FINAL TASK REPORT

CHARACTERIZATION OF THE INHIBITION OF AROMATASE ACTIVITY BY NONYLPHENOL

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Title: **Characterization of the Inhibition of Aromatase Activity by Nonylphenol**

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1.0 Introduction

1.1 Problem Definition

The objective of this task was to determine whether nonylphenol, used in the validation of the aromatase assay, is a competitive inhibitor of aromatase activity.

1.2 Background

Nonylphenol (CAS No. 84852-15-3) was used as one of the reference chemicals for the interlaboratory validation of the estrogen receptor (ER) binding and the placental and recombinant microsomal aromatase assays. Initial studies of nonylphenol during the prevalidation phase of testing gave partial curves in the ER binding assay, demonstrating its activity as an estrogen receptor agonist, but a negative response (a noninhibitor) in the aromatase assay. The EPA was interested when, in subsequent interlaboratory studies, nonylphenol appeared to inhibit aromatase activity. Since the later studies used higher concentrations of nonylphenol than those used during the initial studies, it is not known whether or not nonylphenol is a true competitive inhibitor of aromatase activity or if these data reflect a false positive. No historical data exist to document whether or not nonylphenol is a competitive inhibitor of aromatase activity at these higher concentrations. Therefore, it is necessary to conduct secondary K_i experiments to appropriately classify this chemical as an inhibitor or noninhibitor of aromatase activity.

There are several ways a chemical can inhibit enzymatic activity. For example, a competitive inhibitor binds reversibly to the active site of the enzyme and thereby prevents the binding of the natural substrate. Thus, the binding of the substrate and competitive inhibitor to the active site is mutually exclusive. Noncompetitive inhibitors modify the structure of the enzyme and render it no longer capable of activity. A third type of nonspecific inhibition occurs when the chemical causes physical or chemical changes in the enzyme, resulting in a denaturation of the protein as may occur when high concentrations of a test chemical are used in an *in vitro* assay. When testing environmental chemicals for their ability to inhibit aromatase activity, it is important to understand the type of inhibition, since a nonspecific inhibition would result in a false positive classification for the chemical.

The type of inhibition may be characterized by conducting a series of experiments that evaluate the catalytic activity of the enzyme in the absence or presence of a chemical inhibitor. The experiment is designed to study enzymatic activity at several fixed and nonsaturating concentrations of the test chemical in the presence of varying substrate concentrations. Plotting the data on a double reciprocal plot (1/v versus 1/S; Lineweaver-Burk Plot) provides a pattern of lines that are indicative of competitive or noncompetitive inhibition. The negative x-intercept of a linear replot (secondary plot) of the slopes vs the inhibitor concentration yields an experimental K_i (inhibitor concentration). These relationships can be used to distinguish chemicals which compete for binding with the substrate at the active site of the enzyme from chemicals which interfere with the enzyme through noncompetitive or nonspecific inhibition. Competitive inhibitors cause a change in the apparent K_m but do not affect V_{max}, so the Lineweaver-Burk plot will contain lines

with a common y-intercept ($1/V_{max}$). Noncompetitive inhibitors (those that alter enzyme activity by binding away from the substrate active site) affect V_{max} but do not change K_m and will result in a set of lines with a common x-intercept ($-1/K_m$). Uncompetitive inhibitors (those that bind to the ES complex) change both K_m and V_{max}, so the lines on the Lineweaver-Burk plots have neither common x- or y-intercepts. Therefore, the Lineweaver-Burk plots may be used to distinguish between types of enzyme inhibition.

2.0 Materials and Methods

2.1 Substrate and Test Inhibitors

The substrate nonradiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) were provided by the Chemical Repository (CR). Each inhibitor was supplied neat by the CR. Aminoglutethimide (AG) is a known competitive inhibitor of aromatase and served as the positive control chemical for this task. 4-Nonylphenol (NYP) was the test inhibitor. Lot and source details are presented in Table 1. Supplier's certificates of analysis are presented in Appendix 3.

Table 1. Substrate and Test Inhibitors

Chemical	CAS Number	Molecular Formula (Molecular Weight [g/mol])	Supplier	Lot Number	Stated Purity
ASDN	63-05-8	$C_{19}H_{26}O_2$ (286.4)	Sigma	024K0809	100%
[^{3}H]ASDN	NA	NA	Perkin Elmer	3557306	>97%
AG	125-84-8	$C_{13}H_{16}N_2O_2$ (232.3)	Sigma	06016JS	99%
NYP	84852-15-3	$C_{15}H_{24}O$ (220.4)	Acros Organics	A0192712	>98.5%

2.1.2 Radiochemical Purity

The radiochemical purity of the [^{3}H]ASDN was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consisted of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 µL glass scintillant cell. Data was collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method used a Zorbax SB-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant was monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions were collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN was analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN was confirmed.

2.1.3 Preparation of Substrate Solution

The substrate solution was prepared fresh each day of assay by combining solutions of [^{3}H]ASDN and non-radiolabeled ASDN in order to prepare a solution containing either 0.5 or 2 μM ASDN with ca. 1 μCi/mL. A 1:100 dilution of the radiolabeled [^{3}H] ASDN stock in buffer was prepared fresh each day of assay. A solution (1 mg/mL) of ASDN in ethanol was prepared (fresh each day) and then diluted in buffer to a final concentration of 1 μg/mL. The substrate solution containing 2 μM ASDN was prepared by combining 4.5 mL of the 1 μg/mL solution of ASDN, 800 μL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution. The substrate solution containing 0.5 μM ASDN was prepared by combining 660 μL of the 1 μg/mL solution of ASDN, 500 μL of the [^{3}H]ASDN dilution and 3.84 mL buffer to make 5 mL of substrate solution. The addition of an appropriate amount (usually 50 to 300 μL, depending on the solution concentration and the desired final ASDN concentration) of the substrate solution to each 2 mL assay volume yields the desired final [^{3}H]ASDN concentration of about 0.05 - 0.3 μCi/tube.

2.1.4 Inhibitor Formulation Preparation and Analysis

Preparation of Formulations. Inhibitor stock solutions were prepared and analyzed by RTI. Aminoglutethimide (AG) and 4-nonyphenol (NYP) were formulated (0.1 M) in dimethylsulfoxide (DMSO). The formulations were prepared by weighing 1120.3 and 1097.6 mg, respectively of AG and NYP into 50 mL volumetric flasks and adding DMSO to about 50% of total volume. Each sample was mixed and/or sonicated until the inhibitor was dissolved and the contents of each flask were diluted to volume with DMSO, sealed and mixed well. Inhibitor formulations were stored refrigerated in sealed amber bottles. Under these conditions, stock solutions have been shown to be stable for at least 4 weeks (EPA Contract 68-W-01-023, WA4-17). Formulation data are summarized in Table 2.

Table 2. Inhibitor Formulation Data

Inhibitor	Preparation Date	Formulation ID	Target Conc. of Stock (M)	Molecular Weight (g/mol)	Target conc. (mg/mL)
AG	10/30/06	12507-13A	0.1	232.2	23.2
NYP	10/30/06	12507-13B	0.1	220.4	22.0

Preparation of Calibration Standards and Blanks. For AG, duplicate stock standards were prepared by weighing 20.1 mg samples into two individual 20-mL volumetric flasks, diluting to volume with acetonitrile and mixing until dissolved. This produced stocks AG-1 and AG-2 each at a concentration of 1.0 ± 0.1 mg/mL. Similarly, for NYP, duplicate stock standards were prepared by weighing 19.8 mg samples into two individual 20-mL volumetric flasks, diluting to volume with acetonitrile and mixing until dissolved. This produced stocks NYP-1 and NYP-2 each at a concentration of 1.0 ± 0.1 mg/mL. These stock solutions were used to prepare calibration standards as

described in Table 3 with acetonitrile as solvent. Blanks were prepared by diluting 20 μL DMSO to 10 mL in a volumetric flask with acetonitrile.

Table 3. AG and NYP Calibration Standards Preparation

Calibration Standard ID	Stock Solution ID	Target Conc. (μg/mL)	Volume Stock (mL)	Volume DMSO (μL)	Total Volume (mL)
AG Calibration Standards					
1	AG-1	100	1	20	10
2	AG-2	75	0.75	20	10
3	AG-1	50	0.5	20	10
4	AG-2	25	0.25	20	10
5	AG-1	10	0.1	20	10
NYP Calibration Standards					
1	NYP-1	100	1	20	10
2	NYP-2	75	0.75	20	10
3	NYP-1	50	0.5	20	10
4	NYP-2	25	0.25	20	10
5	NYP-1	10	0.1	20	10

Preparation of Formulation Samples for Analysis. Triplicate dilutions of each inhibitor formulation were prepared by diluting 50 μL of the formulation with acetonitrile to 25 mL in a volumetric flask.

Analysis. All calibration standards, blanks and formulation samples were analyzed by HPLC with UV detection. The HPLC system consisted of a Waters 2690 Separations Module and a Waters 2487 Dual λ Absorbance Detector. Data was collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0. Both analyses were conducted using a Zorbax Rx C18 (5 μm, 4.6 x 250mm) column and a flow rate of 1 mL/min. An isocratic system consisting of 20% acetonitrile in water and a detection wavelength of 224 nm was used for the AG analysis. NYP samples were analyzed using a gradient system with mobile phase A: acetonitrile and mobile phase B: water and a detection wavelength of 254 nm. The mobile phase composition was 70% A for 6 minutes and then changed linearly over 0.2 min to 100% A, the composition was held at 100% A until 13 min when it was returned to the initial conditions linearly over 20 min.

Calculations. Linear regression equations, un-weighted, were calculated relating the peak area to the concentration of the vehicle/calibration standards (x). This regression equation and the peak area were used to calculate the concentration in each vehicle/calibration standard and formulation sample. The percent relative error (% RE) for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. These values were used to calculate the individual and average concentration, % RE, standard deviation (SD), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration. The percent relative error for each

formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100.

Preparation of Inhibitor Dilutions for Use in Aromatase Assay Fresh dilutions of the inhibitor formulations were prepared in DMSO on the day of use such that the target concentration of inhibitor could be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Preparation details for each inhibitor dilution are presented in Table 4.

Table 4. Preparation of Inhibitor Solutions for Aromatase Assay

Inhibitor	Dilution ID	DMSO (µL)	Source Vol. (µL)	Source Used	Solution concentration (M)	Final Concentration in assay (µM)
Pilot						
AG	1	900	100	12507-13A	1.00E-02	100
	2	500	500	1	5.00E-03	50
	3	300	100	1	2.50E-03	25
Replicates 1-4						
	Predilution 1	900	100	12507-13A	1.00E-02	NA
	1	900	300	Predilution 1	2.50E-03	25
	2	400	600	1	1.50E-03	15
	3	400	200	2	5.00E-04	5
Pilot						
NYP	Predilution 1	900	100	12507-13B	1.00E-02	NA
	1	800	200	Predilution 1	2.00E-03	20
	2	400	400	1	1.00E-03	10
	3	200	200	2	5.00E-04	5
Replicates 1-4						
	Predilution 1	900	100	12507-13B	1.00E-02	NA
	1	800	200	Predilution 1	2.00E-03	20
	2[a]	200	600	1	1.50E-03	15
	3[b]	200	200	2	7.50E-04	7.5

[a] For replicate 1 only, predilution 1 was mistakenly used as the source solution to prepare this dilution. This resulted in a final NYP concentration in the assay of 75 µM.

[b] For replicate 1 only, the incorrectly prepared replicate 2 (see footnote a) was used to prepare this dilution. This resulted in a final NYP concentration in the assay of 37.5 µM.

2.2 *Microsomes*

Recombinant microsomes (Human CYP19 + P450 Reductase SUPERSOMES™, catalog number 456260) were purchased from Gentest (Woburn, MA). The microsomes were stored at approximately -70 to -80°C. The approximate protein content of the microsomes, provided by the supplier, was 6.9 mg/mL. The product data sheet for the microsomes is presented in Appendix 3.

Microsomes were thawed, pooled, homogenized, divided into appropriate aliquots for conduct of a single experiment and refrozen (in liquid nitrogen) in order to minimize and standardize the number of freeze/thaw cycles for the preparation

On the day of use, microsomes were thawed quickly in a 37 ± 1 °C water bath and then were immediately transferred to an ice bath. The microsomes were rehomogenized by vortexing about 5 seconds prior to use. The microsomes were diluted in buffer to an approximate protein concentration of 0.008 mg/mL and the dilutions were stored on ice for no more than 1 h before being used in the assay.

2.3 Other Assay Components

In addition to substrate, test inhibitors or vehicle, and microsomes, the aromatase assay contained NADPH, propylene glycol and phosphate buffer. Supplier and lot numbers for other aromatase assay components are presented in Table 5.

Table 5. Supplier and Lot Numbers for Aromatase Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	104K7034
Propylene glycol	J.T.Baker	B37606
Sodium phosphate dibasic	J.T.Baker	A08H50
Sodium phosphate monobasic	J.T.Baker	A12H20
Vehicle (DMSO) for AG and NYP	Battelle CR	2969A46428

2.3.1 NADPH

A solution of NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, Sigma, cat # 1630, 833.4 g/mol, 6 mM) in pH 7.4 sodium phosphate buffer (see Section 2.3.2) was prepared fresh each day of assay and was kept on ice until used.

2.3.2 Assay Buffer

The assay buffer was 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (JT Baker, catalog # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (JT Baker, catalog # 4062-01, 141.96 g/mol) were used in the preparation of the buffer. Solutions of each reagent at 0.1 M were prepared in distilled, deionized water and then the solutions were combined to a final pH of 7.4. The assay buffer was stored for up to one month in the refrigerator (2-8 °C).

2.4 Protein Determination

The protein concentration of the microsome preparation was determined using a DC Protein Assay Kit purchased from BioRad (Hercules, CA). 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 (BSA). QC standards had reported concentrations of 500 and 1000 μg/mL. Unknown and curve standards were run in triplicate and QC standards were run in duplicate. To a 25 μL aliquot of unknown or standard, 125 μL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed. The samples were allowed to sit at room temperature for at least 15 min to allow for color development. Each sample (unknown and standard) was transferred to a disposable polystyrene cuvette and the absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation of the absorbance value using the curve developed using the protein standards.

2.5 Study Design - Inhibition Assays

The effect of two inhibitors on aromatase activity was determined. AG (a known competitive inhibitor of aromatase activity) served as the control competitive inhibitor. The test inhibitor was NYP and the aim of the study was to characterize the inhibition of aromatase by nonylphenol (determine both K_i and inhibition type). There were a pilot study and four definitive replicates for each inhibitor. Each replicate tested the response of aromatase activity (see Section 2.6) to the presence of three concentrations of an inhibitor and included control tubes with no inhibitor. The concentration of substrate also varied (over six concentrations) in each replicate. The initial substrate and inhibitor concentrations are presented in Table 6, and these were used in the pilot study of each respective inhibitor. The inhibitor concentrations were modified for the remaining replicates based on the results of the pilot studies (Table 7). There were two repetitions (tubes) for each condition (substrate/inhibitor concentration combination) of a given replicate. In addition, quadruplicate background activity control tubes were included for each replicate (substrate, propylene glycol, buffer, vehicle [used for preparation of reference chemical solutions] and microsomes). The control tubes were treated the same as the other samples.

Table 6. Initial Substrate and Inhibitor Concentrations

Assay Component	Chemical	Estimated K_m or K_i[a]	Target Concentrations
Substrate	[^{3}H]ASDN	50 nM	15, 25, 35, 50, 100, 300 nM
Test inhibitor	AG	48 μM[b]	0, 25, 50, 100 μM
Test inhibitor	NYP	10 μM	0, 5, 10, 20 μM

[a] AG IC_{50} = 95 μM; NYP IC_{50} = 21 μM (estimates are from aromatase validation studies where substrate ([^{3}H]ASDN) concentration was 100 nM.

[b] This estimate for K_i was in error. It was based on an IC_{50} value of 95 μM for AG from a draft version of the WA4-17 report. The correct IC_{50} value is 4.76 μM and so the correct K_i estimate should have been 2.4 μM.

Table 7. Definitive Inhibitor Concentrations

Chemical	Target Concentrations
AG	0, 5, 15, 25 μM
NYP	0, 7.5, 15, 20 μM

2.6 Aromatase Assay Method

The assays were performed in 13 x 100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube was uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 μL), [^{3}H]ASDN, NADPH (0.3 mM final concentration), inhibitor (AG or NYP) or vehicle, and buffer (0.1 M sodium phosphate buffer, pH 7.4) were combined in the test tubes (total volume 1 mL). The inhibitor and vehicle were added in a volume of 20 μL. The final concentrations for the substrate and inhibitors are presented in Tables 6 and 7. The tubes and the microsomal suspension were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The target final concentration of microsomal protein in the assay was 0.004 mg/mL. The total assay volume was 2 mL, and the tubes were incubated for 15 min. The incubations were stopped by the addition of methylene chloride (2 mL); the tubes were vortex-mixed for ca. 5 s and placed on ice. The tubes were then vortex-mixed an additional 20-25 s. Next, the tubes were centrifuged using a Beckman Allegra X-15R centrifuge with a 4750a rotor for 10 min at a setting of 1000 rpm. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous portions were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot was determined using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented tritiated water formed.

2.7 Data Recording and Analysis

2.7.1 Aromatase Activity

Assay data was recorded on data forms that were designed to capture all required data. LSS data was captured automatically into an electronic file. Relevant data were entered into a verified spreadsheet for calculation of aromatase activity (velocity). Spreadsheets for all assays are presented in Appendix 4.

2.7.2 Simultaneous Nonlinear Regression for K_i Determination

Reaction velocity, substrate and inhibitor concentration data were entered into GraphPad Prism (Version 4.03) for the calculation of K_m, V_{max} and K_i using simultaneous nonlinear regression methods.

2.7.3 Linearized Equations for Characterization of Inhibition

Data were plotted in double reciprocal (Lineweaver-Burk) plots of 1/velocity vs. 1/(substrate concentration) using GraphPad Prism (Version 4.03). Linear regression analysis of the lines of the Lineweaver-Burk plot yielded x- and y-intercepts used in the estimation of K_m and V_{max}. Secondary plots of the slopes from the Lineweaver Burk plots as a function of inhibitor concentration were also prepared in Prism. Linear regression of the data on these plots yielded an x-intercept equal to $-K_i$. The plots were examined visually in order to characterize the type of inhibition.

3.0 Results

3.1 Radiochemical Purity

The radiochemical purity of the [^{3}H]ASDN was determined by HPLC and LSS to be 97%.

3.2 Inhibitor Formulation Analysis

3.2.1 AG Formulation Analysis

Overlaid chromatograms for the high and low calibration standards of AG and a solvent blank are presented in Figure 1. The linear regression results are shown in Figure 2. The precision and accuracy results for the calibration standards are shown in Table 8.

Figure 1. Representative Chromatograms for the Analysis of AG

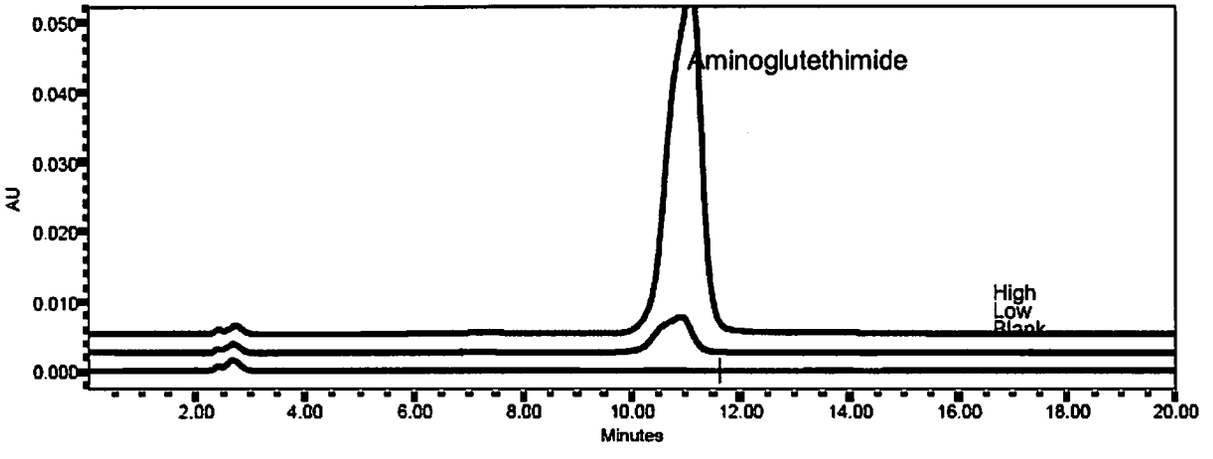


Figure 2. Calibration Curve Relating Peak Area to Amount of AG

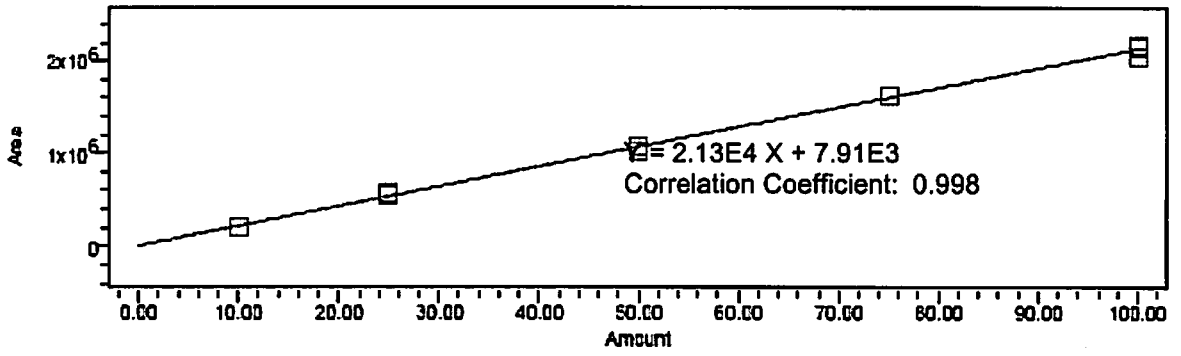


Table 8. AG – Standard Curve Results

Standard	Nominal (µg/mL)	Calc. Value (µg/mL)	%RE	Ave %RE	SD	Mean	%RSD (CV)
1	100	101	0.90	-0.23	2.53	99.8	2.54
		101	0.80				
		101	1.40				
		96.0	-4.00				
2	75	76.2	1.63	1.82			
		76.5	2.00				
3	50	49.5	-0.94	-3.09			
		47.4	-5.24				
4	25	26.1	4.54	3.53			
		25.6	2.52				
5	10	9.86	-1.45	-1.28	0.02	9.87	0.15
		9.88	-1.24				
		9.87	-1.34				
		9.89	-1.09				

The concentration of AG in formulation 12507-13A was determined to be 23.2 mg/mL (Table 9). This matches the target value of 23.2 mg/mL or 0.1 M.

Table 9. AG Formulation Analysis Results

Analysis Date	Formulation ID	Measured Concentration (µg/mL)[a]	Actual Concentration (mg/mL)[b]	Mean (mg/mL)	SD	CV%	% RE
10/31/06	12507-13A	45.5	22.8	23.2	0.9	3.8	0
		45.2	22.6				
		48.4	24.2				

[a]Concentration in analyzed dilution (1:500 dilution of 12507-13A)

[b]Concentration in 12507-13A

3.2.2 NYP Formulation Analysis

Overlaid chromatograms for the high and low calibration standards of NYP and a solvent blank are presented in Figure 3. The linear regression results are shown in Figure 4. The precision and accuracy results for the calibration standards are shown in Table 10.

Figure 3. Representative Chromatograms for the Analysis of NYP

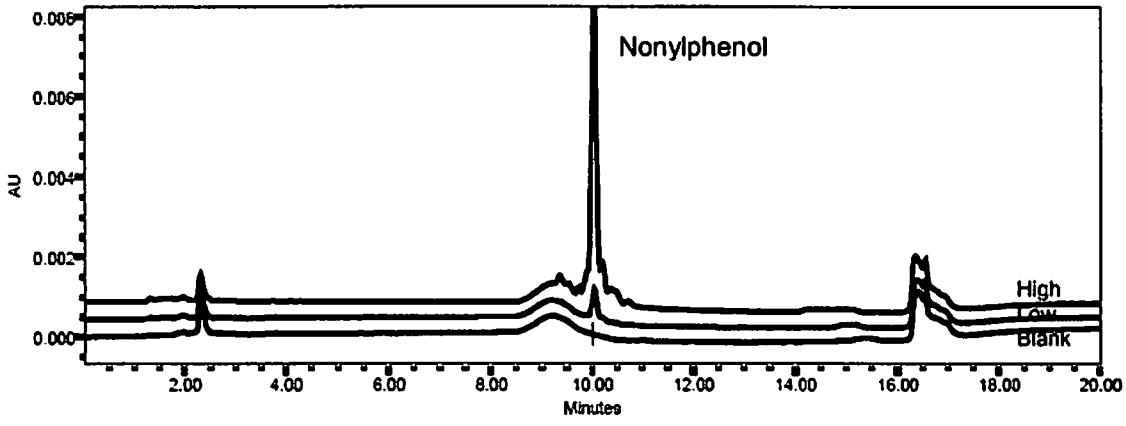
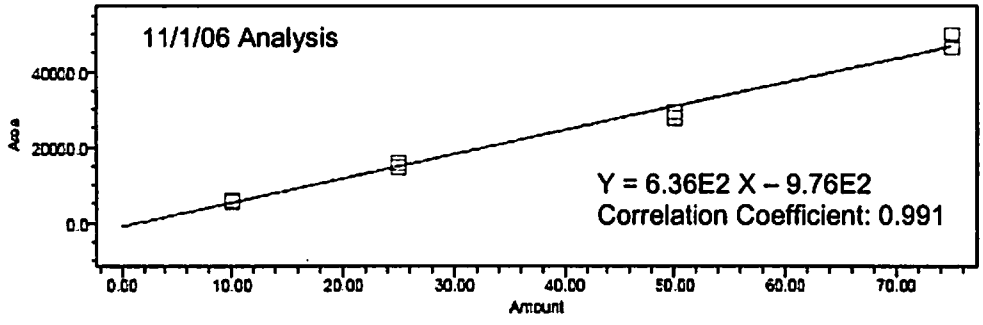


Figure 4. Calibration Curves Relating Peak Area to Amount of NYP



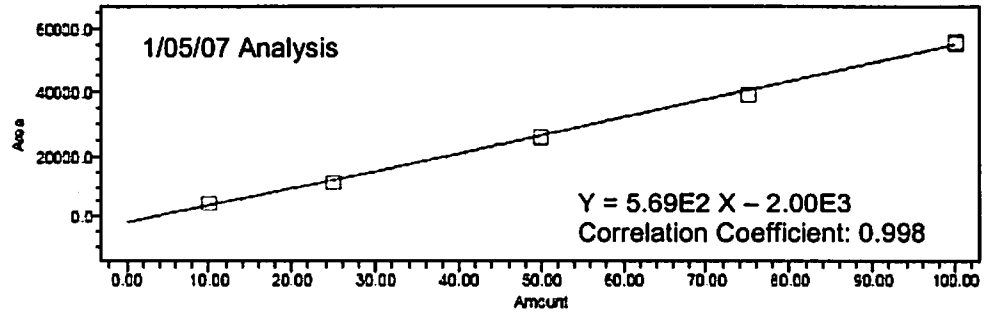


Table 10. NYP – Standard Curve Calculated Value

NYP Standard	Nominal (µg/mL)	Calc. Value (µg/mL)	% RE	Ave % RE	SD	Mean	% RSD (CV)
Analysis of 11/1/06							
2	75	80.0	6.68	2.96			
		74.4	0.75				
3	50	45.2	-9.62	-7.37			
		47.4	-5.11				
4	25	26.0	3.98	0.78			
		24.4	-2.43				
5	10	11.3	13.09	6.34	0.66	10.63	6.24
		11.1	10.98				
		10.1	0.91				
		10.0	0.36				
Analysis of 1/5/07							
1	100	101	1.34	1.14	0.19	101	0.19
		101	1.24				
		101	1.06				
		101	0.92				
2	75	72.7	-3.10	-3.27			
		72.4	-3.44				
3	50	49.3	-1.35	-1.24			
		49.4	-1.13				
4	25	23.6	-5.43	-5.55			
		23.6	-5.67				
5	10	11.0	10.4	10.9	0.06	11.1	0.53
		11.1	10.5				
		11.1	10.9				
		11.2	11.7				

The measured concentration for the NYP formulation at preparation on 11/1/06 was 22.2 mg/mL (Table 11), which represents a relative error of 0.9% from the target concentration of 22.0 mg/mL. The formulation stability was assessed at the completion of the assays on 1/5/07, and the concentration was determined to be 23.5 mg/mL or 106% of the Day 0 value. Thus, the formulation was suitably stable throughout the study period.

Table 11. NYP Formulation Analysis Results

Analysis Date	Formulation ID	Measured Concentration (µg/mL)[a]	Actual Concentration (mg/mL)[b]	Mean (mg/mL)	SD	CV%	%RE	% of Day 0 Concentration
11/1/2006	12507-13B	43.5	21.8	22.2	0.43	1.93	0.9	NA
		45.2	22.6					
		44.2	22.1					
1/5/2007	12507-13B	47.6	23.8	23.5	0.28	1.21	6.8	106
		46.8	23.4					
		46.5	23.3					

[a]Concentration in analyzed dilution (1:500 dilution of 12507-13B)

[b]Concentration in 12507-13B

Formulation preparation, expiration, and use data are summarized in Table 12.

Table 12. Summary of Formulation Preparation and Use Data

Chemical ID	Formulation ID	Formulation Prep Date	Exp. Date	Date of Last Use on TO3
Aminoglutethimide	12507-13A	10/30/06	12/28/06	11/30/06
4-Nonylphenol	12507-13B	10/30/06	1/5/07	1/3/07

3.3 *Protein Content*

The protein content of the recombinant microsomes was determined to be 7.609 ± 0.652 mg/mL.

3.4 *Characterization of Inhibition - AG*

3.4.1 Pilot Study

A pilot study, using AG as the inhibitor, was conducted using substrate and inhibitor concentrations presented in Table 6. The measured aromatase activity for each reaction condition (duplicate determinations per condition) is presented in Table 13. The reaction conditions using 100 nM ASDN and no inhibitor replicated the conditions (except with a new batch of microsomes) that were used in the WA4-17, Task 4 Validation Studies. The aromatase activity (0.2173 and 0.2236 nmol/mg/min) measured in the present study under those conditions was comparable to the validation study data (0.3849 ± 0.1043 nmol/mg/min).

The results of the simultaneous nonlinear regression and linearized equation analysis methods are summarized in Table 14 and Figures 5 and 6. By the SNLR method, the calculated K_m was 52.6 nM, which is in the range of values reported in the

literature, and V_{max} was 0.342 nmol/mg/min. The K_i for AG calculated using this method was 2.50 μM. The value for IC_{50} as determined in WA4-17 was about 4.76 μM and so the expected K_i is about 2.4 μM. The K_i (2.50 μM) calculated from data of the pilot study using SNLR compares favorably with the predicted value. Visual examination of the Lineweaver Burk plot (Figure 6) indicates that the inhibition is competitive. The K_m and V_{max} estimated (Table 14) from the Lineweaver Burk plot (inverses of the x- and y-intercepts of the control set, respectively) were 49.8 nM and 0.333 nmol/mg/min. The secondary plot shows a positive x-intercept, which yields a nonsensical value of -5.98 μM for the K_i.

