 minute intervals, for 10 minutes. Tubes should be vortexed a total of 3 times.
   3. Centrifuge for 10 minutes at 600 × *g* at 4°C.
   4. Carefully decant the entire supernatant into a scintillation vial.
   5. Add 14 ml Formula 989 to each scintillation vial.
   6. Measure the radioactivity from each sample using a liquid scintillation counter.

IX. Data calculations

A. Enter the total radioactivity of each sample into the data calculation spreadsheet prepared by the sponsor for this study.
B. Complete all other fields in the data calculation spreadsheet.
C. Transfer data from spreadsheet into GraphPad Prism tables:
   1. The X-axis is the final log concentration of the non-radioactive compound.
   2. The Y-axis is the DPM.

3. Perform non-linear regression, sigmoidal dose-response analysis on the data.
4. Determine the K_I using the Cheng-Prusoff equation
   (a)
$$K_I = \frac{IC_{50}}{1 + \frac{[\text{radioligand}]}{K_D}}$$

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ANDROGEN RECEPTOR SATURATION BINDING ASSAY USING [^{3}H]-R1881

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I. Introduction

A. This document describes a radioligand binding assay for the evaluation of the ability of test compounds to inhibit binding of [^{3}H]-R1881 to the androgen receptor.

II. Safety Considerations

A. General laboratory safety and radiation safety procedures apply.

III. Materials and Equipment

A. pH meter
B. Timer
C. 20 ml scintillation vials
D. assorted **amber** vials
E. 12 × 75 mm siliconized borosilicate glass test tubes with caps
F. 4°C refrigerator
G. Refrigerated tabletop centrifuge
H. LS6500 T/A Liquid Scintillation Counter
I. Assorted pipettes and tips
J. Shaker
K. Stir/hot plate
L. Assorted graduated cylinders
M. Parafilm®
N. Vacuum concentrator

IV. Reagents and Chemicals (ACS reagent grade)

A. dithiothreitol (DTT), (FW 154.3)
B. hydrochloric acid (HCl) (FW 36.46), 1 N
C. sodium hydroxide (NaOH) (FW 40.0), 1 N
D. absolute ethanol (FW 46.07)
E. [^{3}H]-R1881 (PerkinElmer, purity >97%)
F. deionized water (dH_2O)
G. ethylenediaminetetraacetic acid disodium salt (EDTA) (FW 372.2)
H. glycerol (FW 92.09, 99%+)
I. hydroxyapatite, hydrated (HAP) (BioRad catalog # 130-0151)
J. phenylmethylsulfonyl fluoride (PMSF) (FW 174.2)
K. R1881 (non-radioactive methyltrienolone) (PerkinElmer, FW 284.4, 5 mg/bottle)
L. Formula 989 scintillation cocktail
M. 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris Base) (FW 121.1)
N. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (FW 157.6)
O. Sodium molybdate dihydrate (FW 241.95, Sigma S-6646)
P. Triamcinolone acetonide (FW 434.5, >99%)

V. Preparation of Reagents and Solutions

A. 200 mM EDTA Stock Solution
1. Add 7.444 g EDTA to 50 ml dH_2O.
2. Bring pH to 7.4 with 1N NaOH.
3. When EDTA has dissolved, Q.S. to 95 ml with dH_2O.
4. Adjust pH to 7.4 with 1N NaOH.
5. Q.S. to 100 ml.
6. Sterile filter the solution.
7. Store up to 180 days at 4°C.

B. 100 mM PMSF Stock Solution
1. Dissolve 1.742 g of PMSF in 100 ml of ethanol.
2. Store up to 180 days at 4°C.

C. 1 M Sodium Molybdate Stock Solution
1. Dissolve 2.419 g sodium molybdate to 8 mL of dH_2O
2. Q.S. to 10 mL with dH_2O.
3. Sterile filter the solution.
4. Store up to 180 days at 4°C.

D. 1 M Tris Stock Buffer
1. Dissolve 147.24 g of Tris-HCl and 8 g of Tris base to 800 ml of cold (~4°C) dH_2O.
2. Adjust pH to 7.4 ± 0.1 at 4 (±3) °C using 1N HCl or 1N NaOH
3. Q.S. to 1 L.
4. Sterile filter the buffer.
5. Store up to 180 days at 4°C.

