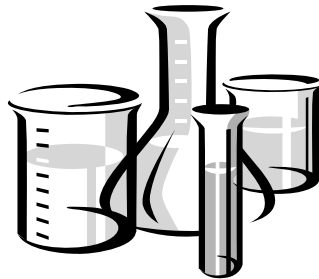


Endocrine Disruptor Screening Program Test Guidelines

OPPTS 890.1200: Aromatase (Human Recombinant)



NOTICE

This guideline is one of a series of test guidelines established by the Office of Prevention, Pesticides and Toxic Substances (OPPTS), United States Environmental Protection Agency for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a).

The OPPTS test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about OPPTS harmonized test guidelines and to access the guidelines electronically, please go to <http://www.epa.gov/oppts> and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in <http://www.regulations.gov> grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OPPTS 890.1200: Aromatase (Human Recombinant)

(a) Scope.

- (1) (1) **Applicability.** This guideline is intended to meet testing requirements of the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).
- (2) **Background.** The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). In general, EPA intends to use the data collected under the EDSP, along with other information, to determine if a pesticide chemical, or other substances, may pose a risk to human health or the environment due to disruption of the endocrine system.

This test guideline is intended to be used in conjunction with other guidelines in the OPPTS 890 series that make up the full screening battery under the EDSP to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone (Tier 1 “screening”). The determination will be made on a weight-of-evidence basis taking into account data from the Tier 1 assays and other scientifically relevant information available. The fact that a substance may interact with a hormone system, however, does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems.

Chemicals that go through Tier 1 screening and are found to have the potential to interact with the estrogen, androgen, or thyroid hormone systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine-related effects caused by the substance, and establish a quantitative relationship between the dose and that endocrine effect.

- (b) **Purpose.** The Aromatase (Human Recombinant) Assay is a screening assay intended to identify chemicals that may affect the endocrine system (*e.g.*, steroidogenesis) by inhibiting catalytic activity of aromatase, the enzyme responsible for the conversion of androgens to estrogens.
- (c) **Introduction.** The objective of this protocol is to describe procedures for conducting the Aromatase (CYP19) Assay for use as a screening tool in EPA’s EDSP Tier I Screening Battery. The assay is based upon the ³H₂O- aromatase assay, an *in vitro* method that has been used extensively for the determination of the presence of aromatase in multiple target tissues in all vertebrates, and has

long been used in pharmaceutical research to identify chemicals that can inhibit the catalytic activity of aromatase through an interaction with the substrate binding site on the enzyme. The assay measures the conversion of androgen to estrogen in microsomes isolated from various target tissues or cell lines containing aromatase, or recombinant aromatase respectively, and cytochrome P450 reductase. In brief, radioactive substrate (³H-androstenedione) and NADPH are added to microsomes containing the aromatase (CYP19) and reductase complex. ³H₂O is released during the conversion of androstenedione to estrone, and can be quantified as a direct measurement of aromatase activity per unit reaction time. Competitive inhibition of aromatase activity by test chemicals can be detected by serial reaction tubes containing increasing concentrations of the chemical of interest.

This protocol has been optimized for use with recombinant microsomes containing human aromatase (CYP19) and cytochrome P450 reductase. This protocol uses relatively large volumes. The assay may be conducted using smaller volumes than stated in this protocol as long as the relative concentrations of each component of the assay remain the same as indicated in Table 2 and results adhere to the performance criteria, as described in this protocol.

(d) **Terms.**

HPLC	High Performance Liquid Chromatography
ADSN	androstenedione (4-Androstene-3,17-dione)
[³ H]ASDN	tritiated androstenedione
LSS	liquid scintillation spectrometry
DMSO	dimethylsulfoxide
NADPH	β-nicotinamide adenine dinucleotide phosphate
4-OH ASDN	4-hydroxy-androstenedione

(e) **Materials Receipt and/or Preparation.**

(1) **Equipment.**

- HPLC (pumps, injector, reversed phase column, uv/vis and radiochemical detectors, data collection/analysis system)
- HPLC column (suggested: Zorbax SB-C18, 4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) and a flow rate of 1 mL/min
- UV spectrophotometer that can read absorbance at 240 nm
- Flow-through radiochemical detector
- Liquid scintillation counter
- Analytical balance
- pH meter
- Shaking water bath capable of maintaining 37°C
- Multi-tube vortex

- Lab timer
- Low speed refrigerated centrifuge
- Micropipettes (various volumes, 1µl–5 mL)
- Glassware free of detergent residue
- Disposable pipet tips
- Disposable test tubes (13 x 100 mm, glass)
- Scintillation vials
- Refrigerated centrifuges

(2) **Reagents**

- Distilled, deionized water
- Tetrahydrofuran, CAS No. 109-99-9 (HPLC grade)
- Absolute methanol (HPLC grade)
- Absolute ethanol
- Dimethylsulfoxide (DMSO)
- 4-hydroxyandrostenedione (4-OH ASDN), CAS# 566-48-3, mw 302.4
- [1-β³H(N)]-Androst-4-ene-3,17-dione, ([³H]ASDN)
- Androstenedione (CAS# # 63-05-8, mw 286.45)
- NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, CAS# 53-59-8)
- Propylene glycol (CAS# 57-55-6)
- Methylene chloride (CAS# 75-009-2)
- Liquid scintillation cocktail
- Reference chemical formulations
- Protein Assay Kit
- Bovine serum albumin
- Microsomes containing human CYP19 + P450 reductase