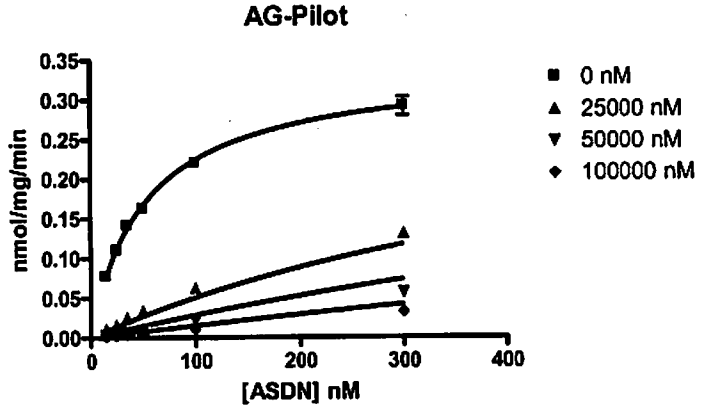
Table 13. AG Pilot Study - Aromatase Activity (nmol/mg/min)

ASDN Concentration (nM)	AG Concentration							
	0 μM		25 μM		50 μM		100 μM	
15	0.078	0.077	0.010	0.011	0.004	0.003	0.001	0.001
25	0.114	0.106	0.016	0.016	0.005	0.005	0.003	0.002
35	0.142	0.140	0.024	0.026	0.008	0.007	0.004	0.004
50	0.169	0.157	0.035	0.033	0.009	0.010	0.005	0.004
100	0.217	0.224	0.060	0.065	0.019	0.021	0.010	0.010
300	0.303	0.280	0.130	0.131	0.053	0.060	0.034	0.030

Table 14. AG Pilot Study - Kinetic Parameters

	SNLR	Linearized
K_m (nM)	52.6	49.8
V_{max} (nmol/mg/min)	0.342	0.333
K_i (μM)	2.50	-5.98

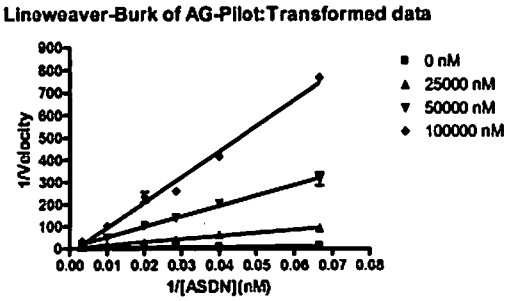
Figure 5. AG Pilot – SNLR Results

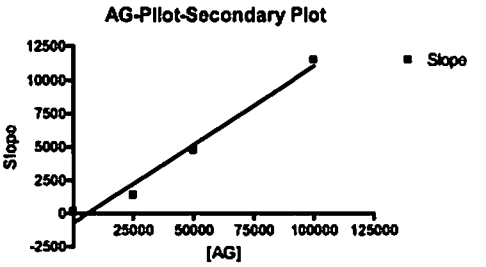


[ASDN] nM	0 nM		25000 nM		50000 nM		100000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0775	0.0769	0.0103	0.0112	0.0035	0.0029	0.0013	0.0013
25	0.1137	0.1059	0.0155	0.0160	0.0046	0.0052	0.0025	0.0023
35	0.1423	0.1399	0.0241	0.0262	0.0076	0.0069	0.0037	0.0040
50	0.1687	0.1571	0.0351	0.0326	0.0094	0.0100	0.0047	0.0039
100	0.2173	0.2236	0.0603	0.0647	0.0194	0.0214	0.0099	0.0097
300	0.3033	0.2786	0.1302	0.1308	0.0527	0.0596	0.0340	0.0296

	0 nM	25000 nM	50000 nM	100000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	52.56	52.56	52.56	52.56	52.56
I	0.0	25000	50000	100000	
KI	2501	2501	2501	2501	2501
VMAX	0.3415	0.3415	0.3415	0.3415	0.3415
Std. Error					
KM	3.139	3.139	3.139	3.139	3.139
KI	151.3	151.3	151.3	151.3	151.3
VMAX	0.008025	0.008025	0.008025	0.008025	0.008025
95% Confidence Intervals					
KM	46.23 to 58.89	46.23 to 58.89	46.23 to 58.89	46.23 to 58.89	46.23 to 58.89
KI	2196 to 2806	2196 to 2806	2196 to 2806	2196 to 2806	2196 to 2806
VMAX	0.3253 to 0.3577	0.3253 to 0.3577	0.3253 to 0.3577	0.3253 to 0.3577	0.3253 to 0.3577
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9919	0.9578	0.8007	0.7775	0.9912
Absolute Sum of Squares	0.0004980	0.0008566	0.0008141	0.0003012	0.002470
Sy.x					0.007409
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 6. AG Pilot – Lineweaver-Burk and Secondary Plot Results





	0 nM	25000 nM	50000 nM	100000 nM
Best-fit values				
Slope	149.7 ± 2.791	1385 ± 61.35	4742 ± 128.2	11470 ± 630.6
Y-intercept when X=0.0	3.007 ± 0.09784	2.724 ± 2.150	4.883 ± 4.493	-19.78 ± 22.10
X-intercept when Y=0.0	-0.02009	-0.001967	-0.001030	0.001724
1/slope	0.006680	0.0007221	0.0002109	0.00008718
95% Confidence Intervals				
Slope	142.0 to 157.5	1215 to 1555	4388 to 5097	9720 to 13220
Y-intercept when X=0.0	2.736 to 3.279	-3.246 to 8.693	-7.591 to 17.36	-81.13 to 41.58
X-intercept when Y=0.0	-0.02294 to -0.01749	-0.006993 to 0.002136	-0.003901 to 0.001511	-0.004144 to 0.006335
Goodness of Fit				
r^2	0.9986	0.9922	0.9971	0.9881
Sy.x	0.1433	3.150	6.581	32.37
Is slope significantly non-zero?				
F	2877	509.6	1368	330.9
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.1175 ± 0.01287
Y-intercept when X=0.0	-701.8 ± 737.4
X-intercept when Y=0.0	5975
1/slope	8.514
95% Confidence Intervals	
Slope	0.06206 to 0.1728
Y-intercept when X=0.0	-3875 to 2471
X-intercept when Y=0.0	-35100 to 25440
Goodness of Fit	
r^2	0.9765
Sy.x	952.0
Is slope significantly non-zero?	
F	83.24
DFn, DFd	1.000, 2.000
P value	0.0118
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

3.4.2 Definitive Study

Four additional replicates of the assay were conducted using AG as the inhibitor at concentrations of 0, 5, 15, and 25 μM. The measured aromatase activity for each reaction condition (duplicate determinations per condition) and each replicate is presented in Table 15. The complete spreadsheets for calculation of aromatase activity are presented in Appendix 4.

The results for the SNLR analysis are summarized in Table 16 and are presented in Figures 7-11. The mean calculated K_m was 50.6 nM, which is in the range of values reported in the literature, and the mean V_{max} was 0.320 nmol/mg/min. The K_i for AG calculated using the SNLR method was 1.62 μM, which falls within the range of values (ca. 0.7 – 2.7 μM) reported in the literature (Brueggemeier, et al., 2005; Kao et al., 2001).

Visual examination of the Lineweaver-Burk and secondary plots (Figures 12-16) indicates that the inhibition is competitive, as evidenced by the common y-intercept for the lines on the Lineweaver-Burk plots and the linear relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots).

The mean K_m and V_{max} estimated (Table 17) from the Lineweaver-Burk plot (from the inverses of the x- and y-intercepts, respectively, of the control runs) were 58.3 nM and 0.343 nmol/mg/min. The mean K_i (extrapolated from the secondary plot as the negative of the x-intercept) was 1.90 μM. The values for K_m, V_{max} and K_i estimated from the plots are in good agreement with those found through SNLR methods.

Table 15. AG Definitive Assays - Aromatase Activity[a]

ASDN Concentration (nM)	Aminoglutethimide Concentration							
	0 µM		5 µM		15 µM		25 µM	
Replicate 1								
15	0.065	0.067	0.018	0.019	0.008	0.007	0.005	0.005
25	0.100	0.094	0.032	0.033	0.014	0.014	0.009	0.008
35	0.115	0.117	0.044	0.048	0.021	0.023	0.014	0.014
50	0.169	0.153	0.058	0.063	0.028	0.029	0.020	0.017
100	0.197	0.197	0.108	0.102	0.053	0.054	0.033	0.032
300	0.252	0.263	0.180	0.183	0.113	0.106	0.081	0.077
Replicate 2								
15	0.070	0.070	0.020	0.018	0.009	0.009	0.005	0.005
25	0.089	0.083	0.028	0.032	0.013	0.014	0.008	0.008
35	0.108	0.121	0.045	0.043	0.019	0.017	0.010	0.012
50	0.153	0.141	0.057	0.059	0.021	0.023	0.015	0.017
100	0.199	0.208	0.095	0.096	0.051	0.051	0.030	0.030
300	0.244	0.238	0.164	0.178	0.117	0.109	0.076	0.072
Replicate 3								
15	0.066	0.066	0.019	0.019	0.007	0.008	0.006	0.005
25	0.106	0.102	0.036	0.037	0.014	0.015	0.010	0.009
35	0.124	0.134	0.049	0.053	0.022	0.020	0.013	0.014
50	0.191	0.174	0.065	0.065	0.030	0.027	0.018	0.019
100	0.239	0.231	0.121	0.119	0.056	0.060	0.036	0.036
300	0.309	0.303	0.213	0.206	0.132	0.125	0.100	0.095
Replicate 4								
15	0.082	0.081	0.023	0.021	0.010	0.009	0.006	0.007
25	0.121	0.130	0.036	0.036	0.016	0.016	0.010	0.011
35	0.155	0.152	0.050	0.050	0.022	0.022	0.015	0.014
50	0.176	0.162	0.069	0.073	0.027	0.026	0.016	0.023
100	0.242	0.210	0.108	0.097	0.055	0.054	0.035	0.032
300	0.279	0.292	0.189	0.183	0.110	0.120	0.088	0.088

[a]nmol ASDN metabolized/mg protein/min

Table 16. AG Definitive Assays - Kinetic Parameters Calculated by SNLR

Replicate	K_m[a]	V_{max}[b]	K_I[c]
1	51.0	0.302	1.70
2	49.8	0.288	1.65
3	60.7	0.372	1.83
4	40.9	0.320	1.29
Mean	50.6	0.320	1.62
SEM	4.0	0.018	0.12

[a]nM

[b]nmol ASDN metabolized/mg protein/min

[c]μM

Table 17. AG Definitive Assays - Kinetic Parameters Calculated from Linearized Equations

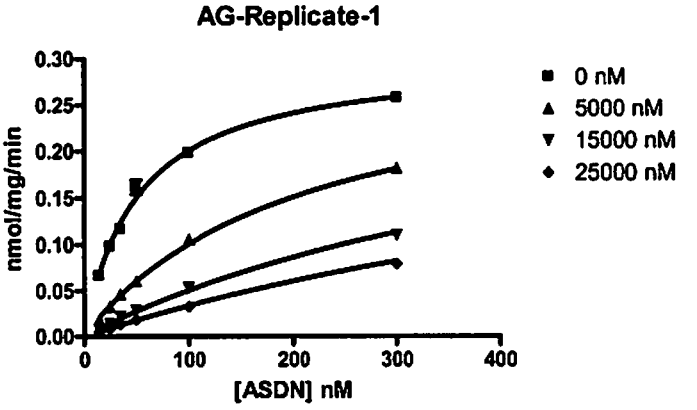
Replicate	K_m[a]	V_{max}[b]	K_I[c]
1	56.2	0.315	1.63
2	46.0	0.273	1.59
3	85.0	0.445	2.49
4	45.8	0.337	1.88
Mean	58.3	0.343	1.90
SEM	9.2	0.037	0.21

[a]nM

[b]nmol ASDN metabolized/mg protein/min

[c]μM

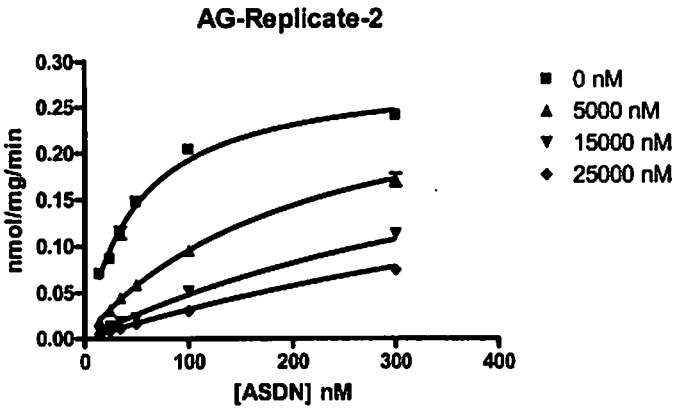
Figure 7. AG Replicate 1 – SNLR Results



[ASDN] nM	0 nM		5000 nM		15000 nM		25000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0650	0.0675	0.0181	0.0188	0.0084	0.0070	0.0047	0.0051
25	0.1002	0.0945	0.0323	0.0328	0.0142	0.0137	0.0085	0.0080
35	0.1150	0.1174	0.0440	0.0481	0.0206	0.0226	0.0138	0.0137
50	0.1689	0.1533	0.0578	0.0628	0.0277	0.0286	0.0199	0.0169
100	0.1973	0.1974	0.1080	0.1020	0.0526	0.0536	0.0332	0.0316
300	0.2522	0.2628	0.1796	0.1835	0.1134	0.1056	0.0807	0.0773

	0 nM	5000 nM	15000 nM	25000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	51.00	51.00	51.00	51.00	51.00
I	0.0	5000	15000	25000	
KI	1697	1697	1697	1697	1697
VMAX	0.3020	0.3020	0.3020	0.3020	0.3020
Std. Error					
KM	1.901	1.901	1.901	1.901	1.901
KI	57.83	57.83	57.83	57.83	57.83
VMAX	0.004310	0.004310	0.004310	0.004310	0.004310
95% Confidence Intervals					
KM	47.17 to 54.83	47.17 to 54.83	47.17 to 54.83	47.17 to 54.83	47.17 to 54.83
KI	1581 to 1814	1581 to 1814	1581 to 1814	1581 to 1814	1581 to 1814
VMAX	0.2933 to 0.3107	0.2933 to 0.3107	0.2933 to 0.3107	0.2933 to 0.3107	0.2933 to 0.3107
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9881	0.9970	0.9940	0.9947	0.9962
Absolute Sum of Squares	0.0005931	0.0001108	8.695e-005	4.027e-005	0.0008311
Sy.x					0.004297
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

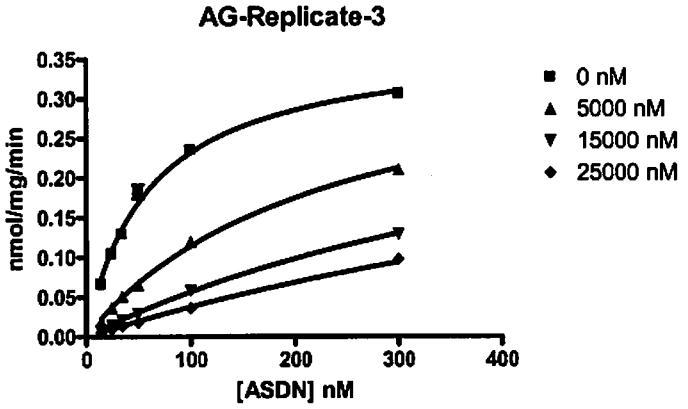
Figure 8. AG Replicate 2 – SNLR Results



[ASDN] nM	0 nM		5000 nM		15000 nM		25000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0699	0.0705	0.0201	0.0179	0.0088	0.0088	0.0051	0.0052
25	0.0893	0.0829	0.0283	0.0325	0.0133	0.0136	0.0082	0.0082
35	0.1085	0.1210	0.0449	0.0432	0.0188	0.0173	0.0103	0.0117
50	0.1528	0.1412	0.0573	0.0595	0.0215	0.0230	0.0151	0.0171
100	0.1994	0.2080	0.0948	0.0956	0.0507	0.0512	0.0299	0.0305
300	0.2440	0.2378	0.1641	0.1777	0.1166	0.1088	0.0763	0.0724

	0 nM	5000 nM	15000 nM	25000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	49.75	49.75	49.75	49.75	49.75
I	0.0	5000	15000	25000	
KI	1648	1648	1648	1648	1648
VMAX	0.2876	0.2876	0.2876	0.2876	0.2876
Std. Error					
KM	2.277	2.277	2.277	2.277	2.277
KI	68.97	68.97	68.97	68.97	68.97
VMAX	0.005008	0.005008	0.005008	0.005008	0.005008
95% Confidence Intervals					
KM	45.16 to 54.34	45.16 to 54.34	45.16 to 54.34	45.16 to 54.34	45.16 to 54.34
KI	1509 to 1787	1509 to 1787	1509 to 1787	1509 to 1787	1509 to 1787
VMAX	0.2775 to 0.2977	0.2775 to 0.2977	0.2775 to 0.2977	0.2775 to 0.2977	0.2775 to 0.2977
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9816	0.9961	0.9913	0.9930	0.9943
Absolute Sum of Squares	0.0008382	0.0001243	0.0001372	4.767e-005	0.001147
Sy.x					0.005049
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

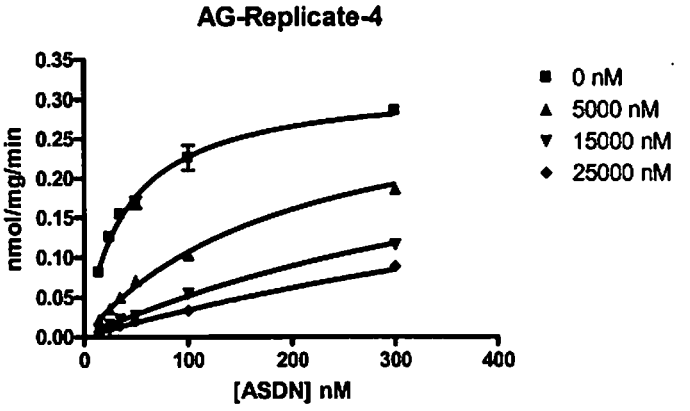
Figure 9. AG Replicate 3 - SNLR Results



[ASDN] nM	0 nM		5000 nM		15000 nM		25000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0657	0.0657	0.0190	0.0185	0.0075	0.0080	0.0058	0.0052
25	0.1061	0.1019	0.0358	0.0368	0.0142	0.0147	0.0101	0.0094
35	0.1238	0.1336	0.0488	0.0528	0.0216	0.0201	0.0130	0.0139
50	0.1912	0.1739	0.0652	0.0650	0.0304	0.0270	0.0176	0.0187
100	0.2390	0.2310	0.1206	0.1188	0.0561	0.0604	0.0365	0.0358
300	0.3094	0.3030	0.2134	0.2055	0.1323	0.1253	0.1003	0.0951

	0 nM	5000 nM	15000 nM	25000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	60.68	60.68	60.68	60.68	60.68
I	0.0	5000	15000	25000	
KI	1834	1834	1834	1834	1834
VMAX	0.3720	0.3720	0.3720	0.3720	0.3720
Std. Error					
KM	2.411	2.411	2.411	2.411	2.411
KI	66.17	66.17	66.17	66.17	66.17
VMAX	0.005936	0.005936	0.005936	0.005936	0.005936
95% Confidence Intervals					
KM	55.82 to 65.54	55.82 to 65.54	55.82 to 65.54	55.82 to 65.54	55.82 to 65.54
KI	1701 to 1968	1701 to 1968	1701 to 1968	1701 to 1968	1701 to 1968
VMAX	0.3601 to 0.3840	0.3601 to 0.3840	0.3601 to 0.3840	0.3601 to 0.3840	0.3601 to 0.3840
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9875	0.9966	0.9965	0.9954	0.9958
Absolute Sum of Squares	0.001003	0.0001708	7.325e-005	5.558e-005	0.001303
Sy.x					0.005381
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 10. AG Replicate 4 – SNLR Results



[ASDN] nM	0 nM		5000 nM		15000 nM		25000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0816	0.0807	0.0228	0.0210	0.0102	0.0091	0.0058	0.0065
25	0.1210	0.1296	0.0356	0.0355	0.0165	0.0160	0.0105	0.0106
35	0.1549	0.1524	0.0502	0.0496	0.0225	0.0222	0.0146	0.0143
50	0.1761	0.1617	0.0689	0.0733	0.0266	0.0261	0.0165	0.0232
100	0.2417	0.2100	0.1082	0.0968	0.0555	0.0540	0.0352	0.0316
300	0.2788	0.2922	0.1892	0.1827	0.1098	0.1200	0.0884	0.0883

	0 nM	5000 nM	15000 nM	25000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	40.94	40.94	40.94	40.94	40.94
I	0.0	5000	15000	25000	
KI	1288	1288	1288	1288	1288
VMAX	0.3197	0.3197	0.3197	0.3197	0.3197
Std. Error					
KM	1.888	1.888	1.888	1.888	1.888
KI	54.39	54.39	54.39	54.39	54.39
VMAX	0.005317	0.005317	0.005317	0.005317	0.005317
95% Confidence Intervals					
KM	37.13 to 44.75	37.13 to 44.75	37.13 to 44.75	37.13 to 44.75	37.13 to 44.75
KI	1179 to 1398	1179 to 1398	1179 to 1398	1179 to 1398	1179 to 1398
VMAX	0.3090 to 0.3304	0.3090 to 0.3304	0.3090 to 0.3304	0.3090 to 0.3304	0.3090 to 0.3304
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9810	0.9913	0.9933	0.9920	0.9944
Absolute Sum of Squares	0.001023	0.0003141	0.0001043	7.569e-005	0.001517
Sy.x					0.005806
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 11. AG Definitive – SNLR Plots

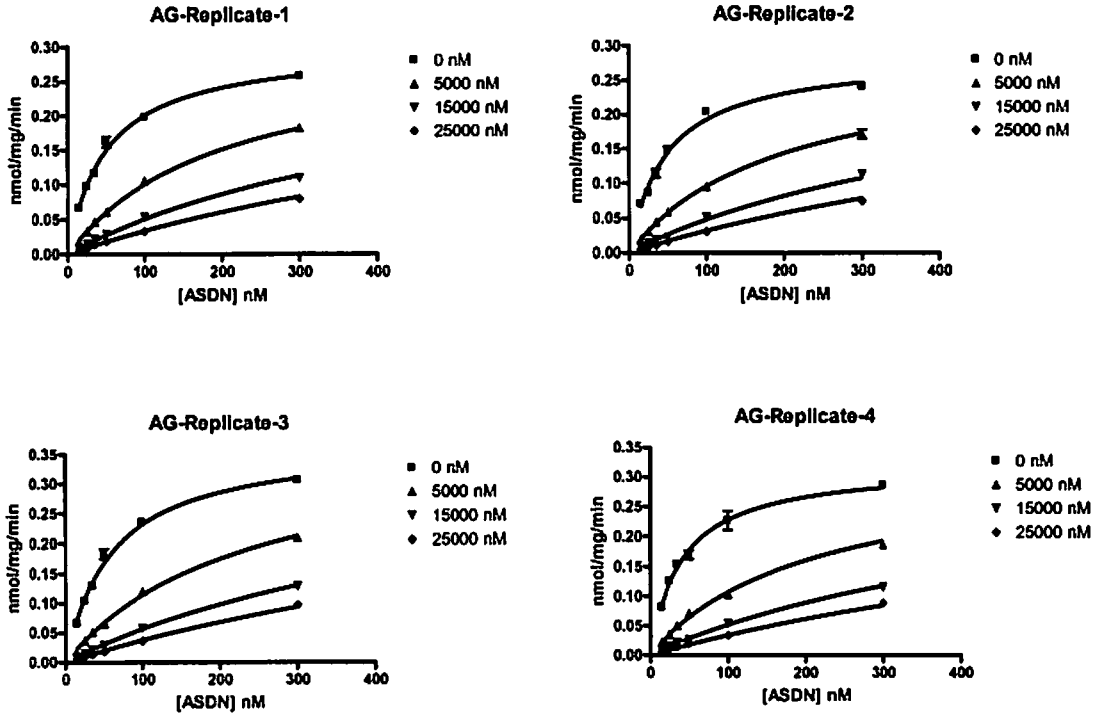
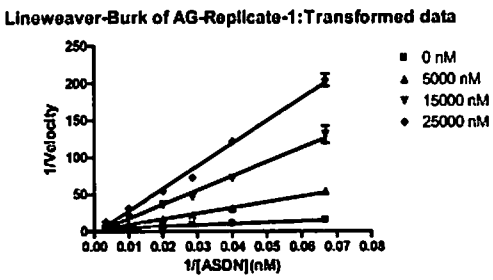
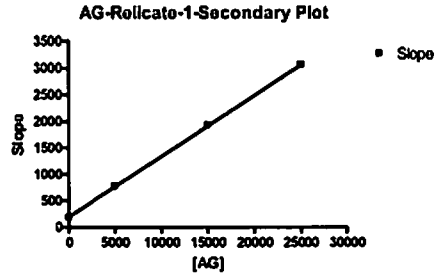


Figure 12. AG Replicate 1 – Lineweaver-Burk and Secondary Plot Results

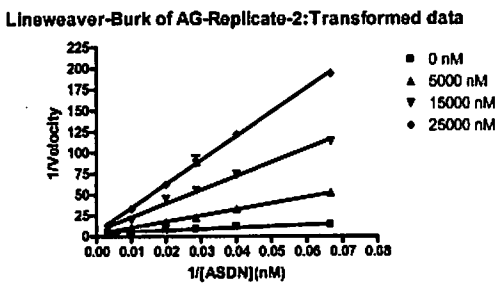


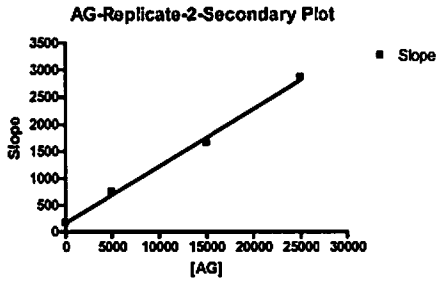


	0 nM	5000 nM	15000 nM	25000 nM
Best-fit values				
Slope	178.6 ± 6.237	767.9 ± 30.28	1924 ± 98.16	3053 ± 139.4
Y-intercept when X=0.0	3.177 ± 0.2186	1.484 ± 1.061	-1.967 ± 3.441	-3.000 ± 4.885
X-intercept when Y=0.0	-0.01779	-0.001933	0.001022	0.0009825
1/slope	0.005600	0.001302	0.0005197	0.0003275
95% Confidence Intervals				
Slope	161.2 to 195.9	683.9 to 852.0	1652 to 2197	2667 to 3440
Y-intercept when X=0.0	2.570 to 3.784	-1.462 to 4.431	-11.52 to 7.584	-16.56 to 10.56
X-intercept when Y=0.0	-0.02316 to -0.01329	-0.006346 to 0.001752	-0.004461 to 0.005397	-0.003860 to 0.004940
Goodness of Fit				
r^2	0.9951	0.9938	0.9897	0.9917
Sy.x	0.3202	1.555	5.039	7.155
Is slope significantly non-zero?				
F	819.7	643.1	384.3	480.0
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.1149 ± 0.0006680
Y-intercept when X=0.0	187.8 ± 9.880
X-intercept when Y=0.0	-1634
1/slope	8.700
95% Confidence Intervals	
Slope	0.1121 to 0.1178
Y-intercept when X=0.0	145.3 to 230.3
X-intercept when Y=0.0	-2044 to -1240
Goodness of Fit	
r^2	0.9999
Sy.x	12.83
Is slope significantly non-zero?	
F	29610
DFn, DFd	1.000, 2.000
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 13. AG Replicate 2 – Lineweaver-Burk and Secondary Plot Results

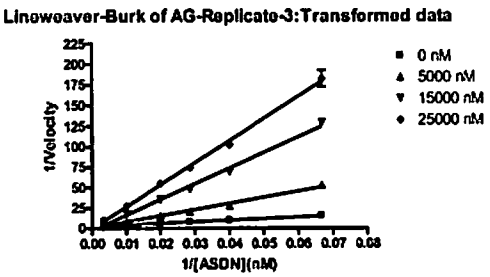


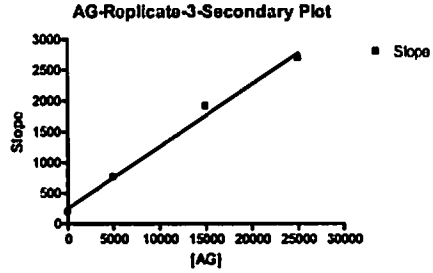


	0 nM	5000 nM	15000 nM	25000 nM
Best-fit values				
Slope	168.9 ± 14.58	746.4 ± 16.63	1652 ± 79.30	2864 ± 51.44
Y-intercept when X=0.0	3.670 ± 0.5111	2.708 ± 0.5829	6.413 ± 2.780	5.601 ± 1.803
X-intercept when Y=0.0	-0.02172	-0.003628	-0.003881	-0.001956
1/slope	0.005920	0.001340	0.0006052	0.0003492
95% Confidence Intervals				
Slope	128.4 to 209.4	700.2 to 792.5	1432 to 1872	2721 to 3007
Y-intercept when X=0.0	2.251 to 5.088	1.090 to 4.326	-1.304 to 14.13	0.5959 to 10.61
X-intercept when Y=0.0	-0.03844 to -0.01108	-0.006109 to -0.001390	-0.009631 to 0.0007131	-0.003862 to -0.0002001
Goodness of Fit				
r^2	0.9711	0.9980	0.9909	0.9987
Sy.x	0.7486	0.8538	4.071	2.641
Is slope significantly non-zero?				
F	134.2	2014	434.1	3099
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	0.0003	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.1058 ± 0.004607
Y-intercept when X=0.0	167.7 ± 68.13
X-intercept when Y=0.0	-1585
1/slope	9.453
95% Confidence Intervals	
Slope	0.08597 to 0.1256
Y-intercept when X=0.0	-125.5 to 460.9
X-intercept when Y=0.0	-5145 to 1041
Goodness of Fit	
r^2	0.9982
Sy.x	88.46
Is slope significantly non-zero?	
F	527.4
DFn, DFd	1.000, 2.000
P value	0.0019
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 14. AG Replicate 3 – Lineweaver-Burk and Secondary Plot Results

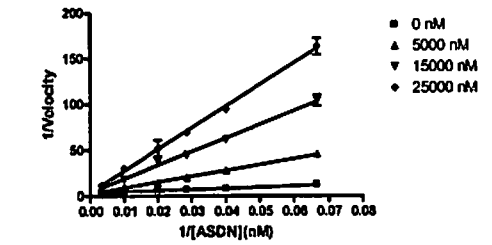


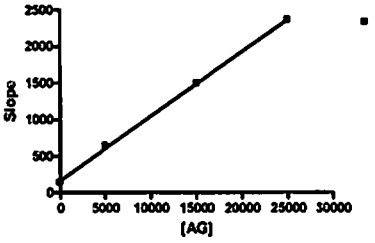


	0 nM	5000 nM	15000 nM	25000 nM
Best-fit values				
Slope	190.8 ± 7.692	759.2 ± 48.42	1913 ± 83.24	2690 ± 63.85
Y-intercept when X=0.0	2.245 ± 0.2696	0.1874 ± 1.697	-2.692 ± 2.918	-0.1616 ± 2.238
X-intercept when Y=0.0	-0.01176	-0.0002468	0.001407	0.00006007
1/slope	0.005241	0.001317	0.0005227	0.0003717
95% Confidence Intervals				
Slope	169.5 to 212.2	624.8 to 893.6	1682 to 2144	2513 to 2868
Y-intercept when X=0.0	1.496 to 2.993	-4.524 to 4.899	-10.79 to 5.407	-6.374 to 6.051
X-intercept when Y=0.0	-0.01737 to -0.007173	-0.007571 to 0.005243	-0.003136 to 0.005160	-0.002376 to 0.002252
Goodness of Fit				
r^2	0.9935	0.9840	0.9925	0.9978
Sy.x	0.3949	2.486	4.273	3.278
Is slope significantly non-zero?				
F	615.4	245.8	528.3	1776
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.1011 ± 0.006652
Y-intercept when X=0.0	251.2 ± 98.38
X-intercept when Y=0.0	-2485
1/slope	9.894
95% Confidence Intervals	
Slope	0.07245 to 0.1297
Y-intercept when X=0.0	-172.1 to 674.6
X-intercept when Y=0.0	-8775 to 1408
Goodness of Fit	
r^2	0.9914
Sy.x	127.7
Is slope significantly non-zero?	
F	230.9
DFn, DFd	1.000, 2.000
P value	0.0043
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 15. AG Replicate 4– Lineweaver-Burk and Secondary Plot Results