E. Low Salt TEDG + PMSF Buffer
1. 871.5 ml dH_2O.
2. 10 ml 1M Tris Stock Buffer
3. 1 mL sodium molybdate stock solution
4. 100 ml glycerol
5. 7.5 ml 200 mM EDTA Stock Solution
6. Store at 4°C until addition of DTT and PMSF.
7. Dissolve 154 mg DTT in the buffer immediately before use.
8. 10 ml 100 mM PMSF Stock Solution
9. Check pH and adjust to 7.4 ± 0.1 (at 4 ± 3°C).
10. Prepare fresh daily.

F. 50 mM Tris Buffer
1. 950 mL of dH_2O
2. 50 mL of 1 M Tris Stock Buffer
3. Check pH and adjust to 7.4 ±0.1 at 4 ±3 °C.
4. Sterile filter the buffer.
5. Store up to 30 days at 4°C.

G. HAP Slurry
1. Begin preparation on the day the assay incubation starts.
2. 100 mL HAP

(a) HAP comes hydrated at 2.5 ml/g (0.4 g/ml)
(b) Mix by gently rolling the bottle on the countertop, shaking will destroy the HAP crystals.
(c) Pour 100 ml into a graduated cylinder.

3. Allow it to settle for at least 2 hours at 4°C.
4. Aspirate the supernatant and discard.
5. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
6. Allow the HAP to settle for at least 2 hours at 4°C.
7. Aspirate the supernatant and discard.
8. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
9. Allow the HAP to settle overnight at 4°C.
10. Aspirate the supernatant and discard.
11. Q.S. to 100 ml of 50 mM Tris buffer, cover with Parafilm® and invert several times to resuspend the HAP.
12. Allow the HAP to settle for at least 2 hours at 4°C.
13. Measure the volume of HAP remaining in the graduated cylinder.
14. Divide that volume by 0.6; this will give you the total volume of HAP slurry to be made.
15. Q.S. to the total volume calculated in step V.G.14.
16. Store at 4°C until ready for use in the extraction.

H. Labelling of assay tubes

1. Each tube will be labelled with a unique identifying code consisting of a position code, a "tube type code", and a replicate code.
2. The position code represents the number in the counting sequence of the final sample in the scintillation vial.
3. The replicate code identifies the tube as being the first, second or third tube of each triplicate.
4. The tube type code identifies the type of sample:
   (a) Incubation tubes
      (i) H = tubes containing cytosol, buffer, and [^{3}H]-R1881
      (ii) HC = tubes containing cytosol, buffer, R1881 and [^{3}H]-R1881
   (b) Dosing samples
      (i) Hot = sample of [^{3}H]-R1881 in scintillation vial containing only the amount of [^{3}H]-R1881 used in the corresponding H and HC tubes.
   (c) HAP tubes
      (i) H = tubes containing cytosol, buffer, and [^{3}H]-R1881
      (ii) HC = tubes containing cytosol, buffer, R1881 and [^{3}H]-R1881

VI. Androgen Receptor Saturation Binding Assay Start Solutions

A. [^{3}H]-R1881 Stock solutions in **amber** vials [Supplied by sponsor]
   1. 0.1 µM [^{3}H]-R1881 Stock Solution (**amber** vial)
      (a) Dispense 1 µL per specific activity unit into a 20 mL **amber** vial and bring the volume to 10 mL with absolute ethanol.
      (b) **Example:** if the specific activity is 86 Ci/mmol, dispense 86 µL of the original bottle into a 20 mL **amber** vial and add 9.914 mL of absolute ethanol.
      (c) Store at -20°C.

   2. 10 nM [^{3}H]-R1881 Stock Solution (**amber** vial)
      (a) 1 mL of 0.1 µM [^{3}H]-R1881 Stock Solution
      (b) 9 mL absolute ethanol
      (c) Store at -20°C in an **amber** vial.

B. 100X R1881 Stock Solutions (non-radioactive) (**amber** vials) [Supplied by sponsor]
   1. Add 5 mL absolute ethanol to 5 mg bottle to yield 3.52 mM R1881.
   2. 10 µM R1881
      (a) 56.82 µL of 3.52 mM R1881
      (b) 19.943 mL absolute ethanol
      (c) Store at -20°C in an amber vial
   3. 1 µM R1881
      (a) 2 mL of 10 µM R1881
      (b) 18 mL of absolute ethanol.
      (c) Store at -20°C in an amber vial.

C. 60 µM triamcinolone acetonide
   1. First prepare 6 mM triamcinolone acetonide
      (a) 2.61 mg
      (b) dissolved in 1 mL absolute ethanol
   2. 60 µM triamcinolone acetonide
      (a) 0.1 mL of 6 mM triamcinolone acetonide
      (b) 9.9 mL absolute ethanol

D. 1X Cytosol
   1. Thaw on ice while the assay tube contents are in the vacuum concentrator.
   2. Dilute to 4 mg of protein per mL in low salt TEDG + PMSF buffer.
   3. Keep on ice until addition to assay tubes.

VII. <u>Androgen receptor saturation binding assay incubation</u>

A. Place labelled assay tubes on ice in a rack.
   1. Add components to the tubes according to the table below.

B. Contents of assay tubes

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1.

Tube #	Tube type	Replicate	[^{3}H]R-1881 initial concentration (nM)	[^{3}H]R-1881 volume (µL)	R1881 initial concentration (µM)	R1881 volume (µL)	Triamcinolone (µL)
1	H	1	10	7.5	—	—	50
2	H	2	10	7.5	—	—	50
3	H	3	10	7.5	—	—	50
4	H	1	10	15	—	—	50
5	H	2	10	15	—	—	50
6	H	3	10	15	—	—	50
7	H	1	10	21	—	—	50
8	H	2	10	21	—	—	50
9	H	3	10	21	—	—	50
10	H	1	10	30	—	—	50
11	H	2	10	30	—	—	50
12	H	3	10	30	—	—	50
13	H	1	10	45	—	—	50
14	H	2	10	45	—	—	50
15	H	3	10	45	—	—	50
16	H	1	100	7.5	—	—	50
17	H	2	100	7.5	—	—	50
18	H	3	100	7.5	—	—	50
19	H	1	100	15	—	—	50
20	H	2	100	15	—	—	50
21	H	3	100	15	—	—	50
22	H	1	100	30	—	—	50
23	H	2	100	30	—	—	50
24	H	3	100	30	—	—	50
25	HC	1	10	7.5	1	7.5	50
26	HC	2	10	7.5	1	7.5	50
27	HC	3	10	7.5	1	7.5	50
28	HC	1	10	15	1	15	50
29	HC	2	10	15	1	15	50
30	HC	3	10	15	1	15	50
31	HC	1	10	21	1	21	50
32	HC	2	10	21	1	21	50
33	HC	3	10	21	1	21	50
34	HC	1	10	30	1	30	50
35	HC	2	10	30	1	30	50
36	HC	3	10	30	1	30	50
37	HC	1	10	45	1	45	50
38	HC	2	10	45	1	45	50
39	HC	3	10	45	1	45	50
40	HC	1	100	7.5	10	7.5	50
Tube #	Tube type	Replicate	[^{3}H]R-1881 initial concentration (nM)	[^{3}H]R-1881 volume (µL)	R1881 initial concentration (µM)	R1881 volume (µL)	Triamcinolone (µL)
41	HC	2	100	7.5	10	7.5	50
42	HC	3	100	7.5	10	7.5	50
43	HC	1	100	15	10	15	50

44	HC	2	100	15	10	15	50
45	HC	3	100	15	10	15	50
46	HC	1	100	30	10	30	50
47	HC	2	100	30	10	30	50
48	HC	3	100	30	10	30	50
49	Hot	1	10	7.5	—	—	—
50	Hot	2	10	7.5	—	—	—
51	Hot	3	10	7.5	—	—	—
52	Hot	1	10	15	—	—	—
53	Hot	2	10	15	—	—	—
54	Hot	3	10	15	—	—	—
55	Hot	1	10	21	—	—	—
56	Hot	2	10	21	—	—	—
57	Hot	3	10	21	—	—	—
58	Hot	1	10	30	—	—	—
59	Hot	2	10	30	—	—	—
60	Hot	3	10	30	—	—	—
61	Hot	1	10	45	—	—	—
62	Hot	2	10	45	—	—	—
63	Hot	3	10	45	—	—	—
64	Hot	1	100	7.5	—	—	—
65	Hot	2	100	7.5	—	—	—
66	Hot	3	100	7.5	—	—	—
67	Hot	1	100	15	—	—	—
68	Hot	2	100	15	—	—	—
69	Hot	3	100	15	—	—	—
70	Hot	1	100	30	—	—	—
71	Hot	2	100	30	—	—	—
72	Hot	3	100	30	—	—	—