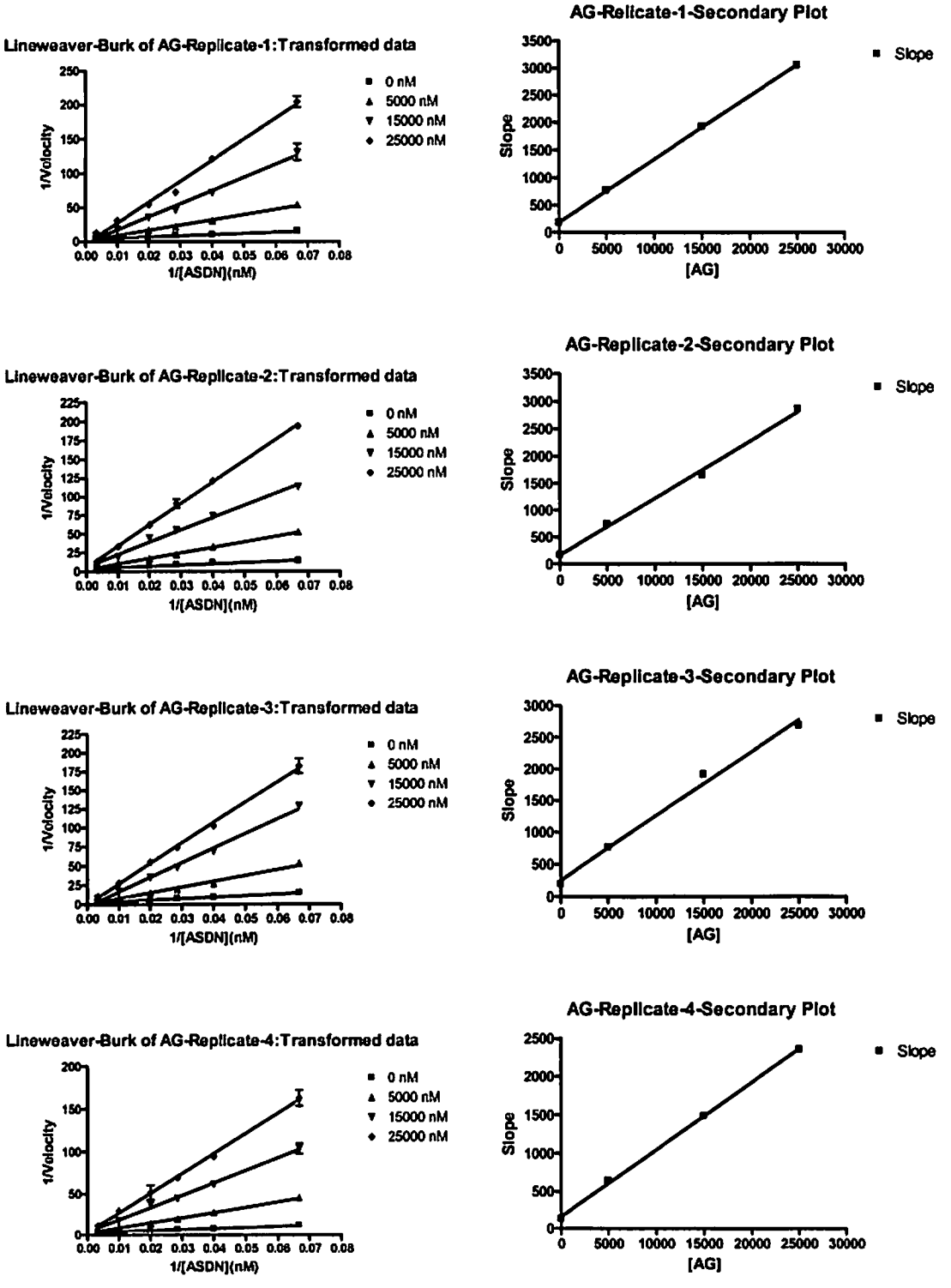




	0 nM	5000 nM	15000 nM	25000 nM
Best-fit values				
Slope	135.9 ± 6.588	639.0 ± 18.74	1484 ± 49.21	2356 ± 50.97
Y-intercept when X=0.0	2.966 ± 0.2309	2.572 ± 0.6570	4.187 ± 1.725	3.861 ± 1.787
X-intercept when Y=0.0	-0.02183	-0.004024	-0.002822	-0.001639
1/slope	0.007358	0.001565	0.0006741	0.0004244
95% Confidence Intervals				
Slope	117.6 to 154.2	587.0 to 691.1	1347 to 1620	2215 to 2498
Y-intercept when X=0.0	2.325 to 3.607	0.7478 to 4.396	-0.6017 to 8.975	-1.098 to 8.821
X-intercept when Y=0.0	-0.03016 to -0.01534	-0.007379 to -0.001098	-0.006551 to 0.0003777	-0.003938 to 0.0004448
Goodness of Fit				
r^2	0.9907	0.9966	0.9956	0.9981
Sy.x	0.3382	0.9624	2.526	2.617
Is slope significantly non-zero?				
F	425.5	1162	908.9	2137
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.08793 ± 0.001715
Y-intercept when X=0.0	165.1 ± 25.38
X-intercept when Y=0.0	-1877
1/slope	11.37
95% Confidence Intervals	
Slope	0.08055 to 0.09531
Y-intercept when X=0.0	55.92 to 274.2
X-intercept when Y=0.0	-3343 to -597.4
Goodness of Fit	
r^2	0.9992
Sy.x	32.93
Is slope significantly non-zero?	
F	2629
DFn, DFd	1.000, 2.000
P value	0.0004
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 16. AG Definitive – Lineweaver-Burk and Secondary Plot Results



3.5 Characterization of Inhibition - NYP

3.5.1 Pilot Study

A pilot study, using NYP as the inhibitor, was conducted using substrate and inhibitor concentrations presented in Table 6. The measured aromatase activity for each reaction condition (duplicate determinations per condition) is presented in Table 18. The results of the analysis by simultaneous nonlinear regression and linearized equations methods are summarized in Table 19 and Figures 17 and 18

The SNLR plots are presented in Figure 17. The calculated K_m was 26.4 nM and V_{max} was 0.241 nmol/mg/min (Table 19). The K_i for NYP calculated using this method was 5.33 μM. The Lineweaver Burk and secondary plots are presented in Figure 18. The K_m and V_{max} estimated (Table 19) from the Lineweaver Burk plot (at NYP = 0 μM) were 30.3 nM and 0.234 nmol/mg/min. The secondary plot yields a value of 2.53 μM for the K_i.

Table 18. NYP Pilot Study - Aromatase Activity[a]

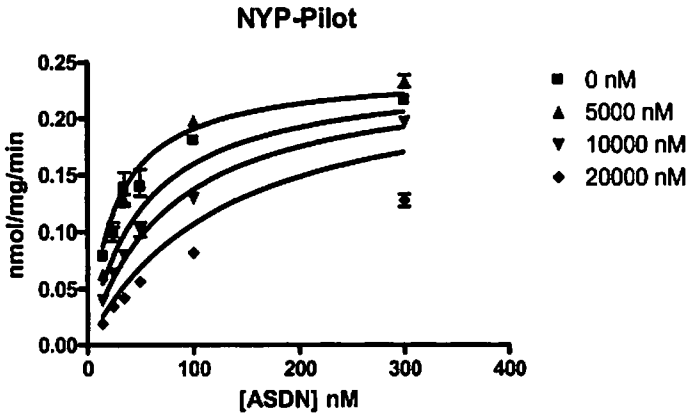
ASDN Concentration (nM)	NYP Concentration							
	0 μM		5 μM		10 μM		20 μM	
15	0.077	0.080	0.064	0.062	0.038	0.042	0.019	0.019
25	0.104	0.094	0.092	0.108	0.061	0.066	0.034	0.034
35	0.125	0.152	0.123	0.133	0.079	0.080	0.042	0.043
50	0.137	0.142	0.155	0.132	0.096	0.109	0.058	0.056
100	0.185	0.176	0.199	0.196	0.130	0.128	0.082	0.081
300	0.215	0.216	0.227	0.238	0.196	0.197	0.132	0.122

[a] nmol/mg/min

Table 19. NYP Pilot Study - Kinetic Parameters

	SNLR	Linearized
K_m (nM)	26.4	30.3
V_{max} (nmol/mg/min)	0.241	0.234
K_i (μM)	5.33	2.53

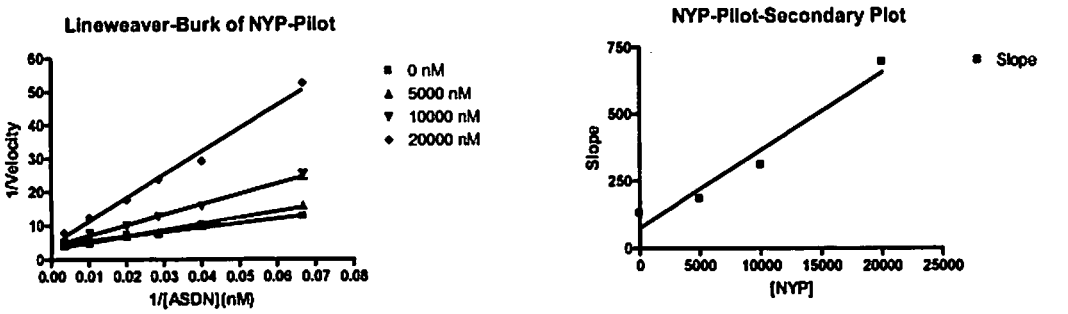
Figure 17. NYP Pilot SNLR Results



[ASDN] nM	0 nM		5000 nM		10000 nM		20000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0771	0.0801	0.0636	0.0622	0.0380	0.0419	0.0188	0.0192
25	0.1042	0.0945	0.0919	0.1081	0.0611	0.0661	0.0345	0.0339
35	0.1246	0.1519	0.1227	0.1327	0.0788	0.0802	0.0417	0.0427
50	0.1370	0.1419	0.1547	0.1315	0.0958	0.1087	0.0577	0.0556
100	0.1848	0.1755	0.1987	0.1959	0.1300	0.1285	0.0821	0.0809
300	0.2154	0.2161	0.2271	0.2378	0.1957	0.1970	0.1324	0.1218

	0 nM	5000 nM	10000 nM	20000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	26.36	26.36	26.36	26.36	26.36
I	0.0	5000	10000	20000	
KI	5325	5325	5325	5325	5325
VMAX	0.2406	0.2406	0.2406	0.2406	0.2406
Std. Error					
KM	4.250	4.250	4.250	4.250	4.250
KI	1062	1062	1062	1062	1062
VMAX	0.01089	0.01089	0.01089	0.01089	0.01089
95% Confidence Intervals					
KM	17.80 to 34.93	17.80 to 34.93	17.80 to 34.93	17.80 to 34.93	17.80 to 34.93
KI	3186 to 7465	3186 to 7465	3186 to 7465	3186 to 7465	3186 to 7465
VMAX	0.2187 to 0.2626	0.2187 to 0.2626	0.2187 to 0.2626	0.2187 to 0.2626	0.2187 to 0.2626
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9157	0.7762	0.9870	0.6332	0.8988
Absolute Sum of Squares	0.002191	0.008817	0.0004029	0.005626	0.01704
Sy.x					0.01946
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 18. NYP Pilot Linearized Plots



	0 nM	5000 nM	10000 nM	20000 nM
Best-fit values				
Slope	129.4 ± 9.667	182.7 ± 10.08	309.4 ± 10.80	694.1 ± 38.09
Y-intercept when X=0.0	4.278 ± 0.3389	3.238 ± 0.3532	3.984 ± 0.3787	4.397 ± 1.335
X-intercept when Y=0.0	-0.03305	-0.01771	-0.01287	-0.006335
1/slope	0.007726	0.005473	0.003232	0.001441
95% Confidence Intervals				
Slope	102.6 to 156.3	154.7 to 210.7	279.5 to 339.4	588.4 to 799.8
Y-intercept when X=0.0	3.337 to 5.218	2.255 to 4.216	2.933 to 5.035	0.6910 to 8.103
X-intercept when Y=0.0	-0.04977 to -0.02183	-0.02669 to -0.01093	-0.01776 to -0.008764	-0.01342 to -0.0008867
Goodness of Fit				
r^2	0.9782	0.9880	0.9951	0.9881
Sy.x	0.4963	0.5174	0.5547	1.955
Is slope significantly non-zero?				
F	179.3	328.8	820.3	332.1
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	0.0002	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.02916 ± 0.004527
Y-intercept when X=0.0	73.78 ± 51.86
X-intercept when Y=0.0	-2530
1/slope	34.30
95% Confidence Intervals	
Slope	0.009878 to 0.04864
Y-intercept when X=0.0	-149.4 to 296.9
X-intercept when Y=0.0	-26770 to 3521
Goodness of Fit	
r^2	0.9540
Sy.x	66.95
Is slope significantly non-zero?	
F	41.48
DFn, DFd	1.000, 2.000
P value	0.0233
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

3.5.2 Definitive Study

Four additional replicates of the assay were conducted using NYP as the inhibitor at concentrations of 0, 7.5, 15, and 20 μM. There were errors in the preparation of the inhibitor dilutions on replicate 1, so that data is excluded from the summary data, although the data from replicate 1 is included in Appendix 4. The measured aromatase activity for each reaction condition (duplicate determinations per condition) and each replicate is presented in Table 20. The complete spreadsheets for calculation of aromatase activity are presented in Appendix 4.

The results for the SNLR analysis are summarized in Table 21 and are presented in Figures 19-22. The mean calculated K_m was 38.9 nM, which is in the range of values reported in the literature, and the mean V_{max} was 0.351 nmol/mg/min. The K_i for NYP calculated using this method was 8.63 μM, which is near the estimate for K_i of 10 μM determined based on IC_{50} data from WA 4-17, Task 4.

Visual examination of the Lineweaver-Burk and secondary plots (Figures 23-26) indicates that the inhibition is primarily competitive, as evidenced by the common y-intercept for the lines on the Lineweaver-Burk plots and the linear relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots). The correlation coefficients for the secondary plots range from 0.817 to 0.946 and may be indicative of a small contribution of another inhibition type to the interaction of NYP and aromatase.

The mean K_m and V_{max} estimated (Table 22) from the Lineweaver-Burk plot (from the inverses of the x- and y-intercepts, respectively, of the control runs) were 53.6 nM and 0.394 nmol/mg/min. The mean K_i (extrapolated from the secondary plot as the negative of the x-intercept), was 6.47 μM. The values for K_m, V_{max} and K_i estimated from the plots are in good agreement with those found through SNLR methods.

Table 20. NYP Definitive Assays - Aromatase Activity*

ASDN Concentration (nM)	NYP Concentration (μM)							
	0		7.5		15		20	
Replicate 1								
15	0.081	0.081	a	a	a	a	0.021	0.021
25	0.117	0.107	a	a	a	a	0.038	0.036
35	0.141	0.156	a	a	a	a	0.051	0.048
50	0.174	0.187	a	a	a	a	0.065	0.075
100	0.226	0.248	a	a	a	a	0.102	0.099
300	0.237	0.257	a	a	a	a	0.157	0.159
Replicate 2								
15	0.081	0.084	0.072	0.062	0.032	0.038	0.031	0.030
25	0.132	0.138	0.099	0.105	0.056	0.056	0.041	0.044
35	0.169	0.176	0.140	0.133	0.078	0.086	0.056	0.056
50	0.217	0.200	0.168	0.162	0.097	0.114	0.077	0.073
100	0.278	0.272	0.225	0.233	0.149	0.146	0.120	0.116
300	0.324	0.318	0.324	0.323	0.241	0.264	0.200	0.194
Replicate 3								
15	0.089	0.086	0.075	0.085	0.057	0.053	0.037	0.035
25	0.136	0.132	0.109	0.110	0.098	0.085	0.061	0.059
35	0.169	0.160	0.152	0.158	0.092	0.096	0.066	0.080
50	0.195	0.211	0.185	0.186	0.130	0.118	0.130	0.126
100	0.263	0.261	0.227	0.257	0.189	0.191	0.157	0.185
300	0.333	0.344	0.333	0.333	0.332	0.273	0.228	0.229
Replicate 4								
15	0.087	0.080	0.061	0.062	0.034	0.031	0.025	0.023
25	0.113	0.124	0.089	0.091	0.055	0.062	0.038	0.034
35	0.139	0.140	0.127	0.127	0.074	0.084	0.061	0.062
50	0.176	0.151	0.134	0.140	0.083	0.075	0.062	0.066
100	0.230	0.183	0.213	0.227	0.194	0.182	0.123	0.115
300	0.269	0.278	0.279	0.239	0.180	0.219	0.177	0.174

* nmol/mg/min

[a]Not reported due to an error in sample preparation

Table 21. NYP Definitive Assays - Kinetic Parameters Calculated by SNLR

Replicate	K_m[a]	V_{max}[b]	K_i[c]
2	39.1	0.374	6.10
3	42.4	0.383	11.79
4	35.1	0.296	8.01
Mean	**38.9**	**0.351**	**8.63**
SEM	**2.1**	**0.028**	**1.67**

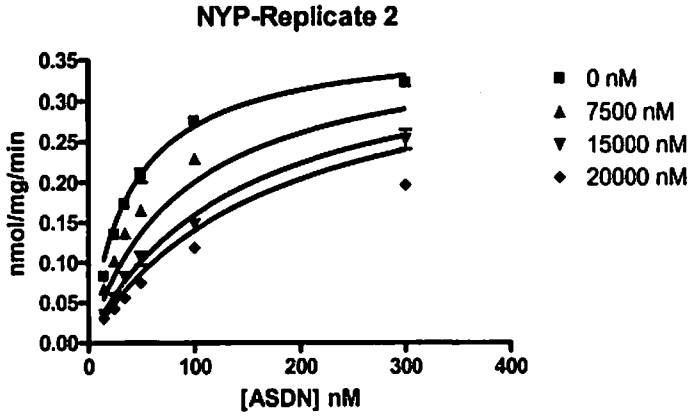
[a]nM
[b]nmol ASDN metabolized/mg protein/min
[c]μM

Table 22. NYP Definitive Assays - Kinetics Parameters Calculated From Linearized Equations

Replicate	K_m[a]	V_{max}[b]	K_i[c]
2	67.4	0.470	7.01
3	56.2	0.422	8.58
4	37.2	0.290	3.82
Mean	**53.6**	**0.394**	**6.47**
SEM	**8.8**	**0.054**	**1.40**

[a]nM
[b]nmol ASDN metabolized/mg protein/min
[c]μM

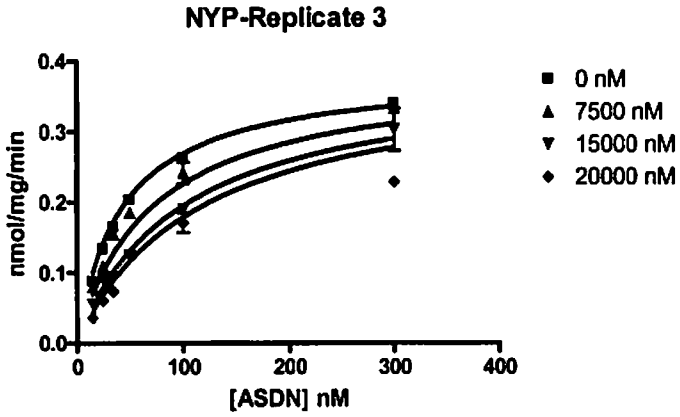
Figure 19. NYP Replicate 2 SNLR Results



[ASDN] nM	0 nM		7500 nM		15000 nM		20000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0806	0.0841	0.0722	0.0617	0.0316	0.0377	0.0307	0.0296
25	0.1315	0.1382	0.0991	0.1052	0.0556	0.0560	0.0406	0.0438
35	0.1687	0.1765	0.1397	0.1333	0.0777	0.0858	0.0556	0.0564
50	0.2165	0.2000	0.1685	0.1618	0.0971	0.1136	0.0766	0.0731
100	0.2785	0.2718	0.2254	0.2333	0.1492	0.1464	0.1202	0.1164
300	0.3242	0.3182	0.3241	0.3234	0.2407	0.2645	0.1995	0.1935

	0 nM	7500 nM	15000 nM	20000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	39.05	39.05	39.05	39.05	39.05
I	0.0	7500	15000	20000	
KI	6097	6097	6097	6097	6097
VMAX	0.3743	0.3743	0.3743	0.3743	0.3743
Std. Error					
KM	4.286	4.286	4.286	4.286	4.286
KI	756.4	756.4	756.4	756.4	756.4
VMAX	0.01362	0.01362	0.01362	0.01362	0.01362
95% Confidence Intervals					
KM	30.41 to 47.69	30.41 to 47.69	30.41 to 47.69	30.41 to 47.69	30.41 to 47.69
KI	4572 to 7622	4572 to 7622	4572 to 7622	4572 to 7622	4572 to 7622
VMAX	0.3468 to 0.4017	0.3468 to 0.4017	0.3468 to 0.4017	0.3468 to 0.4017	0.3468 to 0.4017
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9780	0.9030	0.9856	0.8621	0.9548
Absolute Sum of Squares	0.001729	0.008462	0.0009038	0.005333	0.01643
Sy.x					0.01911
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

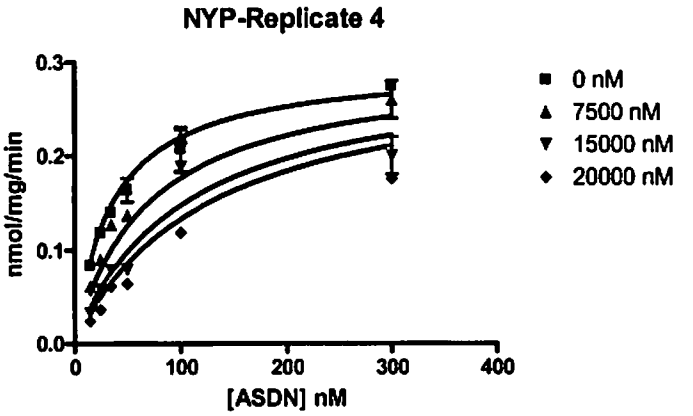
Figure 20. NYP Replicate 3 SNLR Results



[ASDN] nM	0 nM		7500 nM		15000 nM		20000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0890	0.0857	0.0751	0.0846	0.0570	0.0527	0.0366	0.0348
25	0.1363	0.1315	0.1095	0.1098	0.0976	0.0851	0.0615	0.0592
35	0.1693	0.1596	0.1519	0.1576	0.0925	0.0955	0.0663	0.0802
50	0.1951	0.2106	0.1850	0.1863	0.1303	0.1185	0.1297	0.1259
100	0.2634	0.2614	0.2270	0.2568	0.1894	0.1906	0.1571	0.1851
300	0.3333	0.3438	0.3327	0.3332	0.3323	0.2726	0.2283	0.2290

	0 nM	7500 nM	15000 nM	20000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	42.42	42.42	42.42	42.42	42.42
I	0.0	7500	15000	20000	
KI	11791	11791	11791	11791	11791
VMAX	0.3831	0.3831	0.3831	0.3831	0.3831
Std. Error					
KM	4.062	4.062	4.062	4.062	4.062
KI	1664	1664	1664	1664	1664
VMAX	0.01198	0.01198	0.01198	0.01198	0.01198
95% Confidence Intervals					
KM	34.24 to 50.61	34.24 to 50.61	34.24 to 50.61	34.24 to 50.61	34.24 to 50.61
KI	8437 to 15145	8437 to 15145	8437 to 15145	8437 to 15145	8437 to 15145
VMAX	0.3589 to 0.4072	0.3589 to 0.4072	0.3589 to 0.4072	0.3589 to 0.4072	0.3589 to 0.4072
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9881	0.9415	0.9659	0.8828	0.9573
Absolute Sum of Squares	0.0009898	0.005038	0.002854	0.006443	0.01532
Sy.x					0.01845
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 21. NYP Replicate 4 SNLR Results



[ASDN] nM	0 nM		7500 nM		15000 nM		20000 nM	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
15	0.0866	0.0803	0.0610	0.0620	0.0342	0.0306	0.0246	0.0234
25	0.1127	0.1238	0.0885	0.0909	0.0546	0.0623	0.0381	0.0340
35	0.1394	0.1399	0.1274	0.1270	0.0740	0.0837	0.0609	0.0620
50	0.1760	0.1509	0.1341	0.1402	0.0831	0.0751	0.0620	0.0662
100	0.2301	0.1834	0.2129	0.2275	0.1939	0.1822	0.1225	0.1147
300	0.2694	0.2781	0.2792	0.2388	0.1798	0.2191	0.1765	0.1740

	0 nM	7500 nM	15000 nM	20000 nM	Global (shared)
Competitive Inhibition					
Best-fit values					
KM	35.10	35.10	35.10	35.10	35.10
I	0.0	7500	15000	20000	
KI	8012	8012	8012	8012	8012
VMAX	0.2959	0.2959	0.2959	0.2959	0.2959
Std. Error					
KM	5.108	5.108	5.108	5.108	5.108
KI	1479	1479	1479	1479	1479
VMAX	0.01341	0.01341	0.01341	0.01341	0.01341
95% Confidence Intervals					
KM	24.80 to 45.40	24.80 to 45.40	24.80 to 45.40	24.80 to 45.40	24.80 to 45.40
KI	5031 to 10993	5031 to 10993	5031 to 10993	5031 to 10993	5031 to 10993
VMAX	0.2688 to 0.3229	0.2688 to 0.3229	0.2688 to 0.3229	0.2688 to 0.3229	0.2688 to 0.3229
Goodness of Fit					
Degrees of Freedom					45
R^2	0.9490	0.8729	0.8795	0.8658	0.9165
Absolute Sum of Squares	0.002427	0.007483	0.006044	0.004362	0.02032
Sy.x					0.02125
Constraints					
KM	KM is shared	KM is shared	KM is shared	KM is shared	
I	I = column title	I = column title	I = column title	I = column title	
KI	KI is shared	KI is shared	KI is shared	KI is shared	
VMAX	VMAX is shared	VMAX is shared	VMAX is shared	VMAX is shared	
Data					
Number of X values	6	6	6	6	
Number of Y replicates	2	2	2	2	
Total number of values	12	12	12	12	
Number of missing values	0	0	0	0	

Figure 22. NYP SNLR Plots Replicates 2-4

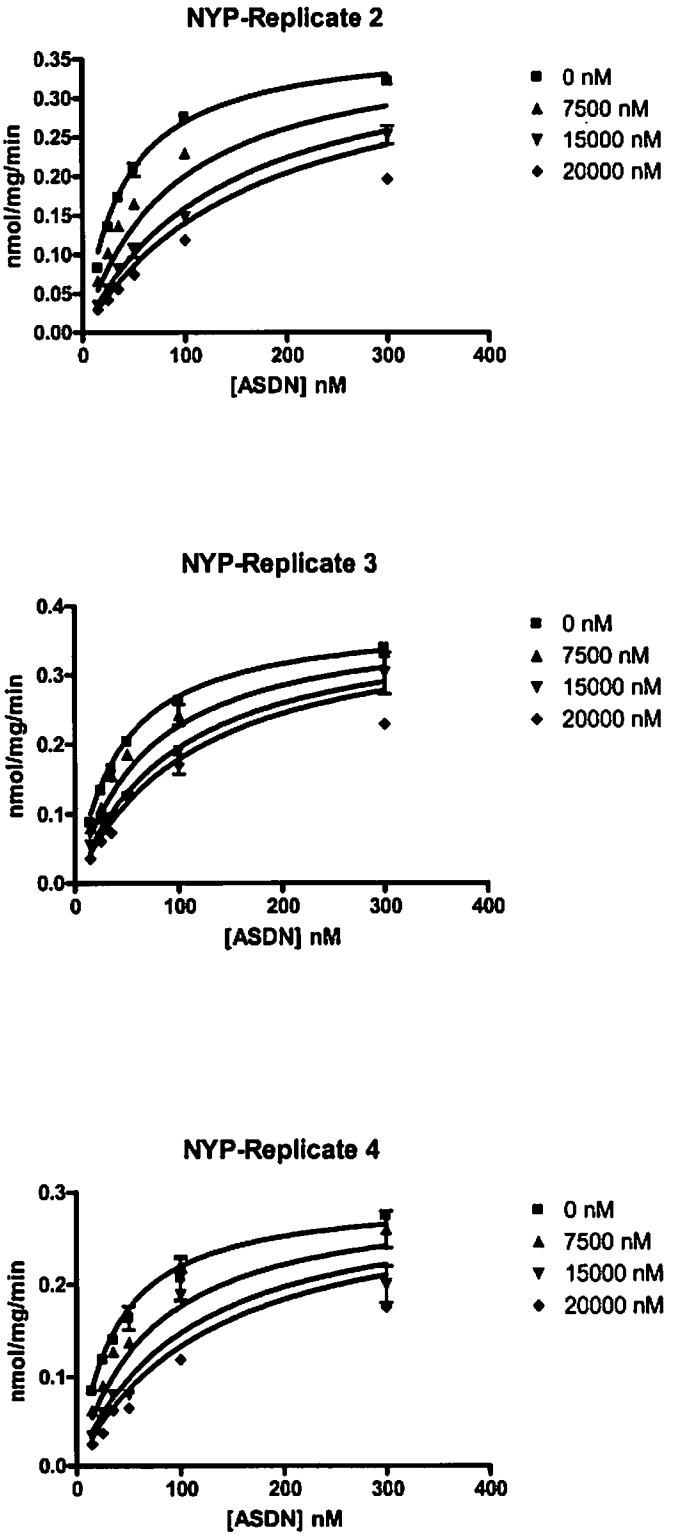
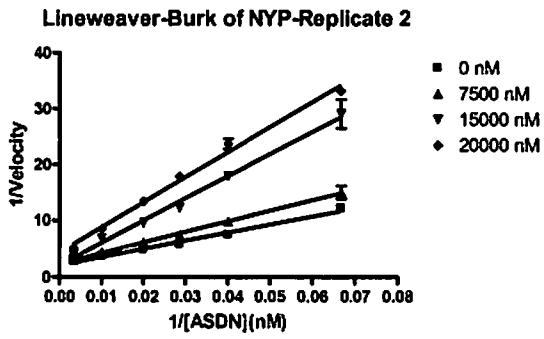
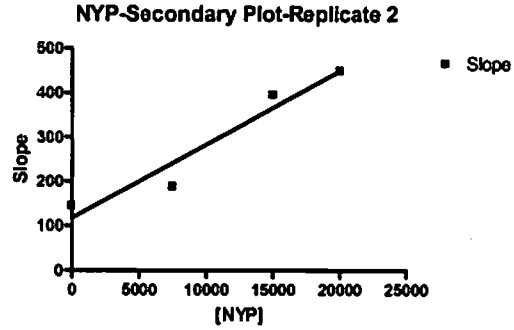


Figure 23. NYP Replicate 2 Linearized Plots

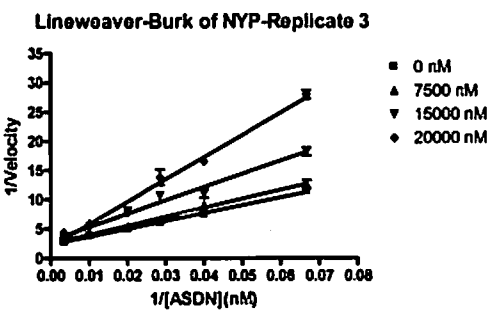


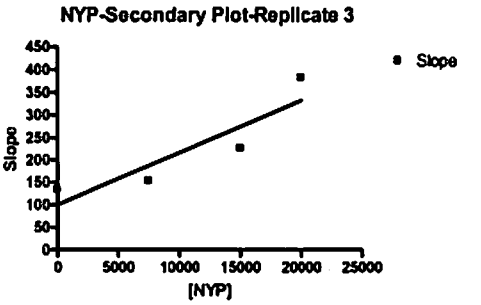


	0 nM	7500 nM	15000 nM	20000 nM
Best-fit values				
Slope	143.4 ± 9.190	187.9 ± 4.457	395.7 ± 15.95	449.0 ± 20.48
Y-intercept when X=0.0	2.126 ± 0.3221	2.331 ± 0.1562	2.141 ± 0.5591	4.332 ± 0.7177
X-intercept when Y=0.0	-0.01483	-0.01240	-0.005411	-0.009648
1/slope	0.006975	0.005321	0.002527	0.002227
95% Confidence Intervals				
Slope	117.9 to 168.9	175.6 to 200.3	351.4 to 440.0	392.2 to 505.8
Y-intercept when X=0.0	1.232 to 3.020	1.897 to 2.764	0.5892 to 3.693	2.339 to 6.324
X-intercept when Y=0.0	-0.02499 to -0.007482	-0.01559 to -0.009563	-0.01031 to -0.001366	-0.01581 to -0.004718
Goodness of Fit				
r^2	0.9838	0.9978	0.9935	0.9917
Sy.x	0.4718	0.2288	0.8188	1.051
Is slope significantly non-zero?				
F	243.3	1778	615.6	480.8
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.01667 ± 0.003112
Y-intercept when X=0.0	116.8 ± 40.61
X-intercept when Y=0.0	-7008
1/slope	59.97
95% Confidence Intervals	
Slope	0.003282 to 0.03007
Y-intercept when X=0.0	-57.92 to 291.6
X-intercept when Y=0.0	-80230 to 2133
Goodness of Fit	
r^2	0.9349
Sy.x	47.17
Is slope significantly non-zero?	
F	28.70
DFn, DFd	1.000, 2.000
P value	0.0331
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 24. NYP Replicate 3 Linearized Plots

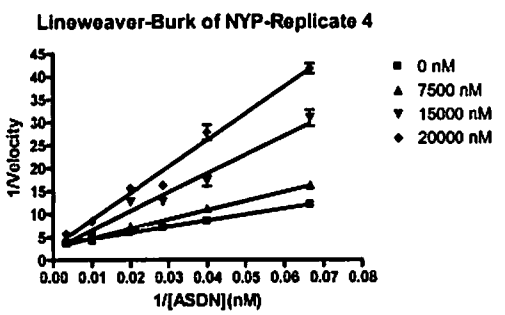


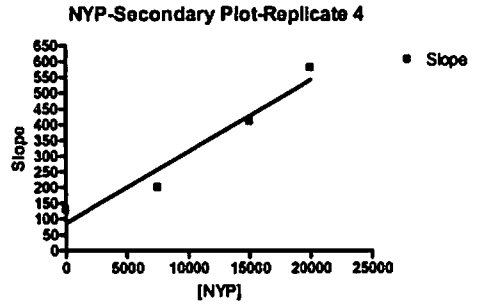


	0 nM	7500 nM	15000 nM	20000 nM
Best-fit values				
Slope	133.3 ± 3.662	153.0 ± 6.719	226.6 ± 16.72	382.0 ± 23.97
Y-intercept when X=0.0	2.372 ± 0.1284	2.483 ± 0.2355	3.061 ± 0.5861	2.012 ± 0.8401
X-intercept when Y=0.0	-0.01779	-0.01622	-0.01351	-0.005266
1/slope	0.007499	0.006535	0.004414	0.002618
95% Confidence Intervals				
Slope	123.2 to 143.5	134.4 to 171.7	180.1 to 273.0	315.5 to 448.5
Y-intercept when X=0.0	2.016 to 2.728	1.829 to 3.137	1.434 to 4.688	-0.3205 to 4.344
X-intercept when Y=0.0	-0.02192 to -0.01419	-0.02295 to -0.01083	-0.02525 to -0.005414	-0.01336 to 0.0007366
Goodness of Fit				
r^2	0.9970	0.9923	0.9787	0.9845
Sy.x	0.1880	0.3450	0.8584	1.231
Is slope significantly non-zero?				
F	1326	518.7	183.6	254.0
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	0.0002	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.01166 ± 0.003901
Y-intercept when X=0.0	99.95 ± 50.90
X-intercept when Y=0.0	-8575
1/slope	85.79
95% Confidence Intervals	
Slope	-0.005128 to 0.02844
Y-intercept when X=0.0	-119.1 to 319.0
X-intercept when Y=0.0	
Goodness of Fit	
r^2	0.8170
Sy.x	59.12
Is slope significantly non-zero?	
F	8.930
DFn, DFd	1.000, 2.000
P value	0.0961
Deviation from zero?	Not Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 25. NYP Replicate 4 Linearized Plots

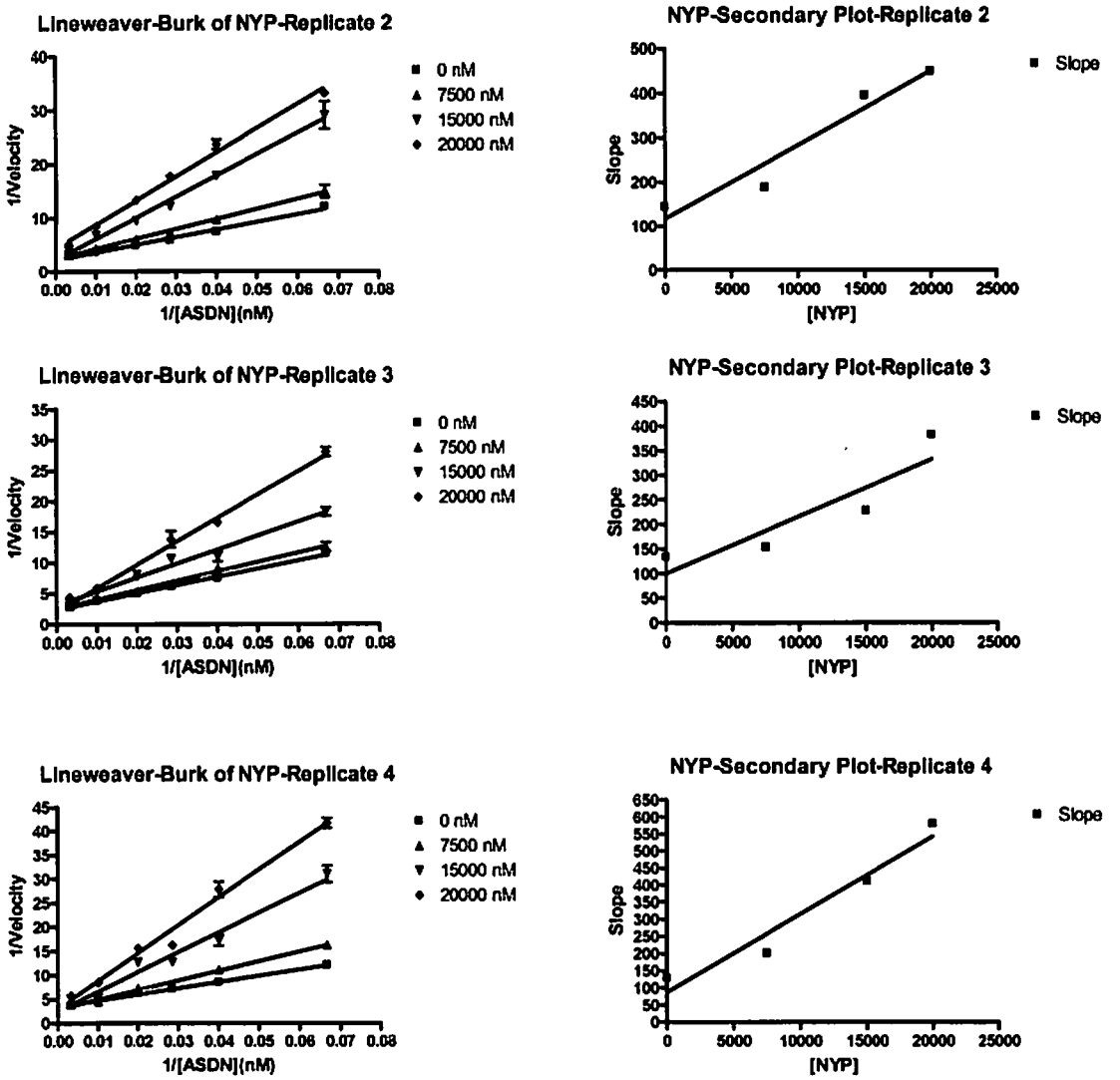




	0 nM	7500 nM	15000 nM	20000 nM
Best-fit values				
Slope	128.3 ± 3.233	199.6 ± 9.785	409.9 ± 35.62	579.2 ± 38.06
Y-intercept when X=0.0	3.452 ± 0.1133	2.891 ± 0.3430	2.472 ± 1.248	2.987 ± 1.334
X-intercept when Y=0.0	-0.02690	-0.01448	-0.006030	-0.005157
1/slope	0.007793	0.005009	0.002439	0.001726
95% Confidence Intervals				
Slope	119.3 to 137.3	172.5 to 226.8	311.1 to 508.8	473.6 to 684.9
Y-intercept when X=0.0	3.138 to 3.767	1.939 to 3.843	-0.9935 to 5.938	-0.7170 to 6.691
X-intercept when Y=0.0	-0.03131 to -0.02304	-0.02185 to -0.008719	-0.01831 to 0.002036	-0.01368 to 0.001081
Goodness of Fit				
r^2	0.9975	0.9905	0.9707	0.9830
Sy.x	0.1660	0.5024	1.829	1.954
Is slope significantly non-zero?				
F	1575	416.3	132.5	231.6
DFn, DFd	1.000, 4.000	1.000, 4.000	1.000, 4.000	1.000, 4.000
P value	< 0.0001	< 0.0001	0.0003	0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	6	6	6	6
Maximum number of Y replicates	1	1	1	1
Total number of values	6	6	6	6
Number of missing values	0	0	0	0

	Slope
Best-fit values	
Slope	0.02280 ± 0.003845
Y-intercept when X=0.0	87.02 ± 50.18
X-intercept when Y=0.0	-3817
1/slope	43.86
95% Confidence Intervals	
Slope	0.006253 to 0.03934
Y-intercept when X=0.0	-128.9 to 302.9
X-intercept when Y=0.0	-43430 to 3655
Goodness of Fit	
r^2	0.9462
Sy.x	58.27
Is slope significantly non-zero?	
F	35.16
DFn, DFd	1.000, 2.000
P value	0.0273
Deviation from zero?	Significant
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0

Figure 26. NYP Linearized Plots Replicates 2-4



4.0 Discussion

This study of the known competitive aromatase inhibitor, AG, has demonstrated the ability of the assay to properly characterize competitive inhibitors of aromatase activity in recombinant microsomes. Estimates for the kinetic parameters K_m, V_{max} and K_i obtained in the present study were in good agreement with literature and historic values. The Lineweaver-Burk plot contained a family of lines of different slopes with a common y-intercept, as is characteristic of competitive inhibition. The secondary plot of the slopes from the Lineweaver-Burk plot versus inhibitor concentration revealed the linear relationship indicative of competitive inhibition with the K_i defined as the negative of the x-intercept.

The interaction of NYP with aromatase activity in recombinant microsomes was studied using the same methods as were used for the study of AG. The K_i measured for NYP in this study (8.63 μM) was similar to the predicted value of 10 μM which was based on the IC_{50} determined in WA4-17, Task 4, assuming competitive inhibition. Visual examination of the Lineweaver-Burk and secondary plots indicates that the inhibition is primarily competitive, as evidenced by the common y-intercept for the lines on the Lineweaver-Burk plots. The relationship between the slopes of the Lineweaver-Burk plot and the inhibitor concentration (shown graphically on the secondary plots) may not be strictly linear which may be indicative of a small contribution of another inhibition type to the interaction of NYP and aromatase. The data obtained indicate that NYP acts primarily as a competitive inhibitor of recombinant aromatase.

5.0 References

Brueggemeier, RW, Hackett, JC and Diaz-Cruz, ES (2005) Aromatase inhibitors in the treatment of breast cancer. *Endocrine Reviews* **26**: 331-345.

Kao, Y-C, Korzekwa, KR, Laughton, CA and Chen, S (2001) Evaluation of the mechanism of aromatase cytochrome P450: a site-directed mutagenesis study. *Eur. J. Biochem.* **268**: 243-251.

RTI INTERNATIONAL

Quality Assurance Statement

Study Title: Characterization of the Inhibition of Aromatase Activity by Nonylphenol

Sponsor: U.S. EPA

EPA Task Order: 03

Protocol Number: RTI-980-AN

This study was audited by the Sciences and Engineering – Health Sciences Quality Assurance Unit and the results of the inspections and audits were reported to the task leader and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader and Management
Protocol Review	September 19, 2006	September 19, 2006
Sample Preparation Inspection	November 30, 2006	November 30, 2006
Data and Report Audit	February 5-8, 2007	February 8, 2007

Prepared by:

K Collier — 3/9/2007

K. Collier
Quality Assurance Specialist

Date

Reviewed by:

Susan J Wade — 3/9/07

Susan Wade
Quality Assurance Specialist

Date

Appendix 1

Study Protocol and Amendments

EPA Contract No.: EP-W-06-026
EPA Task Order No.: 03
RTI Project No.: 0210114.003.004

TITLE: Characterization of the Inhibition of Aromatase Activity by Nonylphenol

SPONSOR: U. S. EPA
U.S. Postal Service address:
OSCP/OPPTS/USEPA
1200 Pennsylvania Ave. NW (7203M)
Washington, DC 20460

Overnight/courier address:
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EPA East Bldg, Room 4106-H
1201 Constitution Ave. NW
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TESTING FACILITY: RTI International
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3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: September 28, 2006
PROPOSED EXPERIMENTAL END DATE: November 15, 2006

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			

sublock 11/27/06

Approved By:

Sherry L Black 10/30/06
Sherry Black, B.S. Date
RTI, Task Order Leader

10/25/06
Gary Timm, M.S., M.A. Date
EPA, Task Order Project Officer (TOPO)

Linda J Phillips 10/25/06
Linda J. Phillips, Ph.D. Date
EPA, Project Officer

Reviewed By:

K Collier 10/31/2006
Kim Collier, M.A. Date
RTI, Quality Assurance Specialist

Thomas McClintock 10/25/06
J. Thomas McClintock, Ph.D Date
EPA, Quality Assurance Manager

Table of Contents

List of Tables

1.0 OBJECTIVES

The objective of this task is to determine whether or not nonylphenol used in the validation of the aromatase (CYP19) assay is a competitive inhibitor of aromatase activity.

1.1 Justification for Test System

The test system for this study is recombinant microsomes from the same source used previously in the validation of the aromatase assay (EPA Contract 68-W-01-023, WA 4-17). This test system was selected because it provides a readily available biological source of the aromatase enzyme and a body of data exists for its performance in this assay.

1.2 Test Method

This *in vitro* test method involves combining microsomes, substrate, appropriate co-factors and potential inhibitors in a common reaction vessel. The effect of the potential inhibitors on microsomal enzyme activity is evaluated by measuring the amount of the product formed.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and recombinant microsomal preparations will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Nonradiolabeled and radiolabeled ASDN will be used. The nonradiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided by the Chemical Repository (CR). The CR will forward all applicable information regarding supplier, lot numbers, and reported/measured purity for the substrate to RTI and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN will be assessed at RTI. If the radiochemical purity is less than 95% the TOPO will be notified.

2.1.2 Radiochemical Purity

The radiochemical purity of the [^{3}H]ASDN will be determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 µL glass scintillant cell. Data will be collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method uses a Zorbax SB-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant will be monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions will be collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN will be analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN will be confirmed.

2.1.3 Preparation of Substrate Solution

Since the specific activity of the stock [^{3}H]ASDN is too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled [^{3}H]ASDN must be prepared so that the desired final concentration of ASDN in the assay can be achieved with about 0.05 – 0.3 µCi radiolabel in each incubation. Initial substrate concentrations to be used in the assay range from 15 to 300 nM; substrate concentrations for remaining replicates may be altered based on pilot study results.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine appropriate amount of the 1 µg/mL solution of ASDN the [^{3}H]ASDN dilution and buffer to make the substrate solution. Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca. 20 µL) and combine with scintillation cocktail for radiochemical content analysis. The addition of an appropriate amount (usually 50 to 300 µL, depending on the solution concentration and the desired final ASDN concentration) of the substrate solution to each 2 mL assay volume yields the desired final [^{3}H]ASDN concentration of about 0.05 - 0.3 µCi/tube. This range of radiolabel concentrations will allow for adequate quantitation of tritiated water, even when aromatase activity is inhibited 90%.

2.2 *Test Inhibitors*

Each inhibitor will be supplied neat by the Chemical Repository or a commercial supplier. Lot and source details will be maintained in the study records.

2.2.1 Aminoglutethimide (AG)

Aminoglutethimide (AG) is a known competitive inhibitor of aromatase and serves as the positive control chemical for this task.

CAS Number: 125-84-8

Molecular Formula: $C_{13}H_{16}N_2O_2$

Molecular Weight (g/mol): 232.3

2.2.2 Nonylphenol (NYP)

CAS Number: 84852-15-3

Molecular Formula: $C_{15}H_{24}O$

Molecular Weight (g/mol): 220.4

2.2.3 Inhibitor Formulation and Analysis

Inhibitor stock solutions will be prepared and analyzed by RTI. Stock solutions of each inhibitor will be analyzed by HPLC for determination of concentration. A standard curve (consisting of at least 5 points) for each inhibitor that relates detector response to known concentrations will be produced and used to calculate concentration of the stock solution. QC samples will be used to demonstrate the validity of the curve. Full details of the analytical method and results will be included in the study report. Inhibitor stock solutions will be stored refrigerated in sealed amber bottles. Under these conditions, stock solutions have been shown to be stable for at least 4 weeks (EPA Contract 68-W-01-023, WA4-17).

AG (0.1 M) and NYP (0.1 M) will be formulated in dimethylsulfoxide (DMSO). Stock solutions will be prepared in 50-mL volumetric flasks. Approximately 1161 ± 58 mg AG or 1102 ± 55 mg NYP will be weighed into a flask and DMSO will be added to 50-mL total volume to produce (after mixing) a 0.1 M solution. The total volume of inhibitor formulation used in each assay should be consistent across all concentrations of inhibitor and no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use such that the target concentration of inhibitor can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

2.3 Microsomes

Recombinant microsomes will be purchased from Gentest (Woburn, MA). The product name is Human CYP19 + P450 Reductase SUPERSOMES™, catalog number 456260. The microsomes must be stored at approximately -70 to -80 °C. The approximate protein content of the microsomes will be provided by the supplier. The product data sheet for the batch of microsomes used will be maintained in the study records.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. that is used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

If the recombinant microsomes are supplied in aliquots in excess of what is required to conduct a single experiment, they will be thawed, pooled, homogenized, divided into appropriate aliquots for conduct of a single experiment and refrozen as described below in order to minimize and standardize the number of freeze/thaw cycles each preparation undergoes. Microsomes will be thawed quickly in a 37 ± 1 °C water bath and then will be immediately transferred to an ice bath. The microsomes will be pooled and rehomogenized using a Potter-Elvejhem homogenizer (about 5–10 passes). The pooled sample will be aliquoted into portions appropriate for use in a single experiment (ca. 160 µL, dependant on the protein concentration of the preparation) and

the samples will be flash frozen and stored at approximately -70 to -80 °C for future use. Each tube will provide enough protein for a single experiment and any excess thawed microsomal preparation will be discarded.

On the day of use, microsomes will be thawed quickly in a 37 ± 1 °C water bath and then will be immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) or by vortexing about 5 seconds prior to use. The microsomes will be diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.008 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.004 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. Microsomes are not to be left on ice for longer than approximately 1 h before proceeding with the assay. Appropriate documentation of time from thaw to use will be maintained.

Diluted microsomes must be used only on the day of preparation. Under no circumstances will diluted microsomes be refrozen for later use in the assay.

2.4 Other Assay Components

2.4.1 Buffer

The assay buffer will be 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (JT Baker, cat # 4062-01, 141.96 g/mol) will be used in the preparation of the buffer. Solutions of each reagent at 0.1 M will be prepared in distilled, deionized water and then the solutions will be combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (2–8 °C).

2.4.2 Propylene Glycol

Propylene glycol (JT Baker, cat # 9402-01, 76.1 g/mol) will be added to the assay directly as described below.

2.4.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, Sigma, cat # 1630, 833.4 g/mol) is the required co-factor for CYP19. The final concentration in the assay is 0.3 mM. Typically, a 6 mM stock solution will be prepared in assay buffer and then 100 µL of the stock will be added to the 2 mL assay volume. NADPH must be prepared fresh each day and will be kept on ice.

3.0 PROTEIN ASSAY

The protein concentration of the microsome preparation will be determined once in order to confirm the protein concentration in the assays. QC standards (nominal protein

concentrations of 10 and 100 µg/mL) will be run in duplicate with each run of the protein assay. A 6-point standard curve will be prepared, ranging from 5 to 250 µg protein/mL. The protein curve standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 200 µL aliquot of unknown, QC or curve standard, 100 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 800 µL of BioRad DC Protein Kit Reagent B will be added to each sample and the samples will be vortex mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow for color development. Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) will be measured using a spectrophotometer. The absorbances are stable for about 1 h. The protein concentration of the microsomal sample will be determined by interpolation of the absorbance value using the curve developed from the protein standards.

4.0 STUDY DESIGN - INHIBITION ASSAYS

The effect of two inhibitors on aromatase activity will be determined. AG (a known competitive inhibitor of aromatase activity) will serve as the control competitive inhibitor and results from experiments with AG will be used to demonstrate that the experimental design can accurately characterize a competitive inhibitor. The test inhibitor will be NYP and the aim of the study is to characterize the inhibition of aromatase by nonylphenol (determine both K_i and inhibition type). There will be four replicates for each inhibitor. Each replicate will test the response of aromatase activity (see Section 6.0) to the presence of three concentrations of an inhibitor and will include control tubes with no inhibitor; the concentration of substrate will also vary (over 6 concentrations) in each replicate. The initial substrate and inhibitor concentrations are presented in Table 1 and these will be used in the pilot study of each respective inhibitor. These concentrations may be modified for the remaining replicates based on the results of the pilot studies. [If there are no changes in concentrations (inhibitor or substrate) required between the pilot study run and the remaining replicates, the pilot study may serve as one of the four replicates.] There will be two repetitions (tubes) for each condition (substrate/inhibitor concentration combination) of a given replicate. In addition, quadruplicate background activity control tubes will be included for each replicate (substrate, propylene glycol, buffer, vehicle [used for preparation of reference chemical solutions] and microsomes). The control tubes will be treated the same as the other samples.

Table 1. Proposed Initial Substrate and Inhibitor Concentrations

Assay Component	Chemical	Estimated K_m or K_i[a]	Proposed Assay Concentrations
Substrate	[^{3}H]ASDN	50 nM	15, 25, 35, 50, 100, 300 nM
Test inhibitor	AG	48 µM	0, 25, 50, 100 µM
Test inhibitor	NYP	10 µM	0, 5, 10, 20 µM

[a] AG IC_{50} = 95 µM; NYP IC_{50} = 21 µM (estimates are from aromatase validation studies where substrate ([^{3}H]ASDN) concentration was 100 nM)

5.0 PILOT STUDY

A single replicate of the inhibition assay using each inhibitor (AG, 4-OH ASDN and NYP) will be conducted using the initial proposed concentrations of inhibitor and substrate. The data will be plotted and reviewed and, if necessary, adjustments to the concentrations of inhibitor and/or substrate will be made prior to the conduct of the remaining replicates.

6.0 AROMATASE ASSAY METHOD

The assays will be performed in 13 x 100 mm test tubes maintained at 37 ± 1 °C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, inhibitor (AG or NYP) or vehicle, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The inhibitor (and vehicle) will be added in a volume not to exceed 20 µL. The final concentrations for the assay components are presented in Tables 1 and 2. The tubes and the microsomal suspension will be placed at 37 ± 1 °C in the water bath for 5 min prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will then be vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman Allegra X-15R centrifuge with a 4750a rotor for 10 min at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers will be extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Table 2. Optimized Aromatase Assay Conditions

Assay factor (units)	Value
Microsomal Protein (mg/mL)[a]	0.004
NADPH (mM)[a]	0.3
Incubation Time (min)	15

[a] Final concentration

Analysis of the samples will be performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represents tritiated water formed.

7.0 DATA RECORDING AND ANALYSIS

7.1 Aromatase Activity

Assay data will be recorded on data forms that are designed to capture all required data.

LSS data is captured automatically into an electronic file. Relevant data are entered into a verified spreadsheet for calculation of aromatase activity (velocity).

7.2 *Lineweaver-Burk Plots*

Data will be plotted in double reciprocal (Lineweaver-Burk) plots of 1/velocity vs. 1/(substrate concentration) using GraphPad Prism, version 4.03. The plots will be examined and the type of inhibition will be characterized. In the case of competitive inhibition, the plots will contain four lines (one for each inhibitor concentration) of different slopes with a common y-intercept. Non-competitive inhibition is evident when the plot contains four lines with a common x-intercept. Uncompetitive inhibition results in a plot containing lines with neither a common x- or y-intercept. V_{max} and K_m will be estimated and reported from the plotted data.

7.3 *Secondary Plots*

The slopes from the Lineweaver Burk plots will be replotted as a function of inhibitor concentration. On these plots, the x-intercept equals $-K_i$ and K_i will be reported.

7.4 *Simultaneous Nonlinear Regression for K_i Determination*

Reaction velocity, substrate and inhibitor concentration data will be entered into GraphPad Prism, version 4.03 for the calculation of K_m, V_{max} and K_i using simultaneous nonlinear regression methods. This method can accurately estimate K_m and V_{max} and generally yields improved estimates of K_i than those obtained through linear transform plotting methods such as those described in Section 7.2 and 7.3.

8.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the RTI archives for the life of the contract.

9.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. This study will not be conducted in strict accordance with the FDA Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58, but will be conducted to the highest standard of record keeping, and in accordance with the applicable Standard Operating Procedures of RTI International.

10.0 REPORTS

10.1 Preliminary Data Submissions

Data from the pilot study for each inhibitor will be submitted to the TOPO for review and approval before proceeding with the remaining replicates for the inhibitor. Data and data analysis (as described in Section 7) from the four replicate assays of AG will be provided to EPA for review and approval before proceeding to the conduct of the replicate assays for inhibition by NYP.

10.2 Final Report

A final report will be prepared that includes (at minimum) a full description of the work performed in the laboratory, summaries of the data in tabular and graphical format (as described in Section 7) and conclusions drawn from the data.

10.3 Electronic Data Submissions

Electronic files containing all raw and analyzed data will be submitted. In addition, Prism data files (from which the reported graphs are derived) will be submitted to allow for any future reanalysis of the data.

11.0 STUDY RECORDS TO BE MAINTAINED

- 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
- QAPP and any Amendments
- List of any QAPP Deviations

EPA Contract No.: EP-W-06-026
EPA Task Order No.: 03
RTI Project No.: 0210114.003.004

Amendment 1

TITLE: Characterization of the Inhibition of Aromatase Activity by Nonylphenol

SPONSOR: U. S. EPA
U.S. Postal Service address:
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1200 Pennsylvania Ave. NW (7203M)
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TESTING FACILITY: RTI International
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3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: September 28, 2006
PROPOSED EXPERIMENTAL END DATE: November 15, 2006

PROTOCOL	RTI International P.O. Box 12194 Research Triangle Park, NC 27709	RTI-980-AN Amendment 1 Page 2 of 3

Approved By:

Sherry Black 12/4/06
Sherry Black, B.S. Date
RTI, Task Order Leader

Gary Timm 12/5/06
Gary Timm, M.S., M.A. Date
EPA, Task Order Project Officer (TOPO)

Linda J. Phillips 12/5/06
Linda J. Phillips, Ph.D. Date
EPA, Project Officer

Reviewed By:

K. Collier 12/4/06
Kim Collier, M.A. Date
RTI, Quality Assurance Specialist

J. Thomas McClintock 12/06/06
J. Thomas McClintock, Ph.D Date
EPA, Quality Assurance Manager

Item 1

Section 3.0 PROTEIN ASSAY, which read:

The protein concentration of the microsome preparation will be determined once in order to confirm the protein concentration in the assays. QC standards (nominal protein concentrations of 10 and 100 • g/mL) will be run in duplicate with each run of the protein assay. A 6-point standard curve will be prepared, ranging from 5 to 250 • g protein/mL. The protein curve standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 200 • L aliquot of unknown, QC or curve standard, 100 • L of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 800 • L of BioRad DC Protein Kit Reagent B will be added to each sample and the samples will be vortex mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow for color development. Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) will be measured using a spectrophotometer. The absorbances are stable for about 1 h. The protein concentration of the microsomal sample will be determined by interpolation of the absorbance value using the curve developed from the protein standards.

Is hereby amended as follows: (changes in **bold**)

3.0 PROTEIN ASSAY

The protein concentration of the microsome preparation will be determined once in order to confirm the protein concentration in the assays. QC standards (nominal protein concentrations of **500** and **1000** • g/mL) will be run in duplicate with each run of the protein assay. A 6-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 (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). **To a 25 • L aliquot of unknown or standard, 125 • L of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or unknown and the samples will be vortex mixed.** The samples will be allowed to sit at room temperature for at least 15 min to allow for color development. Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) will be measured using a spectrophotometer. The absorbances are stable for about 1 h. The protein concentration of the microsomal sample will be determined by interpolation of the absorbance value using the curve developed using the protein standards.

Justification

The expected concentration of protein in the first dilution of the microsome stock is expected to be greater than 250 µg/mL, therefore, the updated procedure is better suited to the accurate determination of protein concentration. The assay using a standard curve ranging from 0.13-1.5 mg/mL is defined as the 'Standard Assay Protocol' for the DC Protein Kit and has been used for many years in our laboratory with good results.

EPA Contract No.: EP-W-06-026
EPA Task Order No.: 03
RTI Project No.: 0210114.003.004

Amendment 2

TITLE: Characterization of the Inhibition of Aromatase Activity by Nonylphenol

SPONSOR: U. S. EPA
U.S. Postal Service address:
OSCP/OPPTS/USEPA
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Washington, DC 20460

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Washington, DC 20004

TESTING FACILITY: RTI International
DMPK
3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: September 28, 2006
PROPOSED EXPERIMENTAL END DATE: November 15, 2006

PROTOCOL	RTI International P.O. Box 12194 Research Triangle Park, NC 27709	RTI-980-AN Amendment 2 Page 2 of 3

Approved By:

Sherry Black, B.S. — 3/1/07 — Date
RTI, Task Order Leader

Gary Timm, M.S., M.A. — 3/07/08 — Date
EPA, Task Order Project Officer (TOPO)

Linda J. Phillips, PH.D. — 3/8/08 — Date
EPA, Project Officer

Reviewed By:

Kim Collier, M.A. — 3/1/2007 — Date
RTI, Quality Assurance Specialist

J. Thomas McClintock, Ph.D — 3/9/07 — Date
EPA, Quality Assurance Manager

The original signature page was lost during shipping. SBlack 3/20/07

Item 1

4.0 STUDY DESIGN - INHIBITION ASSAYS, which read:

Table 1. Proposed Initial Substrate and Inhibitor Concentrations

Assay Component	Chemical	Estimated K_m or K_i[a]	Proposed Assay Concentrations
Substrate	[^{3}H]ASDN	50 nM	15, 25, 35, 50, 100, 300 nM
Test inhibitor	AG	48 μM	0, 25, 50, 100 μM
Test inhibitor	NYP	10 μM	0, 5, 10, 20 μM

[a] AG IC_{50} = 95 μM; NYP IC_{50} = 21 μM (estimates are from aromatase validation studies where substrate ([^{3}H]ASDN) concentration was 100 nM)

Is hereby amended as follows: (changes in **bold**)

3.0 PROTEIN ASSAY

Table 1. Proposed Initial Substrate and Inhibitor Concentrations

Assay Component	Chemical	Estimated K_m or K_i[a]	Proposed Assay Concentrations
Substrate	[^{3}H]ASDN	50 nM	15, 25, 35, 50, 100, 300 nM
Test inhibitor	AG	48 μM	0, 25, 50, 100 μM
Test inhibitor	NYP	10 μM	0, 5, 10, 20 μM

[a] AG IC_{50} = **4.76** μM; NYP IC_{50} = 21 μM (estimates are from aromatase validation studies where substrate ([^{3}H]ASDN) concentration was 100 nM)

Justification

This change is made to correct an error in the cited estimated IC_{50} from WA 4-17, Task 4. Please see the Overall Task Draft Final Report (Battelle) in the study record for an explanation of the discovery and correction of the error.

Appendix 2

QAPP

Quality Assurance Project Plan (QAPP)
For Task Order 3
Characterization of the Inhibition of Aromatase Activity by Nonylphenol

EPA CONTRACT NUMBER EP-W-06-026

October 2006

SIGNATURE PAGE

Quality Assurance Project Plan for Task 3
Characterization of the Inhibition of Aromatase Activity by Nonylphenol
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Concurrences and Approvals

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1.0 PROJECT ORGANIZATION

Table 1 lists RTI International and EPA personnel assigned to this project and their roles.

Table 1. Task Order 3 Staff

Staff	Affiliation	Role for Task Order 3
Gary Timm, M.S., M.A.	EPA	Task Order Project Officer (TOPO)
Shirlee Tan, Ph.D.	EPA	Alternate TOPO
J. Thomas McClintock, Ph.D.	EPA	QA manager
Julia George, PhD	RTI	Program Manager
Bonnie Hamby, B.S.	RTI	Task Order Manager
Sherry Black, B.S.	RTI	Task Order Leader
Purvi Patel, B.S.	RTI	Chemist
James Mathews, Ph.D.	RTI	Consultant
Kimberly Collier, M.A.	RTI	Quality Assurance Specialist
Jennifer Farlow, B.S.	RTI	Financial Specialist
Cathee Winkie	RTI	Administrative Coordinator

1.1 RTI International Personnel

Further descriptions of these roles and the resumes of the RTI team were included in the proposal submitted for this task order. All staff have the training and experience necessary to perform their roles.