C. Place the incubation tubes (#1-48) in the vacuum concentrator and dry according to the instrument instructions.

D. When the tube contents are dry, transfer the tubes to centrifuge rotor buckets on ice.

E. Add 300 µL of 1X Cytosol to all tubes except those labeled "Hot" (#49-72)

F. Incubate for 20 ± 1 hour in the refrigerator.

G. Add 14 mL of Formula 989 to "Hot" tubes #49-72.

VIII. Completion of the androgen receptor binding assay

A. Separation of [^{3}H]-R1881 bound to receptor from free (unbound) [^{3}H]-R1881

1. Add 500 µL of HAP to empty, labeled tubes in an ice-water bath in centrifuge rotor buckets (one HAP tube for each incubation tube--#1-48). NOTE: stir the HAP continuously while adding it to the tubes.

2. Remove the incubation tubes from the refrigerator and place in and ice-water bath.
3. Transfer 100 µL from each incubation tube to its corresponding HAP tube.
4. Vortex the tubes (entire rotor bucket) for 15 ± 5 seconds in 5 minutes intervals for a total of 20 minutes with the tubes remaining on ice between vortexing. Tubes should be vortexed a total of 5 times. (NOTE: cover the tubes with Parafilm when performing any vortexing step to prevent splashing of radioactive material.)
5. Add 2 ml of 50 mM Tris buffer to each tube.
6. Quickly vortex the tubes.
7. Centrifuge the tubes for 2-3 minutes at 600 × *g* at 4°C.
8. Decant or carefully aspirate the supernatant from the tubes.
9. Add 2 ml of 50 mM Tris buffer to each tube.
10. Quickly vortex the tubes.
11. Centrifuge the tubes for 2-3 minutes at 600 × *g* at 4°C.
12. Decant or carefully aspirate the supernatant from the tubes.
13. Repeat steps 7 through 10 two more times.

B. Extraction and quantifying [^{3}H]-R1881 bound to receptor

1. Add 2 ml of absolute ethanol to each HAP tube.
2. Allow the tubes to sit at room temperature, vortexing at 5 minute intervals, for 10 minutes. Tubes should be vortexed a total of 3 times.
3. Centrifuge for 10 minutes at 600 × *g* at 4°C.
4. Carefully decant the entire supernatant into a scintillation vial.
5. Add 14 ml Formula 989 to each scintillation vial.
6. Measure the radioactivity from each sample using a liquid scintillation counter.

IX. Data calculations

A. Enter the total radioactivity of each sample into the data calculation spreadsheet prepared by the sponsor for this study.
B. Complete all other fields in the data calculation spreadsheet.
C. Transfer data from spreadsheet into GraphPad Prism tables:
   1. Saturation isotherm
      (a) Actual concentration of [^{3}H]-R1881 on the X-axis and total binding, specific binding and non-specific binding in DPM or fmol on the Y-axis.
      (b) Perform one-site binding hyperbola regression on total and specific binding, use weighting of $1/Y^2$.
      (c) Perform linear regression on the non-specific binding.
   2. Scatchard analysis
      (a) Bound/Free is plotted on the X-axis
      (b) Bound is plotted on the Y-axis

(c) Perform linear regression. $-1/\text{slope} = K_D$, the X-intercept = B_{max}.

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PREPARATION OF RAT VENTRAL PROSTATE CYTOSOL FOR USE IN ANDROGEN RECEPTOR BINDING ASSAYS

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I. Introduction

A. This document describes the preparation of rat ventral prostate cytosol for use in androgen receptor binding assays.

II. Safety Considerations

A. General laboratory safety procedures apply.

III. Materials and Equipment

A. pH meter
B. 4°C refrigerator
C. Refrigerated centrifuge
D. Assorted graduated cylinders
E. Stir plate
F. Centrifuge tubes/bottles
G. Polytron with 7 mm diameter generator
H. Metzenbaum scissors

IV. Reagents and Chemicals (ACS reagent grade)

A. dithiothreitol (DTT), (FW 154.3)
B. hydrochloric acid (HCl) (FW 36.46), 1 N
C. sodium hydroxide (NaOH) (FW 40.0), 1 N
D. deionized water (dH_2O)
E. ethylenediaminetetraacetic acid disodium salt (EDTA) (FW 372.2)
F. glycerol (FW 92.09, 99%+)
G. phenylmethylsulfonyl fluoride (PMSF) (FW 174.2)
H. absolute ethanol (FW 46.07)
I. 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris Base) (FW 121.1)
J. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (FW 157.6)
K. Sodium molybdate dihydrate (FW 241.95, Sigma S-6646)