Dr. Julia George and Ms. Bonnie Hamby will oversee general task order management. They will be responsible for tracking the expenditure of hours and funds, monitoring task progress, and communicating with the EPA TOPO on all contract issues.

Ms. Sherry Black will be the Task Order Leader, and will be responsible for technical management of the task order. She will oversee and aid in the conduct of the laboratory study. She will oversee and direct the efforts of Ms. Purvi Patel on the laboratory work. Ms. Black will be responsible for data analysis, quality control and reporting of the laboratory work. She will consult with Dr. Mathews and Mr. Timm on technical issues and secure approval of EPA (through Mr. Timm) as required by the Task Order Statement of Work to proceed from one experimental set to the next. Ms. Black will be responsible for responding to data audits and correcting deficiencies.

Ms Purvi Patel will carry out experimental work in accordance with the QAPP, study protocol and instructions of Ms. Black. She will aid in the data analysis, quality control and report preparation.

Kimberly Collier will administer the QAPP at RTI. Her specific responsibilities include:

- Interact with the Task Order Leader to ensure that QA and QC procedures are understood by Task personnel.
- Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the task with respect to the EDSP QMP, the task QAPP and study protocol, and applicable program and facility SOPs.
- Prepare and track reports of deficiencies and submit them to both line and program management.
- Consult with the Task Order Leader and, as necessary, the EPA EDSP QA Manager and RTI Program Manager on actions required to correct deficiencies noted during the conduct of the Task.
- Ensure that all data produced as part of the task are maintained in a secure, environmentally-protected archive.
- Ensure, during the conduct of TSAs, that all staff participating on the task are adequately trained.
- Maintain complete QA records related to the task.
- Submit a QA Statement with the final report that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.
- Maintain effective communication with the EPA QA Manager.

James Mathews will serve as technical consultant for the task and will advise Ms. Black on any problems encountered during task conduct.

1.2 EPA Personnel

Mr. Gary Timm (USEPA) will serve as the Task Order Project Officer (TOPO) for this task. In this role, he will communicate with the RTI Program Manager on matters of task management and scheduling. He will consult with the Task Order Leader on experimental details that have not been defined as of the submission of the Technical Proposal, such as the selection of a control inhibitor and selection of substrate and inhibitor concentrations, etc. He will review data submitted from each group of experiments and provide the necessary approval, as appropriate, for RTI to proceed with the next set of experiments.

Dr. Shirlee Tan is the alternate TOPO for this task and can fulfill all TOPO functions if Mr. Timm is unavailable.

Dr. J. Thomas McClintock is the EPA QA manager for this task. He will review and approve the QAPP before any laboratory work can begin.

2.0 PROBLEM DEFINITION/BACKGROUND

2.1 Problem Definition

The objective of this task is to determine whether nonylphenol used in the validation of the aromatase assay is a competitive inhibitor of aromatase activity.

2.2 Background

Nonylphenol (CAS No. 84852-15-3) was used as one of the reference chemicals for the interlaboratory validation of the estrogen receptor (ER) binding and the placental and recombinant microsomal aromatase assays. Initial studies of nonylphenol during the prevalidation phase of testing gave partial curves in the ER binding assay, demonstrating its activity as an estrogen receptor agonist, but a negative response (a noninhibitor) in the aromatase assay. The EPA was interested when, in subsequent interlaboratory studies, nonylphenol appeared to inhibit aromatase activity. Since the later studies used higher concentrations of nonylphenol than those used during the initial studies, it is not known whether or not nonylphenol is a true competitive inhibitor of aromatase activity or if these data reflect a false positive. No historical data exist to document whether or not nonylphenol is a competitive inhibitor of aromatase activity at these higher concentrations. Therefore, it is necessary to conduct secondary K_i experiments to appropriately classify this chemical as an inhibitor or noninhibitor of aromatase activity.

There are several ways a chemical can inhibit enzymatic activity. For example, a competitive inhibitor binds reversibly to the active site of the enzyme and thereby prevents the binding of the natural substrate. Thus, the binding of the substrate and competitive inhibitor to the active site is mutually exclusive. Noncompetitive inhibitors modify the structure of the enzyme and render it no longer capable of activity. A third type of nonspecific inhibition occurs when the chemical causes physical or chemical changes in the enzyme, resulting in a denaturation of the protein as may occur when high concentrations of a test chemical are used in an *in vitro* assay. When testing environmental chemicals for their ability to inhibit aromatase activity, it is important to understand the type of inhibition, since a nonspecific inhibition would result in a false positive classification for the chemical.

The type of inhibition may be characterized by conducting a series of experiments that evaluate the catalytic activity of the enzyme in the absence or presence of a chemical inhibitor. The experiment is designed to study enzymatic activity at several fixed and nonsaturating concentrations of the test chemical in the presence of varying substrate concentrations. Plotting the data on a double reciprocal plot (1/v versus 1/S; Lineweaver-Burk Plot) provides a pattern of lines that are indicative of competitive or noncompetitive inhibition. The negative x-intercept of a

linear replot of the slopes yields an experimental K_i (inhibitor concentration). These relationships can be used to distinguish chemicals which compete for binding with the substrate at the active site of the enzyme from chemicals which interfere with the enzyme through noncompetitive or nonspecific inhibition.

3.0 TECHNICAL APPROACH

Aminoglutethimide, a known competitive inhibitor of aromatase activity, will be used in this task as the control inhibitor to demonstrate that the proposed methods can be used to characterize a competitive inhibitor for the enzyme. A pilot study (one replicate of the assay) will be conducted first using six substrate and three (plus a no-inhibitor control) concentrations of aminoglutethimide. This data will be analyzed, graphed and kinetic parameters will be estimated according to methods described in the study protocol. The data will be reviewed by Ms. Black and Mr. Timm and appropriate modifications to the assay (perhaps changing the substrate or inhibitor concentrations) will be made before proceeding with the remaining replicates of the assay employing aminoglutethimide as the inhibitor. Data from the four replicates will be examined to determine acceptable limits of variance which can then be applied in the next phase of the study – the investigation of the kinetics of aromatase inhibition by nonylphenol. The entire set of data produced for aminoglutethimide (four replicates) will be reviewed and approved Mr. Timm before experiments using nonylphenol can commence.

The experiments with nonylphenol will proceed in much the same manner as described above for aminoglutethimide. The same substrate concentrations will be used. A pilot study will be conducted using a set of inhibitor concentrations and modifications to the concentrations will be made as necessary prior to the conduct of the other replicates.

Full experimental details (including initial substrate and inhibitor concentrations) are described in the attached study protocol (Appendix).

4.0 QUALITY SYSTEMS

The following general quality systems are in place to ensure that the data produced are reliable, reproducible and of high quality.

- Experimental procedures will be conducted in accordance with the study protocol and applicable SOPs. Any modifications to procedures will be documented as protocol amendments (if they are planned) or as deviations. Amendments are approved by the Task Order Leader and the TOPO. Any deviations are documented and assessed by the Task Order Leader for their impact on the study and are included in the record and the reports.
- Data collection and recording will be done according to study protocol and SOP requirements.

- All instruments/equipment will be maintained and calibrated according to procedures described in applicable SOPs. Any instruments/equipment that do not meet criteria set forth in the applicable SOP will not be used on this task.
- Quality control (QC) procedures will include a 100% check of all transcribed data and the verification of all Excel spreadsheets used for calculation. These QC checks will be documented on printouts by the reviewer's initials and date.
- The Task Order Leader will review the data forms and data for each replicate for completeness and accuracy and will ensure that any errors or omissions are corrected or documented appropriately.
- RTI's QAU will provide data review and audits as described in Section 8.0 and applicable SOPs.

5.0 DATA ACCEPTANCE/REJECTION CRITERIA

In this task, the aromatase activity of recombinant microsomes is measured in the presence of varying concentrations of substrate and inhibitors using a defined experimental protocol. Relevant weights, volumes and conditions (i.e., temperature) of the assay components are collected and recorded using calibrated instruments in accordance with SOPs and the study protocol. The aromatase activity measurement is based on radioactivity remaining in the aqueous portion of the reaction mixture after extraction. Duplicate aliquots of the aqueous portion of the reaction mixture are assayed for radiochemical content using liquid scintillation counters that have been calibrated and performance-verified according to SOPs. Radiochemical content data for each assay tube, along with data on assay contents (solution concentrations, etc) are used to calculate reaction velocity. Reaction velocity and substrate and inhibitor concentration data are used to calculate K_m, V_{max} and K_i. Therefore control of the variance of radiochemical content and reaction velocity data will yield K_m, V_{max} and K_i estimates with low variability.

The following key data will be examined and accepted or rejected as described below:

1. *Radiochemical content of aliquots of extracted reaction mixture (from a given assay tube).* The duplicate aliquots should be within the mean ± 10%. If this is not the case the following actions will be taken: 1) realiquot the reaction mixture and test for acceptable variance; 2) if variance is still too high, exclude the data from that tube from the data set.
2. *Reaction velocity data compared across replicates.* Reaction velocity data will be examined across replicates (two tubes per condition per replicate and four replicates yield a set of 8 data points per condition) and any that appear to be statistical outliers will be subjected to the Q-test (Dean and Dixon, 1951) and excluded as appropriate. Use of this test will eliminate data containing gross errors with 90% confidence. If 3 or more substrate concentration data points for a given inhibitor concentration are eliminated, all data from that inhibitor concentration in that

replicate will not be used in the calculation of K_i. In that case, the TOPO will be consulted as to whether the entire replicate should be rerun.

6.0 DOCUMENTS AND RECORDS

Laboratory and quality management procedures for this task order are described in this QAPP, the study protocol and applicable SOPs. Each of these documents (and any amendments/revisions to them) is distributed to study personnel. It is the responsibility of the Task Order Leader to ensure that all staff have access to the most recent versions of each document. The QAPP and SOPs are controlled by version number in the document header. Protocol amendments are sequentially numbered and distributed upon approval.

Study records to be maintained are detailed in the protocol. Study records will be maintained in the laboratories during the conduct of the study. At the conclusion of the study (i.e., when the final report is signed), records will be transferred to the RTI archives (in accordance with relevant SOPs) and will be retained for the life of the contract. Records may be transferred to the Sponsor if so directed.

Requirements for preliminary data submissions, the final report and electronic data submissions are detailed in the study protocol.

Other reports that will not be included in the above-named reports include QA audit reports and status/progress reports. QA assessment reports are maintained as confidential files in the RTI's QAU. Status/progress reports will be submitted to the EPA TOPO on a monthly basis as stipulated in the contract.

7.0 APPLICABLE SOPS

Table 2 lists applicable SOPs for this Task. The current version of each of these documents is readily available to RTI project staff on RTI's intranet.

Table 2 Applicable SOPs

SOP	Subject
SEG-ADM-003	RTI Notebooks
SEG-ADM-004	Handwritten Records
SEG-ADM-005	Personnel Training Files
SEG-ADM-007	Study Protocols
SEG-EQP-004	Analytical Balances
SEG-EQP-010	pH Meters
SEG-EQP-011	Liquid Scintillation Counters

SOP	Subject
SEG-GLC-002	HPLC: Pumps
SEG-GLC-004	HPLC: UV/Vis detectors
SEG-GLC-006	HPLC: Radioactivity detectors
SEG-GLC-007	HPLC: Autosamplers
SEG-QAU-002	Initiating QAU Audits
SEG-QAU-003	Performing QAU Audits
SEG-QAU-004	Performing QAU Inspections
SEG-QAU-005	Response to QAU Reports
SEG-QAU-007	QA Statements
SEG-QAU-008	Master Schedule
SEG-QAU-010	Archival Storage
DPK-EQP-003	Dubnoff Metabolic Shaking Incubator
DPK-EQP-004	UV Spectrophotometer
DPK-SAM-001	Chemical Receipt
DPK-SAM-002	Test Article Use Log
CCS-EQP-003	Pipettors

8.0 ASSESSMENTS AND RESPONSE ACTIONS

RTI QA team members will perform assessments on task activities and operations affecting data quality and the raw data and final report. They will report any findings to the Task Order Leader and management to ensure that the requirements in relevant SOPs, study protocol and QAPP and the QMP are met.

9.0 RECONCILIATION AND USER REQUIREMENTS

The objective of this study is to determine whether nonylphenol is a competitive inhibitor of aromatase activity in the recombinant microsome model. The data will be analyzed as described in the study protocol in order to determine the type of inhibition present. Because it is possible that a mixed-type or mechanism-based inhibition process is active, the data from the current study design may not definitively determine the inhibition type. Any such findings that cloud the interpretation of the results will be discussed in the study report.

10.0 REFERENCES

Dean, R.B. and Dixon, W.J. (1951) Simplified statistics for small numbers of observations. *Analytical Chem.* **23**: 636-638.

APPENDIX

STUDY PROTOCOL FOR TASK ORDER 3

Appendix 3

Certificates of Analysis

SIGMA-ALDRICH

Certificate of Analysis

Product Name	4-Androstene-3,17-dione
Product Number	A9630
Product Brand	SIGMA
CAS Number	63-05-8
Molecular Formula	$C_{19}H_{26}O_2$
Molecular Weight	286.41

TEST	SPECIFICATION	LOT 024K0809 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF-WHITE POWDER
SOLUBILITY	CLEAR, COLORLESS TO FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF CHLOROFORM	CLEAR VERY FAINT YELLOW
ULTRAVIOLET/VISIBLE SPECTRUM	EMM = 15.9 TO 16.5 AT LAMBDA MAX 239 TO 240 NM IN ETHANOL	EMM = 16.1 AT LAMBDA MAX 240 NM
PURITY BY HPLC	98% MINIMUM	100%
SHELF LIFE	5 YEARS	FEBRUARY 2009
QC ACCEPTANCE DATE		FEBRUARY 2004
PRODUCT CROSS REFERENCE INFORMATION		REPLACEMENT FOR ALDRICH #285137

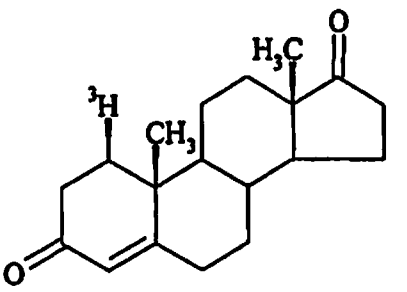
Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

PerkinElmer
PerkinElmer Life and Analytical Sciences
549 Albany Street, Boston, MA 02118

Technical Data
Certificate of Analysis

NET-926 ANDROST-4-ENE-3, 17-DIONE, [1β-^{3}H(N)]-

Lot Number:	3557306
Specific Activity:	23.5 Ci/mmol
	0.87 TBq/mmol
Production Date:	May 18, 2006



M.W. 286
$C_{19}H_{26}O_2$

PACKAGING: 1.0 mCi/ml (37 MBq/ml) in ethanol. Shipped on dry ice.

STABILITY AND STORAGE RECOMMENDATIONS:

When androst-4-ene-3, 17-dione, [1β-^{3}H(N)]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is approximately 1% for 6 months from date of purification. Lot to lot variation may occur, and it is advisable to check purity prior to use.

SPECIFIC ACTIVITY RANGE: 15-30 Ci/mmol (0.55-1.11 TBq/mmol)

RADIOCHEMICAL PURITY: This product was initially found to be greater than 97% when determined by the following methods:

1. High pressure liquid chromatography on a Zorbax ODS column using the following mobile phase:

   water : tetrahydrofuran : methanol (40:15:45)

2. Paper chromatography on Whatman No. 1 treated with 30% formamide in acetone using the following solvent system:

   hexane saturated with formamide.

3. Thin layer chromatography on silica gel using the following solvent system:

   toluene : ethyl acetate, (2:1).

QUALITY CONTROL: The radiochemical purity of androst-4-ene-3, 17-dione, [1β-^{3}H(N)]-is checked at appropriate intervals using the first listed chromatography method. Current purity data is available upon request.

PREPARATIVE PROCEDURE: Androst-4-ene-3, 17-dione, [1β-^{3}H(N)]- is prepared by treatment of androst-4-ene-3, 17-dione, [1β,2β-^{3}H(N)]- with potassium hydroxide under appropriate conditions (1) Purification is by HPLC.

REFERENCE:

1. R. Gore-Langton, et al., *Endocrinology*, 107, 464 (1980).

HAZARD INFORMATION: WARNING:

WARNING:
Harmful by Contact, Ingestion, Inhalation.
Effects of Exposure: Irritating to Eyes; Slightly Toxic; Flammable.
Target Organs: Central Nervous System.

WARNING: This product contains a chemical known to the state of California to cause cancer.

0186:111596

2097 Bat 1 EAC

SIGMA-ALDRICH

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Saint Louis, Missouri 63105 USA
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Certificate of Analysis

BATTELLE NORTHWEST
11222196EAC
MARINE SCIENCES LAB
1529 W SEQUIM BAY RD
SEQUIM WA 98382

PO NBR: 11222196EAC

2097 + 1
expiration date 9/08

PRODUCT NUMBER: 259195-500MG

LOT NUMBER: 06016JS

PRODUCT NAME: AMINOGLUTETHIMIDE, 99%

CAS 125-84-8

FORMULA: C13H16N2O2

FORMULA WEIGHT: 232.29

APPEARANCE	WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 3072B OF EDITION I, VOLUME 2 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
ELEMENTAL ANALYSIS	CARBON 66.60% HYDROGEN 6.93% NITROGEN 11.94%
THIN-LAYER CHROMATOGRAPHY	CONSISTENT WITH 99% PURITY
SOLUBILITY	5% HOAC:MEOH 1:1; SLIGHTLY HAZY, FAINT YELLOW SOLUTION.
QUALITY CONTROL ACCEPTANCE DATE	JULY 1998

ALDRICH CHEMICAL COMPANY
RONNIE MARTIN
SEPTEMBER 4, 2003

ACROS ORGANICS

Certificate of Analysis

Product41624-0000
4-NONYLPHENOL, 99%, MIXTURE OF ISOMERS

General Product Data	
Version	00
CAS No	84852-15-3
Molecular weight	220.35
Molecular formula	C15 H24 O
Linear formula	
Flash point (°C)	141

Lot Specific Data **A0192712**

Appearance	clear viscous liquid
Color scale	<50 APHA
Infrared spectrometry	authentic
Separat. techn. GC	>98.5 %
Water	<0.05 %
Refractive index	1.5119 (20°C, 589 nm)

A BD Biosciences Company
GENTEST
6 Henshaw St., Woburn, MA 01801 USA
Voice: (781) 935-5115, FAX: (781) 938-8644
info@gentest.com
www.gentest.com

Human CYP19 + P450 Reductase SUPERSOMES™

Catalog Number.....456260
Lot Number.....62530

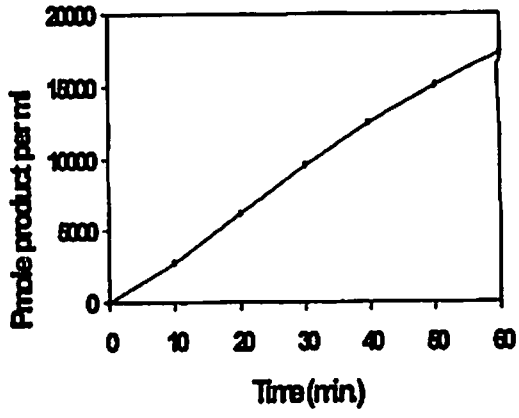
Storage Conditions..STORE AT -80°C
Date Released .....2006 August
Best Used by.....2009 August

Package Contents.....0.5 nmole cytochrome P450 in 0.5 ml
Protein Content.....6.9 mg/ml in 100mM potassium phosphate (pH 7.4)
Cytochrome c Reductase Activity.....280 nmole/(min x mg protein)
Cytochrome P450 Content.....1000 pmol per ml
Aromatase Activity.....4.0 pmol product/(min x pmol P450)

This activity is catalyzed by human CYP19 which is expressed from human CYP19 cDNA using a baculovirus expression system. Baculovirus infected insect cells (BTI-TN-5B1-4) were used to prepare these microsomes. These microsomes also contain cDNA-expressed human P450 reductase. A microsome preparation using wild type virus (GENTEST Catalog No. 456200 or 456201) should be used as a control for native activities.

METHOD: A 0.25 ml reaction mixture containing 25 pmole P450, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.05 mM testosterone in 100 mM potassium phosphate (pH 7.4) was incubated at 37°C for 20 min. After incubation, the reaction was stopped by the addition of 125 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. 50 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and eluted isocratically at 45°C with a mobile phase of 60% water and 40% acetonitrile and at a flow rate of 1.5 ml per min. The product was detected by its absorbance at 200 nm and quantitated by comparing the absorbance to a standard curve of (beta)-estradiol.

Time Course of Product Formation



ADVICE

- Thaw rapidly in a 37°C water bath. Keep on ice until use
- Aliquot to minimize freeze-thawing cycles. Less than 20% of the catalytic activity is lost after 6 freeze thaw cycles.
- Metabolite production is linear with respect to enzyme concentration up to at least 50 pmol P450 per ml.
- Metabolite production with testosterone is approximately linear for 40 minutes (see graph above).

THIS PRODUCT IS SUPPLIED FOR LABORATORY RESEARCH USE ONLY.

A BD Biosciences Company
GENTEST

6 Henshaw St., Woburn, MA 01801 USA
Voice: (781) 935-5115, FAX: (781) 938-8644
info@gentest.com
www.gentest.com

BD Biosciences
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen
BD

INSECT CELL MICROSOMES
SAFETY INFORMATION

HAZARD WARNING:

The product was produced using baculovirus (*Autographa californica*) infected insect cells (BTI-TN-5B1-4). This virus is not known to be pathogenic to humans or other mammals.

SAFETY RECOMMENDATIONS:

When using this product, follow good laboratory safety procedures:

Do not eat, drink or smoke.

Avoid contact with skin or eyes.

Do not inhale aerosols.

Do not pipette by mouth.

Wear suitable protective clothing, gloves and eye protection.

Steam sterilize product or treat product with a 1% solution of sodium hypochlorite prior to disposal.

Appendix 4

Data Spreadsheets

Aromatase Assay Spreadsheet

Assay Date	11/17/2006	Inhibitor	AG-R1	NB page ref	12507-25

Aromatase Assay Spreadsheet

Assay Date	11/17/2006	Test Chemical ID	AG-R1	NB page ref	12507-25

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0193	32345	1675907		
2	0.0191	34778	1820838		
3	0.0192	32315	1683073		
4	0.0199	35606	1789246		
5	0.0200	36448	1822400		
			Average DPM/g soln		1758293
			SD		73187
			CV		4.16
			µCi/g soln		0.792

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1292 g
Mass of dilution B used in substrate prep	4.5886 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.564459 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00965 µg/g soln.
µg/g soln.

a. µCi/g soln	0.792
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.564459 + 0.00965
= 0.574112 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.380 µCi/µg ASDN

877138 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/17/2006	Test Chemical ID	AG-R1	NB page ref	12507-25

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0196	30357	1548827		
2	0.0190	31814	1674421		
3	0.0198	36808	1858990		
4	0.0193	34309	1777668		
5	0.0199	38403	1929799		
			Average DPM/g soln		1757941
			SD		150698
			CV		8.57
			µCi/g soln		0.792

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0696 g
Mass of dilution B used in substrate prep	0.6681 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.131786 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00965 µg/g soln.
µg/g soln.

a. µCi/g soln	0.792
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.131786 + 0.00965
= 0.141436 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.599 µCi/µg ASDN

3559727 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/17/2006	Inhibitor	AG-R1	NB page ref	12507-25

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (μM)
0	0
1	25
2	15
3	5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	11/17/2000	Inhibitor	AG-R1	NB page ref	12507-25

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	105	210	203	406	0.1	A	175629	7	0.0000	15	0.0095	0.0001
				0.5	2	98	196			0.1							
	2	NA	2	0.5	1	103	206	202	404	0.1	A	175629	5	0.0000	15	0.0095	0.0000
				0.5	2	99	198			0.1							
	3	NA	2	0.5	1	100	200	188	376	0.1	A	175629	-23	0.0000	15	0.0095	-0.0002
				0.5	2	88	176			0.1							
	4	NA	2	0.5	1	112	224	205	410	0.1	A	175629	11	0.0000	15	0.0095	0.0001
				0.5	2	93	186			0.1							
AG-R1	15	0	2	0.5	1	8500	17000	16704	33408	0.05	B	105476	33009	0.0093	15	0.0095	0.0650
				0.5	2	8204	16408			0.05							
	15	0	2	0.5	1	8784	17568	17337	34674	0.05	B	105476	34275	0.0096	15	0.0095	0.0675
				0.5	2	8553	17106			0.05							
	15	5	2	0.5	1	2411	4822	4798	9596	0.05	B	105476	9197	0.0026	15	0.0095	0.0181
				0.5	2	2387	4774			0.05							
	15	5	2	0.5	1	2461	4902	4963	9926	0.05	B	105476	9527	0.0027	15	0.0095	0.0188
				0.5	2	2482	4904			0.05							
	15	15	2	0.5	1	1153	2306	2340	4680	0.05	B	105476	4261	0.0012	15	0.0095	0.0084
				0.5	2	1177	2354			0.05							
	15	15	2	0.5	1	993	1986	1971	3942	0.05	B	105476	3543	0.0010	15	0.0095	0.0070
				0.5	2	978	1956			0.05							
	15	25	2	0.5	1	708	1416	1387	2774	0.05	B	105476	2375	0.0007	15	0.0095	0.0047
				0.5	2	679	1358			0.05							
	15	25	2	0.5	1	746	1492	1502	3004	0.05	B	105476	2605	0.0007	15	0.0095	0.0051
				0.5	2	756	1512			0.05							
	25	0	2	0.5	1	12680	25360	25634	51268	0.1	D	175794	50868	0.0143	15	0.0095	0.1002
				0.5	2	12944	25908			0.1							
	25	0	2	0.5	1	12125	24250	24191	48382	0.1	D	175794	47983	0.0135	15	0.0095	0.0945
				0.5	2	12066	24132			0.1							
	25	5	2	0.5	1	4244	8488	8412	16824	0.1	D	175794	16425	0.0046	15	0.0095	0.0323
				0.5	2	4168	8336			0.1							
	25	6	2	0.5	1	4307	8614	8527	17054	0.1	D	175794	16856	0.0047	15	0.0095	0.0328
				0.5	2	4220	8440			0.1							
	25	15	2	0.5	1	1932	3864	3810	7620	0.1	D	175794	7221	0.0020	15	0.0095	0.0142
				0.5	2	1878	3756			0.1							
	25	15	2	0.5	1	1849	3698	3668	7336	0.1	D	175794	6937	0.0019	15	0.0095	0.0137
				0.5	2	1819	3638			0.1							
	25	25	2	0.5	1	1186	2372	2303	4776	0.1	D	175794	4327	0.0012	15	0.0095	0.0085
				0.5	2	1177	2354			0.1							
	25	25	2	0.5	1	1133	2266	2238	4476	0.1	D	175794	4077	0.0011	15	0.0095	0.0080
				0.5	2	1105	2210			0.1							
	35	0	2	0.5	1	14670	29340	29392	58784	0.14	E	248112	56385	0.0164	15	0.0095	0.1150
				0.5	2	14722	29444			0.14							
	35	0	2	0.5	1	14955	29910	30023	60046	0.14	E	248112	58647	0.0155	15	0.0095	0.1174
				0.5	2	15068	30136			0.14							
	35	5	2	0.5	1	9035	11270	11361	22722	0.14	E	248112	22323	0.0063	15	0.0095	0.0446
				0.5	2	8726	11452			0.14							
	35	5	2	0.5	1	6170	12340	12406	24812	0.14	E	248112	24413	0.0069	15	0.0095	0.0481
				0.5	2	6236	12472			0.14							
	35	15	2	0.5	1	2724	5448	5434	10868	0.14	E	248112	10469	0.0029	15	0.0095	0.0206
				0.5	2	2710	5420			0.14							
	35	15	2	0.5	1	3044	6088	5928	11856	0.14	E	248112	11457	0.0032	15	0.0095	0.0226
				0.5	2	2884	5768			0.14							
	35	25	2	0.5	1	1848	3682	3701	7402	0.14	E	248112	7003	0.0020	15	0.0095	0.0138
				0.5	2	1855	3710			0.14							
	35	25	2	0.5	1	1831	3662	3667	7334	0.14	E	248112	6935	0.0019	15	0.0095	0.0137
				0.5	2	1836	3672			0.14							
	50	0	2	0.5	1	5390	10780	10765	21530	0.05	A	87915	21131	0.0241	15	0.0095	0.1659
				0.5	2	5375	10750			0.05							
	50	0	2	0.5	1	4964	9928	9791	19582	0.05	A	87915	19183	0.0219	15	0.0095	0.1533
				0.5	2	4807	9614			0.05							
	50	5	2	0.5	1	1936	3772	3915	7830	0.05	A	87915	7231	0.0082	15	0.0095	0.0578
				0.5	2	1929	3858			0.05							
	50	5	2	0.5	1	2023	4044	4132	8264	0.05	A	87915	7865	0.0090	15	0.0095	0.0628
				0.5	2	2110	4220			0.05							
	50	15	2	0.5	1	978	1956	1934	3868	0.05	A	87915	3469	0.0040	15	0.0095	0.0277
				0.5	2	956	1912			0.05							
	50	15	2	0.5	1	997	1994	1992	3984	0.05	A	87915	3565	0.0041	15	0.0095	0.0285
				0.5	2	995	1990			0.05							
	50	25	2	0.5	1	751	1502	1442	2884	0.05	A	87915	2465	0.0028	15	0.0095	0.0199
				0.5	2	691	1382			0.05							
	50	25	2	0.5	1	618	1232	1266	2510	0.05	A	87915	2111	0.0024	15	0.0095	0.0169
				0.5	2	639	1278			0.05							
	100	0	2	0.5	1	6333	12672	12545	25090	0.1	A	175879	24691	0.0281	15	0.0095	0.1973
				0.5	2	6209	12418			0.1							
	100	0	2	0.5	1	6297	12594	12549	25098	0.1	A	175879	24699	0.0282	15	0.0095	0.1974
				0.5	2	6252	12504			0.1							
	100	5	2	0.5	1	3464	6928	6956	13912	0.1	A	175879	13513	0.0154	15	0.0095	0.1080
				0.5	2	3492	6984			0.1							
	100	5	2	0.5	1	3343	6686	6582	13164	0.1	A	175879	12765	0.0146	15	0.0095	0.1020
				0.5	2	3279	6558			0.1							
	100	15	2	0.5	1	1714	3428	3493	6986	0.1	A	175879	6587	0.0075	15	0.0095	0.0526
				0.5	2	1779	3558			0.1							

Aromatase Assay Spreadsheet

Assay Date: 11/17/2006 Inhibitor: AG-R1 NB page ref: 12507-25

Sample ID			Calculate DPM in aqueous portion after extraction							Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (n/det)	Calculate nmol ^3H_2O formed		Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM				Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed			
	100	15	2	0.5	1	1793	3586	3551	7102	0.1	A	175629	6703	0.0076	15	0.0095	0.0535
				0.5	2	1758	3516			0.1							
	100	25	2	0.5	1	1131	2262	2275	4550	0.1	A	175629	4151	0.0047	15	0.0095	0.0332
				0.5	2	1144	2288			0.1							
	100	25	2	0.5	1	1100	2212	2174	4348	0.1	A	175629	3949	0.0045	15	0.0095	0.0316
				0.5	2	1068	2136			0.1							
	300	0	2	0.5	1	7967	15934	16077	31954	0.3	A	527488	31555	0.0360	15	0.0095	0.2522
				0.5	2	8010	16020			0.3							
	300	0	2	0.5	1	8314	16628	16645	33290	0.3	A	527488	32891	0.0375	15	0.0095	0.2628
				0.5	2	8331	16662			0.3							
	300	5	2	0.5	1	5729	11458	11439	22878	0.3	A	527488	22479	0.0256	15	0.0095	0.1796
				0.5	2	5710	11420			0.3							
	300	5	2	0.5	1	5882	11764	11679	23356	0.3	A	527488	22959	0.0262	15	0.0095	0.1835
				0.5	2	5797	11594			0.3							
	300	15	2	0.5	1	3677	7354	7294	14589	0.3	A	527488	14160	0.0162	15	0.0095	0.1134
				0.5	2	3617	7234			0.3							
	300	15	2	0.5	1	3437	6874	6805	13610	0.3	A	527488	13211	0.0151	15	0.0095	0.1056
				0.5	2	3368	6736			0.3							
	300	25	2	0.5	1	2629	5258	5247	10494	0.3	A	527488	10095	0.0115	15	0.0095	0.0807
				0.5	2	2618	5236			0.3							
	300	25	2	0.5	1	2548	5097	5035	10070	0.3	A	527488	9671	0.0110	15	0.0095	0.0773
				0.5	2	2489	4978			0.3							

Aromatase Assay Spreadsheet

Assay Date	11/17/2006	Inhibitor	AG-R1	NB page ref	12507-25

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		5		15		25	
15	0.0650	0.0675	0.0181	0.0188	0.0084	0.0070	0.0047	0.0051
25	0.1002	0.0945	0.0323	0.0328	0.0142	0.0137	0.0085	0.0080
35	0.1150	0.1174	0.0440	0.0481	0.0206	0.0226	0.0138	0.0137
50	0.1689	0.1533	0.0578	0.0628	0.0277	0.0286	0.0199	0.0169
100	0.1973	0.1974	0.1080	0.1020	0.0526	0.0536	0.0332	0.0316
300	0.2522	0.2628	0.1796	0.1835	0.1134	0.1056	0.0807	0.0773

Aromatase Assay Spreadsheet

Assay Date	11/21/2006	Inhibitor	AG-R2	NB page ref	12507-26

Aromatase Assay Spreadsheet

Assay Date	11/21/2006	Test Chemical ID	AG-R2	NB page ref	12507-26

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0196	35552	1813878		
2	0.0194	34294	1767732		
3	0.0196	38336	1955918		
4	0.0202	39482	1954554		
5	0.0201	37559	1868607		
			Average DPM/g soln		1872138
			SD		83844
			CV		4.48
			µCi/g soln		0.843

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.01	10		1001.00
Dilution A			100	10.01
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	7.9591 g
Mass of dilution B used in substrate prep	4.4218 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.556121 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01028 µg/g soln.
µg/g soln.

a. µCi/g soln	0.843
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.556121 + 0.01028
= 0.566398 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.489 µCi/µg ASDN

946649 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/21/2006	Test Chemical ID	AG-R2	NB page ref	12507-26

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0195	31324	1606359		
2	0.0198	29221	1475808		
3	0.0195	34755	1782308		
4	0.0201	36523	1817065		
5	0.0202	37818	1872178		
			Average DPM/g soln		1710744
			SD		164756
			CV		9.63
			µCi/g soln		0.771

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.01	10		1001.00
Dilution A			100	10.01
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0443 g
Mass of dilution B used in substrate prep	0.6529 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.129563 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00939 µg/g soln.
µg/g soln.

a. µCi/g soln 0.771
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.129563 + 0.00939
= 0.138954 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.546 µCi/µg ASDN

3526032 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/21/2006	Inhibitor	AG-R2	NB page ref	12507-26

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (µM)
0	0
1	25
2	15
3	5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date: 11/21/2006 | Inhibitor: AG-R2 | KB page ref: 12507-26

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration, Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (mCal)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	116	232	223	446	0.1	A	167214	11	0.0000	15	0.0095	0.0001
				0.5	2	107	214			0.1							
	2	NA	2	0.5	1	110	220	206	412	0.1	A	167214	-23	0.0000	15	0.0095	-0.0002
				0.5	2	96	192			0.1							
	3	NA	2	0.5	1	111	222	215	430	0.1	A	167214	-5	0.0000	15	0.0095	0.0000
				0.5	2	104	208			0.1							
	4	NA	2	0.5	1	101	202	226	452	0.1	A	167214	17	0.0000	15	0.0095	0.0001
				0.5	2	125	250			0.1							
AG-R2	15	0	2	0.5	1	8921	17842	17794	35588	0.05	B	102645	35153	0.0100	15	0.0095	0.0699
				0.5	2	8873	17746			0.05							
	15	0	2	0.5	1	8022	16044	17945	35890	0.05	B	102645	35456	0.0101	15	0.0095	0.0706
				0.5	2	8923	17846			0.05							
	15	5	2	0.5	1	2586	5190	5273	10546	0.05	B	102645	10111	0.0029	15	0.0095	0.0201
				0.5	2	2678	5356			0.05							
	15	5	2	0.5	1	2333	4666	4713	9426	0.05	B	102645	8991	0.0025	15	0.0095	0.0179
				0.5	2	2380	4760			0.05							
	15	15	2	0.5	1	1214	2428	2442	4884	0.05	B	102645	4449	0.0013	15	0.0095	0.0083
				0.5	2	1228	2456			0.05							
	15	15	2	0.5	1	1216	2432	2429	4858	0.05	B	102645	4423	0.0013	15	0.0095	0.0068
				0.5	2	1213	2426			0.05							
	15	25	2	0.5	1	734	1468	1489	2976	0.05	B	102645	2541	0.0007	15	0.0095	0.0051
				0.5	2	754	1508			0.05							
	15	25	2	0.5	1	760	1520	1534	3068	0.05	B	102645	2633	0.0007	15	0.0095	0.0052
				0.5	2	774	1548			0.05							
	25	0	2	0.5	1	11355	22710	22671	45342	0.1	B	171074	44907	0.0127	15	0.0095	0.0893
				0.5	2	11316	22632			0.1							
	25	0	2	0.5	1	10378	20756	21065	42130	0.1	B	171074	41095	0.0116	15	0.0095	0.0829
				0.5	2	10687	21374			0.1							
	25	5	2	0.5	1	3692	7384	7348	14696	0.1	B	171074	14261	0.0040	15	0.0095	0.0283
				0.5	2	3656	7312			0.1							
	25	5	2	0.5	1	4148	8296	8388	16778	0.1	B	171074	16741	0.0048	15	0.0095	0.0325
				0.5	2	4242	8484			0.1							
	25	15	2	0.5	1	1775	3550	3575	7150	0.1	B	171074	6715	0.0019	15	0.0095	0.0133
				0.5	2	1800	3600			0.1							
	25	15	2	0.5	1	1764	3528	3629	7252	0.1	B	171074	6817	0.0019	15	0.0095	0.0138
				0.5	2	1832	3664			0.1							
	25	25	2	0.5	1	1152	2304	2272	4544	0.1	B	171074	4109	0.0012	15	0.0095	0.0082
				0.5	2	1120	2240			0.1							
	25	25	2	0.5	1	1104	2208	2275	4550	0.1	B	171074	4115	0.0012	15	0.0095	0.0082
				0.5	2	1171	2342			0.1							
	35	0	2	0.5	1	13780	27520	27490	54980	0.14	B	239504	54563	0.0155	15	0.0095	0.1093
				0.5	2	13739	27478			0.14							
	35	0	2	0.5	1	15459	30918	30663	61326	0.14	B	239504	60591	0.0173	15	0.0095	0.1210
				0.5	2	15704	30408			0.14							
	35	5	2	0.5	1	5781	11562	11510	23020	0.14	B	239504	22565	0.0064	15	0.0095	0.0449
				0.5	2	5729	11458			0.14							
	35	5	2	0.5	1	5598	11196	11080	22160	0.14	B	239504	21725	0.0062	15	0.0095	0.0432
				0.5	2	5482	10964			0.14							
	35	15	2	0.5	1	2453	4906	4936	9872	0.14	B	239504	9437	0.0027	15	0.0095	0.0188
				0.5	2	2483	4966			0.14							
	35	15	2	0.5	1	2281	4562	4576	9152	0.14	B	239504	8717	0.0025	15	0.0095	0.0173
				0.5	2	2295	4590			0.14							
	35	25	2	0.5	1	1395	2790	2817	5634	0.14	B	239504	5199	0.0015	15	0.0095	0.0103
				0.5	2	1422	2844			0.14							
	35	25	2	0.5	1	1569	3138	3160	6320	0.14	B	239504	5685	0.0017	15	0.0095	0.0117
				0.5	2	1591	3182			0.14							
	50	0	2	0.5	1	5435	10870	10536	21072	0.05	A	93607	20837	0.0218	15	0.0095	0.1528
				0.5	2	5101	10202			0.05							
	50	0	2	0.5	1	4896	9792	9756	19512	0.05	A	93607	19077	0.0202	15	0.0095	0.1412
				0.5	2	4860	9720			0.05							
	50	5	2	0.5	1	2091	4182	4086	8172	0.05	A	93607	7737	0.0082	15	0.0095	0.0573
				0.5	2	1995	3990			0.05							
	50	5	2	0.5	1	2118	4232	4234	8465	0.05	A	93607	8033	0.0085	15	0.0095	0.0595
				0.5	2	2116	4236			0.05							
	50	15	2	0.5	1	834	1668	1668	3336	0.05	A	93607	2901	0.0031	15	0.0095	0.0215
				0.5	2	834	1668			0.05							
	50	15	2	0.5	1	886	1772	1763	3536	0.05	A	93607	3101	0.0033	15	0.0095	0.0230
				0.5	2	892	1784			0.05							
	50	25	2	0.5	1	595	1190	1234	2468	0.05	A	93607	2033	0.0021	15	0.0095	0.0151
				0.5	2	639	1278			0.05							
	50	25	2	0.5	1	677	1354	1374	2748	0.05	A	93607	2313	0.0024	15	0.0095	0.0171
				0.5	2	697	1394			0.05							
	100	0	2	0.5	1	6949	13898	13685	27370	0.1	A	167214	26335	0.0255	15	0.0095	0.1904
				0.5	2	6836	13672			0.1							
	100	0	2	0.5	1	7224	14448	14263	28526	0.1	A	167214	28091	0.0297	15	0.0095	0.2060
				0.5	2	7039	14078			0.1							
	100	5	2	0.5	1	3378	6756	6620	13240	0.1	A	167214	12805	0.0135	15	0.0095	0.0948
				0.5	2	3242	6484			0.1							
	100	5	2	0.5	1	3309	6618	6672	13344	0.1	A	167214	12909	0.0136	15	0.0095	0.0956
				0.5	2	3363	6726			0.1							
	100	15	2	0.5	1	1827	3654	3644	7288	0.1	A	167214	6853	0.0072	15	0.0095	0.0507
				0.5	2	1817	3634			0.1							

Aromatase Assay Spreadsheet

Assay Date 11/21/2000 Inhibitor AG-R2 ND page ref 12507-26

Sample ID			Calculate DPM in aqueous portion after extraction							Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Calculate nmol ^3H_2O formed		Incubation time (min)	Protein (mg/inc)	Activity (nmol estrogen formed/mg/min
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (μM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM				Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed			
	100	15	2	0.5	1	1863	3726	3676	7352	0.1	A	187214	6821	0.0073	15	0.0095	0.0512
				0.5	2	1815	3630			0.1							
	100	25	2	0.5	1	1134	2268	2236	4472	0.1	A	187214	4037	0.0043	15	0.0095	0.0299
				0.5	2	1102	2204			0.1							
	100	25	2	0.5	1	1148	2296	2275	4550	0.1	A	187214	4115	0.0043	15	0.0095	0.0305
				0.5	2	1127	2254			0.1							
	300	0	2	0.5	1	8406	16812	16697	33394	0.3	A	561641	32958	0.0346	15	0.0095	0.2440
				0.5	2	8289	16578			0.3							
	300	0	2	0.5	1	8032	16064	16274	32548	0.3	A	561641	32113	0.0339	15	0.0095	0.2378
				0.5	2	8242	16484			0.3							
	300	5	2	0.5	1	5404	10809	11302	22604	0.3	A	561641	22169	0.0234	15	0.0095	0.1641
				0.5	2	5898	11796			0.3							
	300	5	2	0.5	1	6119	12239	12217	24434	0.3	A	561641	23999	0.0254	15	0.0095	0.1777
				0.5	2	6098	12196			0.3							
	300	15	2	0.5	1	4111	8222	8089	16178	0.3	A	561641	15743	0.0166	15	0.0095	0.1165
				0.5	2	3978	7956			0.3							
	300	15	2	0.5	1	3578	7156	7552	15104	0.3	A	561641	14659	0.0155	15	0.0095	0.1085
				0.5	2	3974	7948			0.3							
	300	25	2	0.5	1	2682	5364	5369	10735	0.3	A	561641	10303	0.0109	15	0.0095	0.0763
				0.5	2	2687	5374			0.3							
	300	25	2	0.5	1	2554	5108	5106	10212	0.3	A	561641	9777	0.0103	15	0.0095	0.0724
				0.5	2	2552	5104			0.3							

Aromatase Assay Spreadsheet

Assay Date	11/21/2008	Inhibitor	AG-R2	NB page ref	12507-26

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		5		15		25	
15	0.0699	0.0705	0.0201	0.0179	0.0088	0.0088	0.0051	0.0052
25	0.0893	0.0829	0.0283	0.0325	0.0133	0.0136	0.0082	0.0082
35	0.1085	0.1210	0.0449	0.0432	0.0188	0.0173	0.0103	0.0117
50	0.1528	0.1412	0.0573	0.0595	0.0215	0.0230	0.0151	0.0171
100	0.1994	0.2080	0.0948	0.0956	0.0507	0.0512	0.0299	0.0305
300	0.2440	0.2378	0.1641	0.1777	0.1166	0.1086	0.0763	0.0724

Aromatase Assay Spreadsheet

Assay Date	11/28/2006	Inhibitor	AG-R3	NB page ref	12507-27

Aromatase Assay Spreadsheet

Assay Date	11/28/2006	Test Chemical ID	AG-R3	NB page ref	12507-27

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0200	34785	1739250		
2	0.0196	35717	1822296		
3	0.0196	37865	1931888		
4	0.0200	38160	1908000		
5	0.0196	38870	1983163		
			Average DPM/g soln		1876919
			SD		96452
			CV		5.14
			µCi/g soln		0.845

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	7.9991 g
Mass of dilution B used in substrate prep	4.4692 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.55983 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01030 µg/g soln.
µg/g soln.

a. µCi/g soln	0.845
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.559830 + 0.01030
= 0.570134 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.483 µCi/µg ASDN

942848 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/28/2006	Test Chemical ID	AG-R3	NB page ref	12507-27

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0200	33787	1689350		
2	0.0197	32897	1669898		
3	0.0194	34886	1798247		
4	0.0194	36073	1859433		
5	0.0202	35812	1772871		
			Average DPM/g soln		1757960
			SD		78429
			CV		4.46
			µCi/g soln		0.792

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0426 g
Mass of dilution B used in substrate prep	0.6642 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.131981 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00965 µg/g soln.
µg/g soln.

a. µCi/g soln	0.792
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.131981 + 0.00965
= 0.141632 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.591 µCi/µg ASDN

3554846 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/28/2006	Inhibitor	AG-R3	NB page ref	12507-27

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (µM)
0	0
1	25
2	15
3	5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	11/28/2000	Inhibitor	AG-R3	NB page ref	12507-27

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (mCal)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	101	202	202	404	0.1	A	187692	-16	0.0000	15	0.0095	-0.0001
				0.5	2	101	202			0.1							
	2	NA	2	0.5	1	99	198	202	404	0.1	A	187692	-16	0.0000	15	0.0095	-0.0001
				0.5	2	103	206			0.1							
	3	NA	2	0.5	1	106	212	223	445	0.1	A	187692	25	0.0000	15	0.0095	0.0002
				0.5	2	117	234			0.1							
	4	NA	2	0.5	1	112	224	213	426	0.1	A	187692	0	0.0000	15	0.0095	0.0000
				0.5	2	101	202			0.1							
AG-R3	15	0	2	0.5	1	8470	16940	16881	33762	0.05	B	105478	33342	0.0094	15	0.0095	0.0657
				0.5	2	8411	16822			0.05							
	15	0	2	0.5	1	8431	16862	16883	33766	0.05	B	105478	33346	0.0094	15	0.0095	0.0657
				0.5	2	8452	16904			0.05							
	15	5	2	0.5	1	2534	5068	5024	10048	0.05	B	105478	9626	0.0027	15	0.0095	0.0190
				0.5	2	2490	4980			0.05							
	15	5	2	0.5	1	2453	4906	4910	9820	0.05	B	105478	9400	0.0026	15	0.0095	0.0185
				0.5	2	2457	4914			0.05							
	15	15	2	0.5	1	1078	2152	2101	4202	0.05	B	105478	3782	0.0011	15	0.0095	0.0075
				0.5	2	1025	2050			0.05							
	15	15	2	0.5	1	1096	2192	2235	4470	0.05	B	105478	4050	0.0011	15	0.0095	0.0080
				0.5	2	1139	2278			0.05							
	15	25	2	0.5	1	846	1692	1685	3370	0.05	B	105478	2950	0.0008	15	0.0095	0.0058
				0.5	2	839	1678			0.05							
	15	25	2	0.5	1	773	1546	1527	3054	0.05	B	105478	2634	0.0007	15	0.0095	0.0052
				0.5	2	754	1508			0.05							
	25	0	2	0.5	1	13351	26702	27119	54238	0.1	B	175795	53818	0.0151	15	0.0095	0.1061
				0.5	2	13768	27536			0.1							
	25	0	2	0.5	1	13126	26252	26047	52094	0.1	B	175795	51874	0.0145	15	0.0095	0.1019
				0.5	2	12921	25842			0.1							
	25	5	2	0.5	1	4622	9244	9285	18570	0.1	B	175795	18150	0.0051	15	0.0095	0.0358
				0.5	2	4663	9326			0.1							
	25	5	2	0.5	1	4787	9574	9554	19108	0.1	B	175795	18688	0.0053	15	0.0095	0.0369
				0.5	2	4767	9534			0.1							
	25	15	2	0.5	1	1904	3808	3817	7634	0.1	B	175795	7214	0.0020	15	0.0095	0.0142
				0.5	2	1913	3826			0.1							
	25	15	2	0.5	1	2020	4040	3937	7874	0.1	B	175795	7454	0.0021	15	0.0095	0.0147
				0.5	2	1917	3834			0.1							
	25	25	2	0.5	1	1382	2764	2768	5536	0.1	B	175795	5116	0.0014	15	0.0095	0.0101
				0.5	2	1386	2772			0.1							
	25	25	2	0.5	1	1277	2554	2569	5138	0.1	B	175795	4778	0.0013	15	0.0095	0.0094
				0.5	2	1322	2644			0.1							
	35	0	2	0.5	1	15779	32558	31589	63178	0.14	B	248114	62778	0.0177	15	0.0095	0.1236
				0.5	2	15320	30640			0.14							
	35	0	2	0.5	1	17240	34480	34100	68200	0.14	B	248114	67780	0.0191	15	0.0095	0.1336
				0.5	2	16860	33720			0.14							
	35	5	2	0.5	1	6186	12332	12568	25176	0.14	B	248114	24756	0.0070	15	0.0095	0.0488
				0.5	2	6422	12844			0.14							
	35	5	2	0.5	1	6810	13620	13596	27192	0.14	B	248114	26772	0.0075	15	0.0095	0.0528
				0.5	2	6786	13572			0.14							
	35	15	2	0.5	1	2842	5684	5694	11388	0.14	B	248114	10968	0.0031	15	0.0095	0.0216
				0.5	2	2852	5704			0.14							
	35	15	2	0.5	1	2652	5304	5295	10590	0.14	B	248114	10170	0.0029	15	0.0095	0.0201
				0.5	2	2643	5286			0.14							
	35	25	2	0.5	1	1786	3572	3494	6988	0.14	B	248114	6568	0.0018	15	0.0095	0.0130
				0.5	2	1708	3416			0.14							
	35	25	2	0.5	1	1904	3808	3745	7490	0.14	B	248114	7070	0.0020	15	0.0095	0.0139
				0.5	2	1841	3682			0.14							
	50	0	2	0.5	1	6503	13006	13069	26138	0.05	A	93846	25718	0.0273	15	0.0095	0.1912
				0.5	2	6478	12956			0.05							
	50	0	2	0.5	1	6184	12368	11907	23814	0.05	A	93846	23394	0.0248	15	0.0095	0.1739
				0.5	2	5723	11446			0.05							
	50	5	2	0.5	1	2320	4640	4565	9130	0.05	A	93846	8770	0.0093	15	0.0095	0.0652
				0.5	2	2280	4560			0.05							
	50	5	2	0.5	1	2315	4630	4584	9168	0.05	A	93846	8748	0.0093	15	0.0095	0.0650
				0.5	2	2269	4538			0.05							
	50	15	2	0.5	1	1158	2316	2256	4512	0.05	A	93846	4092	0.0043	15	0.0095	0.0304
				0.5	2	1098	2196			0.05							
	50	15	2	0.5	1	999	1998	2025	4050	0.05	A	93846	3830	0.0039	15	0.0095	0.0270
				0.5	2	1026	2052			0.05							
	50	25	2	0.5	1	694	1388	1392	2784	0.05	A	93846	2364	0.0025	15	0.0095	0.0176
				0.5	2	698	1396			0.05							
	50	25	2	0.5	1	767	1534	1471	2942	0.05	A	93846	2522	0.0027	15	0.0095	0.0187
				0.5	2	704	1408			0.05							
	100	0	2	0.5	1	8311	16622	16267	32574	0.1	A	187692	32154	0.0341	15	0.0095	0.2390
				0.5	2	7978	15952			0.1							
	100	0	2	0.5	1	8018	16036	15747	31494	0.1	A	187692	31074	0.0330	15	0.0095	0.2310
				0.5	2	7729	15458			0.1							
	100	5	2	0.5	1	4153	8306	8324	16648	0.1	A	187692	16228	0.0172	15	0.0095	0.1206
				0.5	2	4171	8342			0.1							
	100	5	2	0.5	1	4175	8350	8197	16394	0.1	A	187692	15974	0.0169	15	0.0095	0.1186
				0.5	2	4022	8044			0.1							
	100	15	2	0.5	1	2014	4028	3952	7904	0.1	A	187692	7544	0.0080	15	0.0095	0.0561
				0.5	2	1950	3900			0.1							

Aromatase Assay Spreadsheet

Assay Date: 11/28/2006 Inhibitor: AG-R3 NB page ref: 12507-27

Sample ID			Calculate DPM in aqueous portion after extraction										Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
	100	15	2	0.5	1	2175	4350	4274	8548	0.1	A	187602	8120	0.0086	15	0.0095	0.0604
				0.5	2	2099	4198			0.1							
	100	25	2	0.5	1	1355	2710	2663	5326	0.1	A	187602	4806	0.0052	15	0.0095	0.0365
				0.5	2	1308	2616			0.1							
	100	25	2	0.5	1	1295	2592	2616	5232	0.1	A	187602	4812	0.0051	15	0.0095	0.0356
				0.5	2	1320	2640			0.1							
	300	0	2	0.5	1	10477	20954	21020	42040	0.3	A	563076	41620	0.0441	15	0.0095	0.3094
				0.5	2	10543	21086			0.3							
	300	0	2	0.5	1	10294	20588	20587	41174	0.3	A	563076	40754	0.0432	15	0.0095	0.3030
				0.5	2	10293	20586			0.3							
	300	5	2	0.5	1	7400	14800	14562	29124	0.3	A	563076	28704	0.0304	15	0.0095	0.2134
				0.5	2	7162	14324			0.3							
	300	5	2	0.5	1	7059	14118	14034	28068	0.3	A	563076	27648	0.0293	15	0.0095	0.2055
				0.5	2	6975	13950			0.3							
	300	15	2	0.5	1	4558	9116	9110	18220	0.3	A	563076	17800	0.0189	15	0.0095	0.1323
				0.5	2	4552	9104			0.3							
	300	15	2	0.5	1	4457	8914	8638	17276	0.3	A	563076	16856	0.0179	15	0.0095	0.1253
				0.5	2	4181	8362			0.3							
	300	25	2	0.5	1	3410	6820	6957	13914	0.3	A	563076	13494	0.0143	15	0.0095	0.1003
				0.5	2	3547	7094			0.3							
	300	25	2	0.5	1	3240	6480	6608	13216	0.3	A	563076	12796	0.0136	15	0.0095	0.0951
				0.5	2	3368	6736			0.3							

Aromatase Assay Spreadsheet

Assay Date	11/28/2006	Inhibitor	AG-R3	NB page ref	12507-27

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		5		15		25	
15	0.0657	0.0657	0.0190	0.0185	0.0075	0.0080	0.0058	0.0052
25	0.1061	0.1019	0.0358	0.0368	0.0142	0.0147	0.0101	0.0094
35	0.1238	0.1336	0.0488	0.0528	0.0216	0.0201	0.0130	0.0139
50	0.1912	0.1739	0.0652	0.0650	0.0304	0.0270	0.0176	0.0167
100	0.2390	0.2310	0.1206	0.1188	0.0561	0.0604	0.0365	0.0358
300	0.3094	0.3030	0.2134	0.2055	0.1323	0.1253	0.1003	0.0951

Aromatase Assay Spreadsheet

Assay Date	11/30/2006	Inhibitor	AG-R4	NB page ref	12507-28

Aromatase Assay Spreadsheet

Assay Date	11/30/2006	Test Chemical ID	AG-R4	NB page ref	12507-28

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0200	36249	1812450		
2	0.0197	34418	1747107		
3	0.0200	39342	1967100		
4	0.0196	37197	1897806		
5	0.0201	49313	2453383		
			Average DPM/g soln		1975569
			SD		279834
			CV		14.16
			µCi/g soln		0.890

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.01	10		1001.00
Dilution A			100	10.01
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1296 g
Mass of dilution B used in substrate prep	4.5743 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.563235 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01085 µg/g soln.
µg/g soln.

a. µCi/g soln	0.890
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.563235 + 0.01085
= 0.574080 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.550 µCi/µg ASDN

985582 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/30/2006	Test Chemical ID	AG-R4	NB page ref	12507-28

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0195	31891	1635436		
2	0.0195	33120	1698462		
3	0.0192	36820	1917708		
4	0.0195	34981	1793897		
5	0.0199	37984	1908744		
			Average DPM/g soln		1790849
			SD		125190
			CV		6.99
			µCi/g soln		0.807

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.01	10		1001.00
Dilution A			100	10.01
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0622 g
Mass of dilution B used in substrate prep	0.6749 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.133455 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00983 µg/g soln.
µg/g soln.

a. µCi/g soln 0.807
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.133455 + 0.00983
= 0.143286 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.630 µCi/µg ASDN

3579546 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	11/30/2006	Inhibitor	AG-R4	NB page ref	12507-28

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (µM)
0	0
1	25
2	15
3	5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date: 11/30/2006 Inhibitor: AG-R4 NB page ref: 12507-28

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (nmol)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	115	230	234	468	0.1	A	197557	20	0.0000	15	0.0095	0.0001
				0.5	2	119	238			0.1							
	2	NA	2	0.5	1	113	226	218	436	0.1	A	197557	-12	0.0000	15	0.0095	-0.0001
				0.5	2	105	210			0.1							
	3	NA	2	0.5	1	121	242	231	462	0.1	A	197557	14	0.0000	15	0.0095	0.0001
				0.5	2	110	220			0.1							
	4	NA	2	0.5	1	99	198	213	426	0.1	A	197557	-22	0.0000	15	0.0095	-0.0002
				0.5	2	114	228			0.1							
AG-R4	15	0	2	0.5	1	10540	21080	21072	42144	0.05	B	107451	41598	0.0115	15	0.0095	0.0316
				0.5	2	10532	20864			0.05							
	15	0	2	0.5	1	10330	20660	20828	41656	0.05	B	107451	41206	0.0115	15	0.0095	0.0307
				0.5	2	10498	20996			0.05							
	15	5	2	0.5	1	3059	6118	6040	12080	0.05	B	107451	11632	0.0032	15	0.0095	0.0228
				0.5	2	2981	5962			0.05							
	15	5	2	0.5	1	2855	5710	5564	11128	0.05	B	107451	10720	0.0030	15	0.0095	0.0210
				0.5	2	2729	5458			0.05							
	15	15	2	0.5	1	1402	2804	2622	5644	0.05	B	107451	5196	0.0015	15	0.0095	0.0102
				0.5	2	1420	2840			0.05							
	15	15	2	0.5	1	1240	2480	2542	5084	0.05	B	107451	4636	0.0013	15	0.0095	0.0091
				0.5	2	1302	2604			0.05							
	15	25	2	0.5	1	859	1718	1710	3420	0.05	B	107451	2972	0.0008	15	0.0095	0.0058
				0.5	2	851	1702			0.05							
	15	25	2	0.5	1	904	1808	1896	3792	0.05	B	107451	3344	0.0009	15	0.0095	0.0065
				0.5	2	942	1884			0.05							
	25	0	2	0.5	1	15727	31454	31129	62258	0.1	B	179085	61810	0.0173	15	0.0095	0.1210
				0.5	2	15402	30804			0.1							
	25	0	2	0.5	1	16715	33430	33313	66626	0.1	B	179085	66178	0.0185	15	0.0095	0.1296
				0.5	2	16568	33196			0.1							
	25	5	2	0.5	1	4691	9382	9311	18622	0.1	B	179085	18174	0.0051	15	0.0095	0.0356
				0.5	2	4620	9240			0.1							
	25	5	2	0.5	1	4655	9310	9294	18588	0.1	B	179085	18140	0.0051	15	0.0095	0.0355
				0.5	2	4639	9278			0.1							
	25	15	2	0.5	1	2131	4262	4427	8854	0.1	B	179085	8406	0.0023	15	0.0095	0.0165
				0.5	2	2246	4492			0.1							
	25	15	2	0.5	1	2151	4302	4311	8622	0.1	B	179085	8174	0.0023	15	0.0095	0.0160
				0.5	2	2160	4320			0.1							
	25	25	2	0.5	1	1442	2884	2900	5800	0.1	B	179085	5352	0.0015	15	0.0095	0.0105
				0.5	2	1458	2916			0.1							
	25	25	2	0.5	1	1491	2982	2929	5858	0.1	B	179085	5410	0.0015	15	0.0095	0.0106
				0.5	2	1438	2876			0.1							
	35	0	2	0.5	1	20114	40228	39784	79568	0.14	B	250719	79120	0.0221	15	0.0095	0.1549
				0.5	2	19670	39340			0.14							
	35	0	2	0.5	1	19833	39666	39142	78284	0.14	B	250719	77836	0.0217	15	0.0095	0.1524
				0.5	2	19509	39018			0.14							
	35	5	2	0.5	1	6541	13082	13035	26070	0.14	B	250719	25622	0.0072	15	0.0095	0.0502
				0.5	2	6494	12988			0.14							
	35	5	2	0.5	1	6565	13130	12889	25778	0.14	D	250719	25330	0.0071	15	0.0095	0.0496
				0.5	2	6324	12648			0.14							
	35	15	2	0.5	1	3023	6046	5928	11856	0.14	B	250719	11465	0.0032	15	0.0095	0.0225
				0.5	2	2945	5890			0.14							
	35	15	2	0.5	1	2909	5818	5896	11792	0.14	B	250719	11344	0.0032	15	0.0095	0.0222
				0.5	2	2987	5974			0.14							
	35	25	2	0.5	1	1947	3894	3964	7928	0.14	B	250719	7480	0.0021	15	0.0095	0.0146
				0.5	2	2017	4034			0.14							
	35	25	2	0.5	1	2029	4058	3879	7758	0.14	D	250719	7310	0.0020	15	0.0095	0.0143
				0.5	2	1850	3700			0.14							
	50	0	2	0.5	1	6237	12474	12606	25212	0.05	A	98778	24784	0.0251	15	0.0095	0.1761
				0.5	2	6369	12738			0.05							
	50	0	2	0.5	1	5756	11512	11590	23180	0.05	A	98778	22732	0.0231	15	0.0095	0.1617
				0.5	2	5834	11668			0.05							
	50	5	2	0.5	1	2562	5124	5067	10134	0.05	A	98778	9686	0.0098	15	0.0095	0.0689
				0.5	2	2505	5010			0.05							
	50	5	2	0.5	1	2730	5460	5390	10780	0.05	A	98778	10312	0.0105	15	0.0095	0.0733
				0.5	2	2650	5300			0.05							
	50	15	2	0.5	1	1074	2148	2096	4192	0.05	A	98778	3744	0.0038	15	0.0095	0.0266
				0.5	2	1022	2044			0.05							
	50	15	2	0.5	1	1053	2106	2056	4112	0.05	A	98778	3664	0.0037	15	0.0095	0.0261
				0.5	2	1003	2006			0.05							
	50	25	2	0.5	1	676	1352	1383	2766	0.05	A	98778	2318	0.0024	15	0.0095	0.0165
				0.5	2	707	1414			0.05							
	50	25	2	0.5	1	922	1844	1852	3704	0.05	A	98778	3256	0.0033	15	0.0095	0.0232
				0.5	2	930	1860			0.05							
	100	0	2	0.5	1	8645	17290	17210	34432	0.1	A	197557	33984	0.0345	15	0.0095	0.2417
				0.5	2	8571	17142			0.1							
	100	0	2	0.5	1	7490	14980	14985	29970	0.1	A	197557	29522	0.0300	15	0.0095	0.2100
				0.5	2	7495	14990			0.1							
	100	5	2	0.5	1	3823	7646	7833	15666	0.1	A	197557	15218	0.0154	15	0.0095	0.1082
				0.5	2	3910	7820			0.1							
	100	5	2	0.5	1	3402	6804	7028	14056	0.1	A	197557	13608	0.0138	15	0.0095	0.0968
				0.5	2	3626	7252			0.1							
	100	15	2	0.5	1	2063	4125	4123	8248	0.1	A	197557	7796	0.0079	15	0.0095	0.0555
				0.5	2	2060	4120			0.1							