V. Preparation of Reagents and Solutions

A. 200 mM EDTA Stock Solution
1. Add 7.444 g EDTA to 50 ml dH_2O.
2. Bring pH to 7.4 with 1N NaOH.
3. When EDTA has dissolved, Q.S. to 95 ml with dH_2O.
4. Adjust pH to 7.4 with 1N NaOH.
5. Q.S. to 100 ml.
1. Sterile filter the solution.
2. Store up to 180 days at 4°C.

B. 100 mM PMSF Stock Solution
1. Dissolve 1.742 g of PMSF in 100 ml of ethanol.

2. Store up to 180 days at 4°C.

C. 1 M Sodium Molybdate Stock Solution
1. Dissolve 2.419 g sodium molybdate to 8 mL of dH_2O
2. Q.S. to 10 mL with dH_2O.
3. Sterile filter the solution.
4. Store up to 180 days at 4°C.

D. 1 M Tris Stock Buffer
1. Dissolve 147.24 g of Tris-HCl and 8 g of Tris base to 800 ml of cold (~4°C) dH_2O.
2. Adjust pH to 7.4 ± 0.1 at 4 (±3) °C using 1N HCl or 1N NaOH
3. Q.S. to 1 L.
4. Sterile filter the buffer.
5. Store up to 180 days at 4°C.

E. Low Salt TEDG + PMSF Buffer
1. 871.5 ml dH_2O.
2. 10 ml 1M Tris Stock Buffer
3. 1 mL sodium molybdate stock solution
4. 100 ml glycerol
5. 7.5 ml 200 mM EDTA Stock Solution
6. Store at 4°C until addition of DTT and PMSF.
7. Dissolve 154 mg DTT in the buffer immediately before use.
8. 10 ml 100 mM PMSF Stock Solution
9. Check pH and adjust to 7.4 ± 0.1 (at 4 ± 3°C).
10. Prepare fresh daily.

VI. Preparation of rat ventral prostate cytosol

A. Harvest of ventral prostate from castrated rats will be performed by the vendor.
1. Obtain male Sprague-Dawley rats from vendor.
   (a) The rats must be 60-90 days of age. (90 days of age is preferred)
   (b) The rats must be castrated 24 hours prior to harvesting the ventral prostates.
2. Sacrifice the rats by carbon dioxide asphyxiation.
3. Remove the ventral prostates and snap-freeze in liquid nitrogen.
4. Store at -70°C.
5. Ship on dry ice.

B. Cytosol preparation
1. Thaw ventral prostates in a beaker containing ice cold Low Salt TEDG + PMSF buffer
2. Weigh the tissue.

3. Mince the ventral prostates with Metzenbaum scissors until all the pieces are 1 to 2 mm cubes.
4. Distribute the minced tissue into an empty, glass beaker on ice. (more than one beaker may be needed)
5. Add Low Salt TEDG + PMSF buffer to the beaker such that there is 10 mL of buffer for every 1 g of tissue. (e.g. 2 g of tissue with 20 mL of buffer.) Fill beaker(s) to no more than 75% of capacity.
6. Cool the Polytron generator in a container of ice cold Low Salt TEDG + PMSF buffer on ice.
7. Homogenize at full speed in 5 second bursts. The beaker containing the tissue should be kept on ice during the homogenization procedure. Cool the homogenizer generator as in step VI.B.6 between bursts. It will take 3 to 5 bursts to homogenize the tissue.
8. Transfer the homogenate to pre-cooled centrifuge tubes/bottles.
9. Centrifuge at 30,000 × *g* at 4°C for 30 minutes.
10. Combine the supernatants from step VI.B.9 into one container on ice and stir gently to mix. (Discard the pellets.)
11. Aliquot the combined supernatant into pre-labelled vials at 5 mL per vial.
12. Store at –70°C to –80°C.
13. The cytosol expires 180 days from date of preparation.
14. Perform a protein assay as per biological method B020.A (Colorimetric Quantification of Total Protein Using BioRad® Protein Assay Kit II in Microplate Format). This can be done on the day of preparation or at a later date.

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Appendix 8: Battelle QAU Statement