Aromatase Assay Spreadsheet

Assay Date 11/30/2006 Inhibitor AG-R4 NB page ref 12507-28

Sample ID			Calculate DPM in aqueous portion after extraction							Volume of substrate solution used/assay tube (mL)	Substrate ID	Calculate nmol ^3H_2O formed			Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (μM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM			total DPM in assay tube (mdef)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed			
	100	15	2	0.5	1	2020	4040	4023	8046	0.1	A	197667	7586	0.0077	15	0.0095	0.0540
				0.5	2	2003	4006			0.1							
	100	25	2	0.5	1	1363	2726	2700	5400	0.1	A	197667	4952	0.0050	15	0.0095	0.0352
				0.5	2	1337	2674			0.1							
	100	25	2	0.5	1	1248	2496	2446	4892	0.1	A	197667	4444	0.0045	15	0.0095	0.0316
				0.5	2	1198	2396			0.1							
	300	0	2	0.5	1	9905	19810	19827	39654	0.3	A	592671	39200	0.0395	15	0.0095	0.2788
				0.5	2	9922	19844			0.3							
	300	0	2	0.5	1	10457	20914	20767	41534	0.3	A	592671	41080	0.0417	15	0.0095	0.2922
				0.5	2	10310	20620			0.3							
	300	5	2	0.5	1	6947	13894	13528	27056	0.3	A	592671	26508	0.0270	15	0.0095	0.1892
				0.5	2	6581	13162			0.3							
	300	5	2	0.5	1	6448	12896	13071	26142	0.3	A	592671	25684	0.0261	15	0.0095	0.1827
				0.5	2	6623	13246			0.3							
	300	15	2	0.5	1	3911	7822	7843	15686	0.3	A	592671	15433	0.0157	15	0.0095	0.1098
				0.5	2	4032	8064			0.3							
	300	15	2	0.5	1	4276	8552	8661	17322	0.3	A	592671	15874	0.0171	15	0.0095	0.1200
				0.5	2	4385	8770			0.3							
	300	25	2	0.5	1	3217	6434	6437	12874	0.3	A	592671	12426	0.0126	15	0.0095	0.0884
				0.5	2	3220	6440			0.3							
	300	25	2	0.5	1	3233	6466	6429	12858	0.3	A	592671	12410	0.0126	15	0.0095	0.0883
				0.5	2	3196	6392			0.3							

Aromatase Assay Spreadsheet

Assay Date	11/30/2006	Inhibitor	AG-R4	NB page ref	12507-28

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		5		15		25	
15	0.0816	0.0807	0.0228	0.0210	0.0102	0.0091	0.0058	0.0065
25	0.1210	0.1298	0.0356	0.0355	0.0165	0.0160	0.0105	0.0108
35	0.1549	0.1524	0.0502	0.0496	0.0225	0.0222	0.0146	0.0143
50	0.1761	0.1617	0.0689	0.0733	0.0266	0.0261	0.0165	0.0232
100	0.2417	0.2100	0.1082	0.0968	0.0555	0.0540	0.0352	0.0316
300	0.2788	0.2922	0.1892	0.1827	0.1098	0.1200	0.0884	0.0883

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Inhibitor	NYP-Pilot	NB page ref	12507-30

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Test Chemical ID	NYP-Pilot	NB page ref	12507-30

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0202	38141	1888168		
2	0.0193	36657	1899326		
3	0.0195	40235	2063333		
4	0.0195	39894	2045846		
5	0.0199	39628	1991357		
			Average DPM/g soln		1977606
			SD		81120
			CV		4.10
			µCi/g soln		0.891

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1399 g
Mass of dilution B used in substrate prep	4.5857 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.564487 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01086 µg/g soln.
µg/g soln.

a. µCi/g soln	0.891
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.564487 + 0.01086
= 0.575344 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.548 µCi/µg ASDN

984431 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Test Chemical ID	NYP-Pilot	NB page ref	12507-30

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0199	32949	1655729	
2	0.0192	29671	1545365	
3	0.0201	35539	1768109	
4	0.0198	32678	1650404	
5	0.0202	39885	1974505	
			Average DPM/g soln	1718822
			SD	163212
			CV	9.50
			µCi/g soln	0.774

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0532 g
Mass of dilution B used in substrate prep	0.6706 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.132973 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00944 µg/g soln.
µg/g soln.

a. µCi/g soln 0.774
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.132973 + 0.00944
= 0.142409 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.437 µCi/µg ASDN

3456732 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Inhibitor	NYP-Pilot	NB page ref	12507-30

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (µM)
0	0
1	20
2	10
3	5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Inhibitor	NYP-Pilot	NB page ref	12507-30

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (uM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	102	204	208	412	0.1	A	197761	-23	0.0000	15	0.0095	-0.0002
				0.5	2	104	208			0.1							
	2	NA	2	0.5	1	98	196	202	404	0.1	A	197761	-31	0.0000	15	0.0095	-0.0002
				0.5	2	104	208			0.1							
	3	NA	2	0.5	1	114	228	230	460	0.1	A	197761	25	0.0000	15	0.0095	0.0002
				0.5	2	116	232			0.1							
	4	NA	2	0.5	1	114	228	232	464	0.1	A	197761	29	0.0000	15	0.0095	0.0002
				0.5	2	118	236			0.1							
NYP-Pilot	15	0	2	0.5	1	9839	19358	19224	38448	0.05	B	1031129	38013	0.0110	15	0.0095	0.0771
				0.5	2	9545	19090			0.05							
	15	0	2	0.5	1	10021	20042	19959	39918	0.05	B	1031129	39483	0.0114	15	0.0095	0.0801
				0.5	2	9938	19876			0.05							
	15	5	2	0.5	1	7865	15730	15593	31186	0.05	B	1031129	31351	0.0091	15	0.0095	0.0636
				0.5	2	8028	16056			0.05							
	15	5	2	0.5	1	7912	15824	15580	31170	0.05	B	1031129	30825	0.0089	15	0.0095	0.0622
				0.5	2	7648	15296			0.05							
	15	10	2	0.5	1	4978	9956	9579	19158	0.05	B	1031129	18723	0.0054	15	0.0095	0.0380
				0.5	2	4601	9202			0.05							
	15	10	2	0.5	1	5403	10806	10546	21086	0.05	B	1031129	20581	0.0060	15	0.0095	0.0419
				0.5	2	5145	10290			0.05							
	15	20	2	0.5	1	2416	4832	4849	9698	0.05	B	1031129	9253	0.0027	15	0.0095	0.0188
				0.5	2	2433	4866			0.05							
	15	20	2	0.5	1	2449	4898	4962	9924	0.05	B	1031129	9489	0.0027	15	0.0095	0.0192
				0.5	2	2513	5026			0.05							
	25	0	2	0.5	1	13010	26020	25907	51814	0.1	B	171882	51379	0.0149	15	0.0095	0.1042
				0.5	2	12797	25794			0.1							
	25	0	2	0.5	1	11751	23502	23527	47054	0.1	B	171882	46819	0.0135	15	0.0095	0.0945
				0.5	2	11776	23552			0.1							
	25	5	2	0.5	1	11456	22912	22880	45760	0.1	B	171882	45325	0.0131	15	0.0095	0.0919
				0.5	2	11432	22864			0.1							
	25	5	2	0.5	1	13465	26930	20871	53742	0.1	B	171882	53307	0.0154	15	0.0095	0.1081
				0.5	2	13406	26812			0.1							
	25	10	2	0.5	1	7532	15064	15282	30564	0.1	B	171882	30179	0.0087	15	0.0095	0.0611
				0.5	2	7750	15500			0.1							
	25	10	2	0.5	1	8366	16716	16505	33010	0.1	B	171882	32575	0.0094	15	0.0095	0.0661
				0.5	2	8147	16294			0.1							
	25	20	2	0.5	1	4357	8714	8720	17440	0.1	B	171882	17005	0.0049	15	0.0095	0.0345
				0.5	2	4363	8726			0.1							
	25	20	2	0.5	1	4346	8692	8504	17196	0.1	B	171882	16733	0.0048	15	0.0095	0.0339
				0.5	2	4238	8476			0.1							
	35	0	2	0.5	1	15541	31082	30946	61892	0.14	B	240835	61457	0.0178	15	0.0095	0.1246
				0.5	2	15405	30810			0.14							
	35	0	2	0.5	1	18978	37956	37680	75320	0.14	B	240835	74925	0.0217	15	0.0095	0.1519
				0.5	2	18702	37404			0.14							
	35	5	2	0.5	1	15370	30740	30477	60954	0.14	B	240835	60519	0.0175	15	0.0095	0.1227
				0.5	2	15107	30214			0.14							
	35	5	2	0.5	1	16344	32688	32836	65672	0.14	B	240835	65437	0.0189	15	0.0095	0.1327
				0.5	2	16492	32984			0.14							
	35	10	2	0.5	1	9752	19504	19849	39298	0.14	B	240835	38583	0.0112	15	0.0095	0.0788
				0.5	2	9897	19794			0.14							
	35	10	2	0.5	1	10021	20042	19886	39972	0.14	B	240835	39537	0.0114	15	0.0095	0.0802
				0.5	2	9863	19730			0.14							
	35	20	2	0.5	1	5183	10366	10491	20982	0.14	B	240835	20547	0.0059	15	0.0095	0.0417
				0.5	2	5309	10618			0.14							
	35	20	2	0.5	1	5403	10806	10736	21472	0.14	B	240835	21037	0.0061	15	0.0095	0.0427
				0.5	2	5333	10666			0.14							
	50	0	2	0.5	1	4977	9954	9837	19674	0.05	A	98580	19239	0.0195	15	0.0095	0.1370
				0.5	2	4860	9720			0.05							
	50	0	2	0.5	1	5006	10012	10182	20364	0.05	A	98580	19929	0.0202	15	0.0095	0.1419
				0.5	2	5176	10352			0.05							
	50	5	2	0.5	1	5484	10968	11084	22168	0.05	A	98580	21733	0.0221	15	0.0095	0.1547
				0.5	2	5600	11200			0.05							
	50	5	2	0.5	1	4755	9510	9455	18910	0.05	A	98580	18475	0.0188	15	0.0095	0.1315
				0.5	2	4676	9350			0.05							
	50	10	2	0.5	1	3449	6898	6945	13890	0.05	A	98580	13455	0.0137	15	0.0095	0.0958
				0.5	2	3496	6992			0.05							
	50	10	2	0.5	1	3903	7806	7854	15708	0.05	A	98580	15273	0.0155	15	0.0095	0.1087
				0.5	2	3951	7902			0.05							
	50	20	2	0.5	1	2149	4298	4271	8542	0.05	A	98580	8107	0.0082	15	0.0095	0.0577
				0.5	2	2122	4244			0.05							
	50	20	2	0.5	1	2080	4160	4121	8242	0.05	A	98580	7807	0.0079	15	0.0095	0.0556
				0.5	2	2020	4040			0.05							
	100	0	2	0.5	1	6443	12886	13194	26388	0.1	A	197761	25653	0.0260	15	0.0095	0.1848
				0.5	2	6751	13502			0.1							
	100	0	2	0.5	1	6291	12582	12542	25084	0.1	A	197761	24849	0.0252	15	0.0095	0.1755
				0.5	2	6251	12502			0.1							
	100	5	2	0.5	1	7174	14348	14174	28348	0.1	A	197761	27913	0.0284	15	0.0095	0.1987
				0.5	2	7000	14000			0.1							
	100	5	2	0.5	1	7003	14006	13976	27952	0.1	A	197761	27517	0.0280	15	0.0095	0.1958
				0.5	2	6973	13946			0.1							
	100	10	2	0.5	1	4752	9504	9350	18700	0.1	A	197761	18265	0.0186	15	0.0095	0.1300
				0.5	2	4598	9196			0.1							

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Inhibitor	MYP-Pilot	NB page ref	12507-30

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nonenzyme total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (nmol)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
	100	10	2	0.5	1	4617	9234	9238	18476	0.1	A	197761	16041	0.0183	15	0.0095	0.1265
				0.5	2	4621	9242			0.1							
	100	20	2	0.5	1	2978	5956	5966	11972	0.1	A	197761	11537	0.0117	15	0.0095	0.0821
				0.5	2	3011	6022			0.1							
	100	20	2	0.5	1	2902	5824	5900	11800	0.1	A	197761	11365	0.0116	15	0.0095	0.0809
				0.5	2	2908	5876			0.1							
	300	0	2	0.5	1	7697	15394	15344	30688	0.3	A	593252	30253	0.0307	15	0.0095	0.2154
				0.5	2	7647	15294			0.3							
	300	0	2	0.5	1	7709	15538	15391	30782	0.3	A	593252	30347	0.0308	15	0.0095	0.2161
				0.5	2	7622	15244			0.3							
	300	5	2	0.5	1	8340	16680	16166	32332	0.3	A	593252	31837	0.0324	15	0.0095	0.2271
				0.5	2	7826	15652			0.3							
	300	5	2	0.5	1	8519	17038	16917	33834	0.3	A	593252	33399	0.0339	15	0.0095	0.2378
				0.5	2	8398	16796			0.3							
	300	10	2	0.5	1	6978	13956	13962	27924	0.3	A	593252	27489	0.0279	15	0.0095	0.1957
				0.5	2	6984	13968			0.3							
	300	10	2	0.5	1	6916	13832	14049	28098	0.3	A	593252	27663	0.0281	15	0.0095	0.1970
				0.5	2	7133	14266			0.3							
	300	20	2	0.5	1	4784	9568	9517	19034	0.3	A	593252	18599	0.0189	15	0.0095	0.1324
				0.5	2	4733	9466			0.3							
	300	20	2	0.5	1	4523	9046	8774	17548	0.3	A	593252	17113	0.0174	15	0.0095	0.1218
				0.5	2	4251	8502			0.3							

Aromatase Assay Spreadsheet

Assay Date	12/8/2006	Inhibitor	NYP-Pilot	NB page ref	12507-30

Substrate Concentration (nM)	Inhibitor Concentration (µM)							
	0		5		10		20	
15	0.0771	0.0801	0.0636	0.0622	0.0380	0.0419	0.0188	0.0192
25	0.1042	0.0945	0.0919	0.1081	0.0611	0.0661	0.0345	0.0339
35	0.1246	0.1519	0.1227	0.1327	0.0788	0.0802	0.0417	0.0427
50	0.1370	0.1419	0.1547	0.1315	0.0958	0.1087	0.0577	0.0556
100	0.1848	0.1755	0.1987	0.1959	0.1300	0.1285	0.0821	0.0809
300	0.2154	0.2161	0.2271	0.2378	0.1957	0.1970	0.1324	0.1218

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Inhibitor	NYP-R1	NB page ref	12507-31

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Test Chemical ID	NYP-R1	NB page ref	12507-31

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0199	35166	1767136	
2	0.0194	35467	1828196	
3	0.0194	34484	1777526	
4	0.0199	37053	1861960	
5	0.0200	35555	1777750	
			Average DPM/g soln	1802513
			SD	40874
			CV	2.27
			µCi/g soln	0.812

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1654 g
Mass of dilution B used in substrate prep	4.5816 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.561099 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00990 µg/g soln.
µg/g soln.

a. µCi/g soln 0.812
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.561099 + 0.00990
= 0.570995 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.422 µCi/µg ASDN

904106 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Test Chemical ID	NYP-R1	NB page ref	12507-31

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0199	29380	1476382		
2	0.0195	30395	1558718		
3	0.0198	32684	1650707		
4	0.0199	30367	1525980		
5	0.0203	34384	1693793		
			Average DPM/g soln		1581116
			SD		89512
			CV		5.66
			µCi/g soln		0.712

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0372 g
Mass of dilution B used in substrate prep	0.6647 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.131958 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00868 µg/g soln.
µg/g soln.

a. µCi/g soln 0.712
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.131958 + 0.00868
= 0.140638 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.064 µCi/µg ASDN

3219835 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Inhibitor	NYP-R1	NB page ref	12507-31

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (µM)
0	0
1	37.5
2	75
3	20

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Inhibitor	NYP-R1	NB page ref	12507-31

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Avg DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	Total DPM in assay tube (mdaf)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	103	206	203	406	0.1	A	180251	-30	0.0000	15	0.0095	-0.0002
				0.5	2	100	200			0.1							
	2	NA	2	0.5	1	102	204	224	448	0.1	A	180251	13	0.0000	15	0.0095	0.0001
				0.5	2	122	244			0.1							
	3	NA	2	0.5	1	113	226	221	442	0.1	A	180251	7	0.0000	15	0.0095	0.0001
				0.5	2	108	216			0.1							
	4	NA	2	0.5	1	116	232	223	446	0.1	A	180251	11	0.0000	15	0.0095	0.0001
				0.5	2	107	214			0.1							
NYP-R1	15	0	2	0.5	1	9468	18936	18849	37698	0.08	B	94867	37763	0.0116	15	0.0095	0.0811
				0.5	2	9381	18762			0.08							
	15	0	2	0.5	1	9376	18752	18819	37638	0.08	B	94867	37703	0.0116	15	0.0095	0.0810
				0.5	2	9443	18886			0.08							
	15	20	2	0.5	1	2578	5156	5019	10038	0.08	B	94867	9603	0.0030	15	0.0095	0.0209
				0.5	2	2441	4882			0.08							
	15	20	2	0.5	1	2593	5186	5083	10166	0.08	B	94867	9731	0.0030	15	0.0095	0.0212
				0.5	2	2490	4980			0.08							
	15	75	2	0.5	1	263	526	564	1128	0.08	B	94867	693	0.0002	15	0.0095	0.0015
				0.5	2	275	550			0.08							
	15	75	2	0.5	1	455	910	936	1872	0.08	B	94867	1437	0.0004	15	0.0095	0.0031
				0.5	2	481	962			0.08							
	15	37.5	2	0.5	1	968	1976	1966	3936	0.08	B	94867	3501	0.0011	15	0.0095	0.0076
				0.5	2	980	1960			0.08							
	15	37.5	2	0.5	1	1067	2174	2216	4432	0.08	B	94867	3997	0.0012	15	0.0095	0.0087
				0.5	2	1129	2258			0.08							
	25	0	2	0.5	1	13503	27206	26994	53988	0.1	B	158112	53553	0.0156	15	0.0095	0.1166
				0.5	2	13391	26782			0.1							
	25	0	2	0.5	1	12463	24926	24877	49754	0.1	B	158112	49319	0.0153	15	0.0095	0.1074
				0.5	2	12414	24828			0.1							
	25	20	2	0.5	1	4356	8712	8846	17692	0.1	B	158112	17257	0.0054	15	0.0095	0.0376
				0.5	2	4490	8980			0.1							
	25	20	2	0.5	1	4239	8478	8483	16966	0.1	B	158112	16531	0.0051	15	0.0095	0.0360
				0.5	2	4244	8488			0.1							
	25	75	2	0.5	1	455	910	883	1766	0.1	B	158112	1331	0.0004	15	0.0095	0.0029
				0.5	2	428	856			0.1							
	25	75	2	0.5	1	484	968	950	1900	0.1	B	158112	1465	0.0005	15	0.0095	0.0032
				0.5	2	466	932			0.1							
	25	37.5	2	0.5	1	1662	3324	3289	6578	0.1	B	158112	6143	0.0019	15	0.0095	0.0134
				0.5	2	1627	3254			0.1							
	25	37.5	2	0.5	1	1699	3398	3387	6774	0.1	B	158112	6339	0.0020	15	0.0095	0.0138
				0.5	2	1688	3376			0.1							
	35	0	2	0.5	1	16480	32960	32715	65430	0.14	B	221356	65001	0.0202	15	0.0095	0.1415
				0.5	2	16238	32476			0.14							
	35	0	2	0.5	1	17999	35998	35984	71968	0.14	B	221356	71533	0.0222	15	0.0095	0.1557
				0.5	2	17963	35970			0.14							
	35	20	2	0.5	1	5860	11720	11827	23654	0.14	B	221356	23219	0.0072	15	0.0095	0.0505
				0.5	2	5967	11934			0.14							
	35	20	2	0.5	1	5608	11216	11190	22380	0.14	B	221356	21945	0.0068	15	0.0095	0.0478
				0.5	2	5582	11164			0.14							
	35	75	2	0.5	1	695	1390	1422	2844	0.14	B	221356	2409	0.0007	15	0.0095	0.0052
				0.5	2	727	1454			0.14							
	35	75	2	0.5	1	642	1284	1255	2510	0.14	B	221356	2075	0.0006	15	0.0095	0.0045
				0.5	2	613	1226			0.14							
	35	37.5	2	0.5	1	2932	5864	5862	11724	0.14	B	221356	11289	0.0035	15	0.0095	0.0246
				0.5	2	2930	5860			0.14							
	35	37.5	2	0.5	1	2629	5258	5228	10456	0.14	B	221356	10021	0.0031	15	0.0095	0.0218
				0.5	2	2599	5198			0.14							
	50	0	2	0.5	1	5777	11554	11441	22882	0.05	A	90126	22447	0.0248	15	0.0095	0.1740
				0.5	2	5664	11328			0.05							
	50	0	2	0.5	1	6337	12674	12291	24582	0.05	A	90126	24147	0.0267	15	0.0095	0.1872
				0.5	2	5954	11908			0.05							
	50	20	2	0.5	1	2203	4406	4390	8780	0.05	A	90126	8345	0.0092	15	0.0095	0.0647
				0.5	2	2187	4374			0.05							
	50	20	2	0.5	1	2492	4984	5023	10046	0.05	A	90126	9611	0.0106	15	0.0095	0.0745
				0.5	2	2531	5062			0.05							
	50	75	2	0.5	1	312	624	628	1256	0.05	A	90126	821	0.0009	15	0.0095	0.0064
				0.5	2	316	632			0.05							
	50	75	2	0.5	1	323	646	664	1328	0.05	A	90126	893	0.0010	15	0.0095	0.0069
				0.5	2	341	682			0.05							
	50	37.5	2	0.5	1	1142	2284	2291	4582	0.05	A	90126	4147	0.0046	15	0.0095	0.0321
				0.5	2	1149	2298			0.05							
	50	37.5	2	0.5	1	1222	2444	2396	4792	0.05	A	90126	4357	0.0048	15	0.0095	0.0338
				0.5	2	1174	2348			0.05							
	100	0	2	0.5	1	7326	14652	14805	29610	0.1	A	180251	29175	0.0323	15	0.0095	0.2262
				0.5	2	7479	14958			0.1							
	100	0	2	0.5	1	8167	16334	16223	32446	0.1	A	180251	32011	0.0354	15	0.0095	0.2482
				0.5	2	8056	16112			0.1							
	100	20	2	0.5	1	3446	6892	6785	13570	0.1	A	180251	13135	0.0145	15	0.0095	0.1018
				0.5	2	3339	6678			0.1							
	100	20	2	0.5	1	3314	6628	6618	13236	0.1	A	180251	12801	0.0142	15	0.0095	0.0992
				0.5	2	3304	6608			0.1							
	100	75	2	0.5	1	433	866	824	1648	0.1	A	180251	1213	0.0013	15	0.0095	0.0094
				0.5	2	391	782			0.1							

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Inhibitor	NYP-R1	NB page ref	12507-31

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (μM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
	100	75	2	0.5	1	435	870	920	1840	0.1	A	180251	1405	0.0016	15	0.0095	0.0109
				0.5	2	485	970			0.1							
	100	37.5	2	0.5	1	1878	3756	3750	7500	0.1	A	180251	7065	0.0078	15	0.0095	0.0548
				0.5	2	1872	3744			0.1							
	100	37.5	2	0.5	1	1857	3714	3673	7348	0.1	A	180251	6911	0.0076	15	0.0095	0.0536
				0.5	2	1816	3632			0.1							
	300	0	2	0.5	1	7833	15666	15526	31052	0.3	A	540754	30617	0.0339	15	0.0095	0.2374
				0.5	2	7693	15386			0.3							
	300	0	2	0.5	1	6504	17008	16795	33590	0.3	A	540754	33156	0.0367	15	0.0095	0.2570
				0.5	2	8291	16582			0.3							
	300	20	2	0.5	1	5077	10154	10318	20636	0.3	A	540754	20201	0.0223	15	0.0095	0.1566
				0.5	2	5241	10482			0.3							
	300	20	2	0.5	1	5261	10522	10455	20910	0.3	A	540754	20475	0.0226	15	0.0095	0.1587
				0.5	2	5194	10388			0.3							
	300	7.5	2	0.5	1	1786	3572	3519	7039	0.3	A	540754	6585	0.0073	15	0.0095	0.0510
				0.5	2	1724	3448			0.3							
	300	7.5	2	0.5	1	1857	3714	3795	7590	0.3	A	540754	7155	0.0079	15	0.0095	0.0565
				0.5	2	1938	3876			0.3							
	300	3.75	2	0.5	1	3220	6440	6299	12598	0.3	A	540754	12163	0.0135	15	0.0095	0.0943
				0.5	2	3079	6158			0.3							
	300	3.75	2	0.5	1	3244	6488	6482	12964	0.3	A	540754	12529	0.0139	15	0.0095	0.0971
				0.5	2	3238	6476			0.3							

Aromatase Assay Spreadsheet

Assay Date	12/21/2006	Inhibitor	NYP-R1	NB page ref	12507-31

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		20		75		37.5	
15	0.0811	0.0810	0.0209	0.0212	0.0015	0.0031	0.0076	0.0087
25	0.1166	0.1074	0.0376	0.0360	0.0029	0.0032	0.0134	0.0138
35	0.1415	0.1557	0.0505	0.0478	0.0052	0.0045	0.0246	0.0218
50	0.1740	0.1872	0.0647	0.0745	0.0064	0.0069	0.0321	0.0338
100	0.2262	0.2482	0.1018	0.0992	0.0094	0.0109	0.0548	0.0538
300	0.2374	0.2570	0.1566	0.1587	0.0510	0.0555	0.0943	0.0971

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Inhibitor	NYP-R2	NB page ref	12507-32

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Test Chemical ID	NYP-R2	NB page ref	12507-32

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0201	34647	1723731		
2	0.0196	32575	1661990		
3	0.0204	38964	1910000		
4	0.0201	39063	1943433		
5	0.0198	38770	1958081		
			Average DPM/g soln		1839447
			SD		136699
			CV		7.43
			µCi/g soln		0.829

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.05	10		1005.00
Dilution A			100	10.05
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.138 g
Mass of dilution B used in substrate prep	4.5931 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.567224 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01010 µg/g soln.
µg/g soln.

a. µCi/g soln	0.829
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.567224 + 0.01010
= 0.577322 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.435 µCi/µg ASDN

912520 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Test Chemical ID	NYP-R2	NB page ref	12507-32

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0201	34144	1698706	
2	0.0199	31244	1570050	
3	0.0197	38386	1948528	
4	0.0202	37500	1856436	
5	0.0202	38644	1913069	
			Average DPM/g soln	1797358
			SD	159007
			CV	8.85
			µCi/g soln	0.810

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.05	10		1005.00
Dilution A			100	10.05
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0807 g
Mass of dilution B used in substrate prep	0.6711 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.132749 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00987 µg/g soln.
µg/g soln.

a. µCi/g soln 0.810
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.132749 + 0.00987
= 0.142616 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.677 µCi/µg ASDN

3609447 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Inhibitor	NYP-R2	NB page ref	12507-32

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (μM)
0	0
1	20
2	15
3	7.5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Inhibitor	NYP-R2	NB page ref	12507-32

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (mSet)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	118	236	210	430	0.1	A	183945	-22	0.0000	15	0.0095	-0.0002
				0.5	2	97	194			0.1							
	2	NA	2	0.5	1	119	238	234	468	0.1	A	183945	17	0.0000	15	0.0095	0.0001
				0.5	2	115	230			0.1							
	3	NA	2	0.5	1	110	220	224	448	0.1	A	183945	-4	0.0000	15	0.0095	0.0000
				0.5	2	114	228			0.1							
	4	NA	2	0.5	1	115	230	230	460	0.1	A	183945	9	0.0000	15	0.0095	0.0001
				0.5	2	111	222			0.1							
NYP-R2	15	0	2	0.5	1	10808	21616	20968	41936	0.05	B	107841	41485	0.0115	15	0.0095	0.0808
				0.5	2	10160	20320			0.05							
	15	0	2	0.5	1	11028	22056	21873	43746	0.05	B	107841	43295	0.0120	15	0.0095	0.0841
				0.5	2	10845	21690			0.05							
	15	7.5	2	0.5	1	9450	18900	18806	37612	0.05	B	107841	37151	0.0103	15	0.0095	0.0722
				0.5	2	9356	18712			0.05							
	15	7.5	2	0.5	1	8016	16032	16102	32204	0.05	B	107841	31753	0.0088	15	0.0095	0.0617
				0.5	2	8086	16172			0.05							
	15	15	2	0.5	1	4233	8476	8374	16748	0.05	B	107841	16297	0.0045	15	0.0095	0.0316
				0.5	2	4136	8272			0.05							
	15	15	2	0.5	1	4981	9962	9940	19920	0.05	B	107841	19429	0.0054	15	0.0095	0.0377
				0.5	2	4959	9918			0.05							
	15	20	2	0.5	1	4089	8178	8121	16242	0.05	B	107841	15791	0.0044	15	0.0095	0.0307
				0.5	2	4032	8064			0.05							
	15	20	2	0.5	1	3977	7954	7833	15676	0.05	B	107841	15225	0.0042	15	0.0095	0.0296
				0.5	2	3891	7782			0.05							
	25	0	2	0.5	1	17107	34214	34097	68194	0.1	D	179736	67743	0.0188	15	0.0095	0.1315
				0.5	2	16980	33980			0.1							
	25	0	2	0.5	1	17915	35830	35802	71604	0.1	D	179736	71153	0.0197	15	0.0095	0.1382
				0.5	2	17887	35774			0.1							
	25	7.5	2	0.5	1	12789	25578	25734	51466	0.1	D	179736	51017	0.0141	15	0.0095	0.0991
				0.5	2	12945	25890			0.1							
	25	7.5	2	0.5	1	13670	27352	27322	54644	0.1	D	179736	54193	0.0150	15	0.0095	0.1052
				0.5	2	13640	27292			0.1							
	25	15	2	0.5	1	7322	14644	14552	29104	0.1	D	179736	28653	0.0079	15	0.0095	0.0556
				0.5	2	7230	14460			0.1							
	25	15	2	0.5	1	7338	14676	14647	29294	0.1	D	179736	28843	0.0080	15	0.0095	0.0560
				0.5	2	7309	14618			0.1							
	25	20	2	0.5	1	5324	10648	10675	21350	0.1	D	179736	20599	0.0056	15	0.0095	0.0400
				0.5	2	5351	10702			0.1							
	25	20	2	0.5	1	5820	11640	11494	22988	0.1	D	179736	22537	0.0062	15	0.0095	0.0438
				0.5	2	5674	11348			0.1							
	35	0	2	0.5	1	21726	43452	43674	87348	0.14	D	251530	86897	0.0241	15	0.0095	0.1687
				0.5	2	21948	43896			0.14							
	35	0	2	0.5	1	23004	46008	45666	91332	0.14	D	251530	90881	0.0252	15	0.0095	0.1765
				0.5	2	22662	45324			0.14							
	35	7.5	2	0.5	1	18455	36910	36199	72398	0.14	D	251530	71947	0.0199	15	0.0095	0.1397
				0.5	2	17791	35582			0.14							
	35	7.5	2	0.5	1	17273	34546	34545	69090	0.14	D	251530	68639	0.0190	15	0.0095	0.1333
				0.5	2	17272	34544			0.14							
	35	15	2	0.5	1	9520	19040	20232	40464	0.14	D	251530	40013	0.0111	15	0.0095	0.0777
				0.5	2	10712	21424			0.14							
	35	15	2	0.5	1	11128	22256	22321	44642	0.14	D	251530	44191	0.0122	15	0.0095	0.0858
				0.5	2	11193	22386			0.14							
	35	20	2	0.5	1	7316	14632	14531	29062	0.14	D	251530	28611	0.0079	15	0.0095	0.0556
				0.5	2	7215	14430			0.14							
	35	20	2	0.5	1	7317	14634	14749	29498	0.14	D	251530	29047	0.0080	15	0.0095	0.0564
				0.5	2	7432	14864			0.14							
	50	0	2	0.5	1	7146	14292	14319	28638	0.05	A	91972	28187	0.0309	15	0.0095	0.2165
				0.5	2	7171	14342			0.05							
	50	0	2	0.5	1	6540	13080	13243	26486	0.05	A	91972	26035	0.0285	15	0.0095	0.2000
				0.5	2	6703	13406			0.05							
	50	7.5	2	0.5	1	5654	11308	11191	22382	0.05	A	91972	21931	0.0240	15	0.0095	0.1685
				0.5	2	5537	11074			0.05							
	50	7.5	2	0.5	1	5340	10680	10760	21520	0.05	A	91972	21069	0.0231	15	0.0095	0.1618
				0.5	2	5420	10840			0.05							
	50	15	2	0.5	1	3243	6486	6548	13096	0.05	A	91972	12645	0.0139	15	0.0095	0.0971
				0.5	2	3305	6610			0.05							
	50	15	2	0.5	1	3783	7566	7623	15246	0.05	A	91972	14795	0.0162	15	0.0095	0.1136
				0.5	2	3830	7660			0.05							
	50	20	2	0.5	1	2618	5232	5214	10428	0.05	A	91972	9977	0.0109	15	0.0095	0.0766
				0.5	2	2560	5196			0.05							
	50	20	2	0.5	1	2068	5012	4987	9974	0.05	A	91972	9523	0.0104	15	0.0095	0.0731
				0.5	2	2481	4962			0.05							
	100	0	2	0.5	1	9272	18544	18354	36708	0.1	A	183945	36257	0.0397	15	0.0095	0.2783
				0.5	2	9082	18164			0.1							
	100	0	2	0.5	1	8544	17088	17919	35838	0.1	A	183945	35337	0.0386	15	0.0095	0.2710
				0.5	2	8963	17930			0.1							
	100	7.5	2	0.5	1	7470	14940	14795	29780	0.1	A	183945	29339	0.0322	15	0.0095	0.2254
				0.5	2	7425	14850			0.1							
	100	7.5	2	0.5	1	7868	15736	15413	30826	0.1	A	183945	30375	0.0333	15	0.0095	0.2333
				0.5	2	7545	15090			0.1							
	100	15	2	0.5	1	5035	10070	9839	19678	0.1	A	183945	19427	0.0213	15	0.0095	0.1492
				0.5	2	4904	9808			0.1							

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Inhibitor	NYP-R2	NB page ref	12507-32

Sample ID			Calculate DPM in aqueous portion after extraction							Volume of substrate solution used/assay tube (mL)	Substrate ID	Total DPM in assay tube (nmol)	Calculate nmol ^3H_2O formed		Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (μM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM				Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed			
	100	15	2	0.5	1	4691	9782	9756	19516	0.1	A	183945	19005	0.0209	15	0.0095	0.1464
				0.5	2	4877	9754			0.1							
	100	20	2	0.5	1	4127	8254	8053	16106	0.1	A	183945	15656	0.0172	15	0.0095	0.1202
				0.5	2	3926	7852			0.1							
	100	20	2	0.5	1	3890	7780	7805	15610	0.1	A	183945	15159	0.0166	15	0.0095	0.1164
				0.5	2	3906	7812			0.1							
	300	0	2	0.5	1	10703	21406	21328	42656	0.3	A	551834	42205	0.0463	15	0.0095	0.3242
				0.5	2	10625	21250			0.3							
	300	0	2	0.5	1	10504	21008	20941	41882	0.3	A	551834	41431	0.0454	15	0.0095	0.3182
				0.5	2	10437	20874			0.3							
	300	7.5	2	0.5	1	10673	21346	21324	42648	0.3	A	551834	42197	0.0462	15	0.0095	0.3241
				0.5	2	10651	21302			0.3							
	300	7.5	2	0.5	1	10758	21516	21278	42556	0.3	A	551834	42105	0.0461	15	0.0095	0.3234
				0.5	2	10520	21040			0.3							
	300	15	2	0.5	1	7871	15742	15891	31782	0.3	A	551834	31331	0.0343	15	0.0095	0.2407
				0.5	2	8020	16040			0.3							
	300	15	2	0.5	1	8568	17172	17443	34886	0.3	A	551834	34435	0.0377	15	0.0095	0.2645
				0.5	2	8867	17714			0.3							
	300	20	2	0.5	1	6645	13290	13214	26428	0.3	A	551834	25977	0.0285	15	0.0095	0.1995
				0.5	2	6519	13038			0.3							
	300	20	2	0.5	1	6395	12790	12822	25644	0.3	A	551834	25193	0.0276	15	0.0095	0.1935
				0.5	2	6427	12854			0.3							

Aromatase Assay Spreadsheet

Assay Date	12/22/2006	Inhibitor	NYP-R2	NB page ref	12507-32

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		7.5		15		20	
15	0.0806	0.0841	0.0722	0.0617	0.0316	0.0377	0.0307	0.0296
25	0.1315	0.1382	0.0991	0.1052	0.0556	0.0560	0.0406	0.0438
35	0.1687	0.1785	0.1397	0.1333	0.0777	0.0858	0.0556	0.0564
50	0.2165	0.2000	0.1685	0.1618	0.0971	0.1138	0.0766	0.0731
100	0.2785	0.2718	0.2254	0.2333	0.1492	0.1464	0.1202	0.1164
300	0.3242	0.3182	0.3241	0.3234	0.2407	0.2645	0.1995	0.1935

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Inhibitor	NYP-R3	NB page ref	12507-33

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Test Chemical ID	NYP-R3	NB page ref	12507-33

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0198	36888	1863030		
2	0.0192	35222	1834479		
3	0.0199	40390	2029648		
4	0.0195	37514	1923795		
5	0.0199	41460	2083417		
			Average DPM/g soln		1946874
			SD		106877
			CV		5.49
			µCi/g soln		0.877

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	7.9123 g
Mass of dilution B used in substrate prep	4.3871 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.555575 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01069 µg/g soln.
µg/g soln.

a. µCi/g soln 0.877
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.555575 + 0.01069
= 0.566263 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.549 µCi/µg ASDN

984675 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Test Chemical ID	NYP-R3	NB page ref	12507-33

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0201	28384	1412139	
2	0.0199	27062	1359899	
3	0.0205	31406	1532000	
4	0.0197	29936	1519594	
5	0.0202	32591	1613416	
			Average DPM/g soln	1487410
			SD	101027
			CV	6.79
			µCi/g soln	0.670

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.0158 g
Mass of dilution B used in substrate prep	0.6798 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.135803 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00817 µg/g soln.
µg/g soln.

a. µCi/g soln 0.670
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 23500000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.135803 + 0.00817
= 0.143968 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 4.654 µCi/µg ASDN

2958944 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Inhibitor	NYP-R3	NB page ref	12507-33

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (μM)
0	0
1	20
2	15
3	7.5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Inhibitor	NYP-R3	NB page ref	12507-33

Sample ID			Calculate DPM in aqueous portion after extraction										Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	120	240	248	496	0.1	A	194687	20	0.0000	15	0.0095	0.0001
				0.5	2	128	256			0.1							
	2	NA	2	0.5	1	117	234	225	450	0.1	A	194687	-27	0.0000	15	0.0095	-0.0002
				0.5	2	108	216			0.1							
	3	NA	2	0.5	1	125	250	256	512	0.1	A	194687	35	0.0000	15	0.0095	0.0003
				0.5	2	131	262			0.1							
	4	NA	2	0.5	1	110	220	224	448	0.1	A	194687	-29	0.0000	15	0.0095	-0.0002
				0.5	2	114	228			0.1							
NYP-R3	15	0	2	0.5	1	9531	19062	19033	38066	0.06	B	89245	37590	0.0127	15	0.0095	0.0890
				0.5	2	9502	19004			0.06							
	15	0	2	0.5	1	9169	18338	18333	36676	0.06	B	89245	36200	0.0122	15	0.0095	0.0857
				0.5	2	9159	18338			0.06							
	15	7.5	2	0.5	1	8107	16214	16096	32196	0.06	B	89245	31720	0.0107	15	0.0095	0.0751
				0.5	2	7991	15982			0.06							
	15	7.5	2	0.5	1	9015	18030	18086	36172	0.06	B	89245	35696	0.0121	15	0.0095	0.0848
				0.5	2	9071	18142			0.06							
	15	15	2	0.5	1	6112	12224	12265	24530	0.06	B	89245	24054	0.0081	15	0.0095	0.0570
				0.5	2	6153	12306			0.06							
	15	15	2	0.5	1	5725	11450	11366	22732	0.06	B	89245	22256	0.0075	15	0.0095	0.0527
				0.5	2	5641	11282			0.06							
	15	20	2	0.5	1	3919	7838	7961	15922	0.06	B	89245	15446	0.0052	15	0.0095	0.0366
				0.5	2	4042	8084			0.06							
	15	20	2	0.5	1	3800	7600	7577	15154	0.06	B	89245	14678	0.0050	15	0.0095	0.0348
				0.5	2	3777	7554			0.06							
	25	0	2	0.5	1	14811	29622	29010	58020	0.1	B	148741	57544	0.0194	15	0.0095	0.1363
				0.5	2	14399	28798			0.1							
	25	0	2	0.5	1	14219	28438	28004	56008	0.1	B	148741	56632	0.0188	15	0.0095	0.1315
				0.5	2	13785	27570			0.1							
	25	7.5	2	0.5	1	11632	23264	23342	46684	0.1	B	148741	46208	0.0156	15	0.0095	0.1095
				0.5	2	11710	23420			0.1							
	25	7.5	2	0.5	1	11731	23462	23418	46838	0.1	B	148741	46360	0.0157	15	0.0095	0.1008
				0.5	2	11687	23374			0.1							
	25	15	2	0.5	1	10433	20866	20845	41690	0.1	B	148741	41214	0.0139	15	0.0095	0.0976
				0.5	2	10412	20824			0.1							
	25	15	2	0.5	1	9095	18190	18204	36408	0.1	B	148741	35932	0.0121	15	0.0095	0.0851
				0.5	2	9109	18218			0.1							
	25	20	2	0.5	1	6734	13468	13216	26432	0.1	B	148741	25956	0.0088	15	0.0095	0.0615
				0.5	2	6482	12964			0.1							
	25	20	2	0.5	1	6406	12812	12744	25488	0.1	B	148741	25012	0.0085	15	0.0095	0.0592
				0.5	2	6338	12676			0.1							
	35	0	2	0.5	1	18045	36090	35974	71948	0.14	B	208237	71472	0.0242	15	0.0095	0.1693
				0.5	2	17929	35858			0.14							
	35	0	2	0.5	1	17157	34314	33922	67844	0.14	B	208237	67368	0.0228	15	0.0095	0.1596
				0.5	2	16765	33530			0.14							
	35	7.5	2	0.5	1	16152	32304	32309	64618	0.14	B	208237	64142	0.0217	15	0.0095	0.1519
				0.5	2	16157	32314			0.14							
	35	7.5	2	0.5	1	16816	33636	33510	67020	0.14	B	208237	66644	0.0225	15	0.0095	0.1578
				0.5	2	16692	33384			0.14							
	35	15	2	0.5	1	9920	19840	19760	39520	0.14	B	208237	39044	0.0132	15	0.0095	0.0925
				0.5	2	9840	19680			0.14							
	35	15	2	0.5	1	10298	20596	20395	40790	0.14	B	208237	40314	0.0136	15	0.0095	0.0955
				0.5	2	10099	20198			0.14							
	35	20	2	0.5	1	7157	14314	14240	28480	0.14	B	208237	28004	0.0095	15	0.0095	0.0663
				0.5	2	7083	14166			0.14							
	35	20	2	0.5	1	8538	17072	17165	34330	0.14	B	208237	33854	0.0114	15	0.0095	0.0802
				0.5	2	8629	17258			0.14							
	50	0	2	0.5	1	7045	14090	13941	27882	0.05	A	97344	27406	0.0278	15	0.0095	0.1951
				0.5	2	6896	13792			0.05							
	50	0	2	0.5	1	7524	15048	15032	30064	0.05	A	97344	29588	0.0300	15	0.0095	0.2108
				0.5	2	7408	14816			0.05							
	50	7.5	2	0.5	1	6674	13348	13233	26466	0.05	A	97344	25990	0.0264	15	0.0095	0.1850
				0.5	2	6559	13118			0.05							
	50	7.5	2	0.5	1	6748	13496	13323	26646	0.05	A	97344	26170	0.0266	15	0.0095	0.1863
				0.5	2	6575	13150			0.05							
	50	15	2	0.5	1	4806	9612	9394	18788	0.05	A	97344	18312	0.0186	15	0.0095	0.1303
				0.5	2	4588	9176			0.05							
	50	15	2	0.5	1	4324	8648	8560	17120	0.05	A	97344	16644	0.0169	15	0.0095	0.1185
				0.5	2	4236	8472			0.05							
	50	20	2	0.5	1	4664	9328	9351	18702	0.05	A	97344	18226	0.0185	15	0.0095	0.1297
				0.5	2	4687	9374			0.05							
	50	20	2	0.5	1	4588	9176	9085	18170	0.05	A	97344	17694	0.0180	15	0.0095	0.1259
				0.5	2	4497	8994			0.05							
	100	0	2	0.5	1	9538	19076	18739	37478	0.1	A	194687	37002	0.0376	15	0.0095	0.2634
				0.5	2	9201	18402			0.1							
	100	0	2	0.5	1	9362	18724	18600	37200	0.1	A	194687	36724	0.0373	15	0.0095	0.2614
				0.5	2	9238	18476			0.1							
	100	7.5	2	0.5	1	7967	15934	16181	32362	0.1	A	194687	31686	0.0324	15	0.0095	0.2270
				0.5	2	8214	16428			0.1							
	100	7.5	2	0.5	1	9233	18466	18277	36554	0.1	A	194687	36076	0.0366	15	0.0095	0.2568
				0.5	2	9044	18088			0.1							
	100	15	2	0.5	1	6822	13644	13543	27086	0.1	A	194687	26610	0.0270	15	0.0095	0.1894
				0.5	2	6721	13442			0.1							

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Inhibitor	NYP-R3	NB page ref	12507-33

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed				
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (n/def)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mc)	Activity (nmol estrogen formed/mg/min
	100	15	2	0.5	1	6950	13700	13623	27246	0.1	A	194087	26770	0.0272	15	0.0095	0.1900
				0.5	2	6773	13546			0.1							
	100	20	2	0.5	1	5663	11326	11271	22542	0.1	A	194087	22058	0.0224	15	0.0095	0.1571
				0.5	2	5608	11216			0.1							
	100	20	2	0.5	1	6655	13310	13241	26482	0.1	A	194087	26008	0.0264	15	0.0095	0.1851
				0.5	2	6568	13172			0.1							
	300	0	2	0.5	1	11955	23910	23649	47298	0.3	A	584062	46822	0.0476	15	0.0095	0.3333
				0.5	2	11694	23388			0.3							
	300	0	2	0.5	1	12315	24630	24329	48778	0.3	A	584062	48302	0.0491	15	0.0095	0.3438
				0.5	2	12074	24148			0.3							
	300	7.5	2	0.5	1	11743	23486	23610	47220	0.3	A	584062	46744	0.0475	15	0.0095	0.3327
				0.5	2	11867	23734			0.3							
	300	7.5	2	0.5	1	11749	23498	23640	47280	0.3	A	584062	46804	0.0475	15	0.0095	0.3332
				0.5	2	11891	23782			0.3							
	300	15	2	0.5	1	11834	23668	23582	47164	0.3	A	584062	46688	0.0474	15	0.0095	0.3323
				0.5	2	11748	23496			0.3							
	300	15	2	0.5	1	9409	18818	19386	38776	0.3	A	584062	38300	0.0389	15	0.0095	0.2726
				0.5	2	9979	19958			0.3							
	300	20	2	0.5	1	8141	16282	16271	32542	0.3	A	584062	32056	0.0326	15	0.0095	0.2283
				0.5	2	8130	16260			0.3							
	300	20	2	0.5	1	8175	16350	16324	32648	0.3	A	584062	32172	0.0327	15	0.0095	0.2290
				0.5	2	8149	16298			0.3							

Aromatase Assay Spreadsheet

Assay Date	1/2/2007	Inhibitor	NYP-R3	NB page ref	12507-33

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		7.5		15		20	
15	0.0890	0.0857	0.0751	0.0848	0.0570	0.0527	0.0366	0.0348
25	0.1363	0.1315	0.1095	0.1098	0.0976	0.0851	0.0615	0.0592
35	0.1693	0.1596	0.1519	0.1576	0.0925	0.0955	0.0663	0.0802
50	0.1951	0.2106	0.1850	0.1863	0.1303	0.1185	0.1297	0.1259
100	0.2634	0.2614	0.2270	0.2568	0.1894	0.1906	0.1571	0.1851
300	0.3333	0.3438	0.3327	0.3332	0.3323	0.2726	0.2283	0.2290

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Inhibitor	NYP-R4	NB page ref	12507-34

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Test Chemical ID	NYP-R4	NB page ref	12507-34

Substrate Solution A

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0203	36664	1806108		
2	0.0198	32418	1637273		
3	0.0201	40562	2018010		
4	0.0200	38992	1949600		
5	0.0203	39962	1968571		
			Average DPM/g soln		1875912
			SD		154943
			CV		8.26
			µCi/g soln		0.845

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1427 g
Mass of dilution B used in substrate prep	4.5871 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.564466 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01030 µg/g soln.
µg/g soln.

a. µCi/g soln	0.845
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.564466 + 0.01030
= 0.574764 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.470 µCi/µg ASDN

934751 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Test Chemical ID	NYP-R4	NB page ref	12507-34

Substrate Solution B

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	32393	1644315	
2	0.0197	31270	1587310	
3	0.0197	37492	1903147	
4	0.0200	35580	1779000	
5	0.0194	38882	2004227	
			Average DPM/g soln	1783600
			SD	173867
			CV	9.75
			µCi/g soln	0.803

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.02	10		1002.00
Dilution A			100	10.02
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	5.117 g
Mass of dilution B used in substrate prep	0.7091 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.138854 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00979 µg/g soln.
µg/g soln.

a. µCi/g soln	0.803
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	23500000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.138854 + 0.00979
= 0.148646 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 5.405 µCi/µg ASDN

3436508 dpm/nmol

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Inhibitor	NYP-R4	NB page ref	12507-34

Microsome Dilution Details

Dilution A	0.1	mL microsome Stock used
	2	mL total volume
	20	dilution factor
Dilution B	1.75	mL microsome Dilution A used
	70	mL total volume
	40	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	800	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.61
Protein Concentration (dilution added to assay, mg/mL):	0.009511

Inhibitor Concentrations	
Level	Final Concentration (μM)
0	0
1	20
2	15
3	7.5

Substrate Concentrations	
Level	Final Concentration (nM)
1	15
2	25
3	35
4	50
5	100
6	300

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Inhibitor	NYP-R4	NB parp ref	12507-34

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (initial)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
Background control	1	NA	2	0.5	1	116	232	215	430	0.1	A	187591	-51	-0.0001	15	0.0095	-0.0004
				0.5	2	99	198			0.1							
	2	NA	2	0.5	1	126	252	252	504	0.1	A	187591	23	0.0000	15	0.0095	0.0002
				0.5	2	126	252			0.1							
	3	NA	2	0.5	1	123	246	227	454	0.1	A	187591	-27	0.0000	15	0.0095	-0.0002
				0.5	2	104	208			0.1							
	4	NA	2	0.5	1	133	266	268	536	0.1	A	187591	55	0.0001	15	0.0095	0.0004
				0.5	2	135	270			0.1							
NYP-R4	15	0	2	0.5	1	10629	21258	21463	42926	0.05	B	107016	42445	0.0124	15	0.0095	0.0866
				0.5	2	10764	21528			0.05							
	15	0	2	0.5	1	9962	19924	19914	39828	0.05	B	107016	39347	0.0114	15	0.0095	0.0803
				0.5	2	9952	19904			0.05							
	15	7.5	2	0.5	1	7692	15384	15190	30380	0.05	B	107016	25899	0.0087	15	0.0095	0.0610
				0.5	2	7498	14996			0.05							
	15	7.5	2	0.5	1	7657	15314	15431	30862	0.05	B	107016	30381	0.0088	15	0.0095	0.0620
				0.5	2	7774	15548			0.05							
	15	15	2	0.5	1	4336	8772	8627	17254	0.05	B	107016	16773	0.0049	15	0.0095	0.0342
				0.5	2	4241	8482			0.05							
	15	15	2	0.5	1	3839	7778	7740	15480	0.05	B	107016	14999	0.0044	15	0.0095	0.0306
				0.5	2	3851	7702			0.05							
	15	20	2	0.5	1	3203	6406	6270	12540	0.05	B	107016	12059	0.0035	15	0.0095	0.0248
				0.5	2	3067	6134			0.05							
	15	20	2	0.5	1	2978	5956	5976	11952	0.05	B	107016	11471	0.0033	15	0.0095	0.0234
				0.5	2	2998	5996			0.05							
	25	0	2	0.5	1	13830	27660	27858	55716	0.1	B	178360	55235	0.0161	15	0.0095	0.1127
				0.5	2	13978	27956			0.1							
	25	0	2	0.5	1	15204	30408	30594	61188	0.1	B	178360	60707	0.0177	15	0.0095	0.1238
				0.5	2	15390	30780			0.1							
	25	7.5	2	0.5	1	11012	22024	21947	43894	0.1	B	178360	43413	0.0126	15	0.0095	0.0885
				0.5	2	10835	21670			0.1							
	25	7.5	2	0.5	1	11295	22590	22519	45033	0.1	B	178360	44557	0.0130	15	0.0095	0.0909
				0.5	2	11224	22448			0.1							
	25	15	2	0.5	1	6832	13664	13634	27268	0.1	B	178360	26787	0.0078	15	0.0095	0.0548
				0.5	2	6802	13604			0.1							
	25	15	2	0.5	1	7849	15698	15511	31022	0.1	B	178360	30541	0.0089	15	0.0095	0.0623
				0.5	2	7662	15324			0.1							
	25	20	2	0.5	1	4903	9806	9573	19146	0.1	B	178360	18665	0.0054	15	0.0095	0.0381
				0.5	2	4670	9340			0.1							
	25	20	2	0.5	1	4326	8652	8565	17130	0.1	B	178360	16849	0.0048	15	0.0095	0.0340
				0.5	2	4239	8478			0.1							
	35	0	2	0.5	1	17021	34042	34404	68808	0.14	B	249704	68327	0.0199	15	0.0095	0.1394
				0.5	2	17383	34766			0.14							
	35	0	2	0.5	1	17327	34654	34530	69060	0.14	B	249704	68579	0.0200	15	0.0095	0.1399
				0.5	2	17203	34406			0.14							
	35	7.5	2	0.5	1	15597	31194	31465	62930	0.14	B	249704	62449	0.0182	15	0.0095	0.1274
				0.5	2	15868	31736			0.14							
	35	7.5	2	0.5	1	15751	31502	31382	62764	0.14	B	249704	62283	0.0181	15	0.0095	0.1270
				0.5	2	15631	31262			0.14							
	35	15	2	0.5	1	9159	18318	18387	36774	0.14	B	249704	36293	0.0105	15	0.0095	0.0740
				0.5	2	9228	18456			0.14							
	35	15	2	0.5	1	10297	20594	20758	41516	0.14	B	249704	41035	0.0119	15	0.0095	0.0837
				0.5	2	10461	20922			0.14							
	35	20	2	0.5	1	7604	15208	15159	30318	0.14	B	249704	29837	0.0087	15	0.0095	0.0609
				0.5	2	7555	15110			0.14							
	35	20	2	0.5	1	7702	15404	15440	30880	0.14	B	249704	30399	0.0088	15	0.0095	0.0620
				0.5	2	7738	15476			0.14							
	50	0	2	0.5	1	6006	12012	11978	23956	0.05	A	93796	23475	0.0251	15	0.0095	0.1760
				0.5	2	5970	11940			0.05							
	50	0	2	0.5	1	5215	10430	10302	20604	0.05	A	93796	20123	0.0215	15	0.0095	0.1509
				0.5	2	5087	10174			0.05							
	50	7.5	2	0.5	1	4564	9128	9182	18364	0.05	A	93796	17853	0.0191	15	0.0095	0.1341
				0.5	2	4618	9236			0.05							
	50	7.5	2	0.5	1	4849	9698	9586	19172	0.05	A	93796	18691	0.0200	15	0.0095	0.1402
				0.5	2	4737	9474			0.05							
	50	15	2	0.5	1	2923	5846	5782	11564	0.05	A	93796	11083	0.0119	15	0.0095	0.0831
				0.5	2	2859	5718			0.05							
	50	15	2	0.5	1	2648	5292	5249	10498	0.05	A	93796	10017	0.0107	15	0.0095	0.0751
				0.5	2	2603	5206			0.05							
	50	20	2	0.5	1	2185	4370	4373	8746	0.05	A	93796	8265	0.0088	15	0.0095	0.0620
				0.5	2	2188	4376			0.05							
	50	20	2	0.5	1	2306	4612	4657	9314	0.05	A	93796	8833	0.0094	15	0.0095	0.0662
				0.5	2	2351	4702			0.05							
	100	0	2	0.5	1	7789	15578	15586	31172	0.1	A	187591	30691	0.0328	15	0.0095	0.2301
				0.5	2	7797	15594			0.1							
	100	0	2	0.5	1	5831	11662	12467	24934	0.1	A	187591	24453	0.0262	15	0.0095	0.1834
				0.5	2	6636	13272			0.1							
	100	7.5	2	0.5	1	7076	14152	14438	28876	0.1	A	187591	28396	0.0304	15	0.0095	0.2129
				0.5	2	7362	14724			0.1							
	100	7.5	2	0.5	1	7775	15550	15410	30820	0.1	A	187591	30339	0.0325	15	0.0095	0.2275
				0.5	2	7635	15270			0.1							
	100	15	2	0.5	1	6764	13528	13168	26336	0.1	A	187591	25656	0.0277	15	0.0095	0.1939
				0.5	2	6404	12808			0.1							

Aromatase Assay Spreadsheet

Assay Date: 1/3/2007 Inhibitor: NYP-R4 NB page ref: 12507-34

Sample ID			Calculate DPM in aqueous portion after extraction									Calculate nmol ^3H_2O formed					
Sample type	Replicate/Substrate Concentration	Inhibitor Concentration Level (µM)	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Substrate ID	total DPM in assay tube (mCal)	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Incubation time (min)	Protein (mg/mL)	Activity (nmol estrogen formed/mg/min
	100	15	2	0.5	1	6117	12234	12339	24778	0.1	A	187591	24797	0.0200	15	0.0095	0.1822
				0.5	2	6272	12544			0.1							
	100	20	2	0.5	1	4192	8384	8412	16834	0.1	A	187591	16343	0.0175	15	0.0095	0.1225
				0.5	2	4220	8440			0.1							
	100	20	2	0.5	1	3823	7646	7890	15780	0.1	A	187591	15299	0.0164	15	0.0095	0.1147
				0.5	2	4067	8134			0.1							
	300	0	2	0.5	1	9172	18344	18306	36612	0.3	A	562774	35931	0.0384	15	0.0095	0.2694
				0.5	2	9034	18068			0.3							
	300	0	2	0.5	1	9504	19008	18783	37566	0.3	A	562774	37005	0.0397	15	0.0095	0.2781
				0.5	2	9279	18558			0.3							
	300	7.5	2	0.5	1	9253	18506	18861	37722	0.3	A	562774	37241	0.0399	15	0.0095	0.2792
				0.5	2	9608	19216			0.3							
	300	7.5	2	0.5	1	8309	16618	16161	32322	0.3	A	562774	31841	0.0341	15	0.0095	0.2388
				0.5	2	7852	15704			0.3							
	300	15	2	0.5	1	6064	12128	12228	24456	0.3	A	562774	23975	0.0256	15	0.0095	0.1798
				0.5	2	6144	12288			0.3							
	300	15	2	0.5	1	7474	14948	14852	29704	0.3	A	562774	29223	0.0313	15	0.0095	0.2191
				0.5	2	7378	14756			0.3							
	300	20	2	0.5	1	6014	12028	12010	24020	0.3	A	562774	23539	0.0252	15	0.0095	0.1765
				0.5	2	5996	11992			0.3							
	300	20	2	0.5	1	5767	11534	11842	23684	0.3	A	562774	23203	0.0248	15	0.0095	0.1740
				0.5	2	6075	12150			0.3							

Aromatase Assay Spreadsheet

Assay Date	1/3/2007	Inhibitor	NYP-R4	NB page ref	12507-34

Substrate Concentration (nM)	Inhibitor Concentration (μM)							
	0		7.5		15		20	
15	0.0866	0.0803	0.0610	0.0620	0.0342	0.0308	0.0246	0.0234
25	0.1127	0.1238	0.0885	0.0909	0.0546	0.0623	0.0381	0.0340
35	0.1394	0.1399	0.1274	0.1270	0.0740	0.0837	0.0609	0.0620
50	0.1760	0.1509	0.1341	0.1402	0.0831	0.0751	0.0520	0.0662
100	0.2301	0.1834	0.2129	0.2275	0.1939	0.1822	0.1225	0.1147
300	0.2694	0.2781	0.2792	0.2388	0.1798	0.2191	0.1765	0.1740

Appendix 5

Study Protocol Deviations

TO3 Protocol Deviation

ITEM 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 8, Section 4.0 STUDY DESIGN - INHIBITION ASSAYS

Each replicate will test the response of aromatase activity (see Section 6.0) to the presence of three concentrations of an inhibitor and will include control tubes with no inhibitor; the concentration of substrate will also vary (over 6 concentrations) in each replicate.

DEVIATION:

Because of an error in sample preparation, which resulted in an inhibitor concentration of 75 µM, rather than 7.5 µM being tested, data from only two inhibitor concentrations was valid and reported for the first definitive replicate.

REASON/IMPACT OF CHANGE:
No discernable impact.

Sherry J Black

Sherry Black, Task Order Leader

2/15/07

Date

TO3 Protocol Deviation

ITEM 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 8, 6.0 **AROMATASE ASSAY METHOD**

The assays will be performed in 13 x 100 mm test tubes maintained at 37 ± 1 °C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, inhibitor (AG or NYP) or vehicle, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The inhibitor (and vehicle) will be added in a volume not to exceed 20 µL. The final concentrations for the assay components are presented in Tables 1 and 2. The tubes and the microsomal suspension will be placed at 37 ± 1 °C in the water bath for 5 min prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will then be vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman Allegra X-15R centrifuge with a 4750a rotor for 10 min at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers will be extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

DEVIATION:

Tubes were vortexed for approximately 10-15 seconds at each extraction step instead of for 20-30 seconds.

REASON/IMPACT OF CHANGE:
This change in procedure had no impact on the results. One can see that the extraction of unreacted [^{3}H]ASDN was complete under the reduced vortexing time by examining the background control data which shows that essentially no radioactivity remained in these samples after extraction.

Sherry Black, Task Order Leader — Date: 3/1/07

TO3 Protocol Deviation

ITEM 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 8, Section 4.0 STUDY DESIGN - INHIBITION ASSAYS

Each replicate will test the response of aromatase activity (see Section 6.0) to the presence of three concentrations of an inhibitor and will include control tubes with no inhibitor; the concentration of substrate will also vary (over 6 concentrations) in each replicate.

DEVIATION:

Because of errors in sample preparation, only one inhibitor concentration was correct for the first definitive replicate. Therefore, all data from this replicate will be excluded from the data set.

REASON/IMPACT OF CHANGE:
It is not appropriate to calculate K_i using inhibitor concentrations as far above the K_m as were inadvertently tested in replicate 1, therefore the data from replicate 1 are not reported. Three replicates remain in the data set.

Sherry L Black
Sherry Black, Task Order Leader

3/20/07
Date