(3) **Substrate.**

- (i) **Substrate Names and Purity.** The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled androstenedione ([1β-³H]- Androst-4-ene-3,17-dione), [³H]ASDN) are be used in this assay. Obtain a non-radiolabeled ASDN that is ≥ 98% pure. Include all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate in study reports.
- (ii) **Radiochemical Purity.** The radiochemical purity of the [³H]ASDN is recommended to be greater than 95 percent. This is usually

supplied at a specific activity of 20-30 Ci/mmol as specified by the vendor. Determine the radiochemical purity of the [³H]ASDN using high performance liquid chromatography (HPLC) and liquid scintillation counting.

The HPLC method uses a 4.6 x 250 mm column 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. Collect eluant fractions manually into vials and assay for radiochemical content by liquid scintillation spectrometry (LSS). Include a reference standard of nonradiolabeled ASDN, analyzed by the same method, to confirm coelution of the nonradiolabeled and radiolabeled ASDN.

If the radiochemical purity is less than 95 percent, then it suggested that a new batch of radiochemical be obtained.

(iii) **Preparation of Substrate Solution for use in Aromatase Assay.**

Since the specific activity of the stock [³H]ASDN is too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled [³H]ASDN is prepared such that the final concentration of ASDN in the assay is 100 nM, and the amount of tritium added to each incubation tube is about 0.1 μ Ci.

The following *example* illustrates the preparation of a substrate solution using a stock of [³H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Calculations may change based on the specific activity and concentration of the stock purchased. The following procedure will reduce the specific activity to 0.3-0.5 Ci/mmol.

- Prepare a 1:100 dilution of the radiolabeled stock in buffer (final concentration = 10 μ Ci/mL) (see 2.5.1).
- Prepare a 1 mg/mL solution of ASDN (MW 286.45 g/mol) in ethanol and then prepare dilutions in buffer to a final concentration of 1 μ g/mL.
- Combine 4.6 mL of the 1 μ g/mL solution of ASDN, 800 μ L of the [³H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for approximate 70-80 tubes).
 - This substrate solution will have a concentration of 2 μ M ASDN with a radiochemical content of about 1 μ Ci/mL.
- After mixing the solution well, confirm radiochemical content by adding 20 μ L to scintillation cocktail and determining total dpms for radiochemical content analysis.

- ❑ Add 100 µL of the substrate solution to each 2 mL assay volume to yield a final ASDN concentration of 100 nM and a final [³H]ASDN content of 0.1 µCi/tube.
- ❑ Calculate the lower specific activity for each preparation of substrate solution based upon the original specific activity and appropriate decay of radioactivity on the day the assay is performed. The agency intends to provide a suggested data entry template, which will contain a calculation check for specific activity on the day of the assay, with the posting of this guideline on the Agency's Web site (**Ref. 1**).

(4) **Test Chemicals.** Please provide the information listed below for each test chemical:

- CAS Number
- Molecular Formula/Weight
- Supplier/source
- Lot Number
- Purity
- Storage Conditions
- Solvent
- Solubility Limit (if insoluble at any concentrations)
- Highest Concentration Tested
- Description of how stock solution was prepared

Prepare test chemical stock solutions and analyze as required by good laboratory practice (GLP) standards described in 40 CFR part 160 (**Ref. 2**). Make the test chemicals in buffer, absolute ethanol or dimethylsulfoxide (DMSO). Selection of the solvent is based upon the physical properties of each test chemical (*e.g.*, partition coefficient, hydrophobicity, solubility, etc.), as well as indicators that a chemical is precipitating out of solution after being added to the assay tube/buffer (*e.g.*, U-shaped inhibition curves with increasing chemical concentrations, solubility test using light scattering techniques, etc.). In general, it is expected that either ethanol or DMSO will be used as the primary solvents to facilitate solubility of the test chemical at the higher dose concentrations for the assay.

It is recommended that the total volume of test chemical formulation used in each assay be no more than 1 percent 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 serial 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 test chemical can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions and stability of stock solutions will be reported.

- (5) **Positive Control.** The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as the positive control. Table 1 contains identity and property information for 4-OH ASDN.

Table 1. Positive Control Substance.

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)
4-OH ASDN	566-48-3	C ₁₉ H ₂₆ O ₃	302.4

Prepare a stock solution and report storage conditions for this positive control substance stock. Conduct and report methods for purity analysis according to GLP standards. The 4-OH ASDN is formulated in either absolute ethanol or DMSO, depending upon which solvent is being used for the test chemicals on a given day. It is recommended that the total volume of control substance formulation used in each assay be no more than 1 percent 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. Dilutions will be prepared such that the target concentrations of the positive control substance (0.01–10,000 nM; Table 4) can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

- (6) **Microsomes.** Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware and other equipment used for microsome preparations be free of detergent residue. New disposable test tubes, bottles, vials, pipettes and pipette tips may be used directly in the assay. Rinse durable labware that may have been exposed to detergents with water and/or buffer prior to use in the assay.
- (i) **Source of the Human Recombinant Microsomes.** Any source of human recombinant microsomes containing CYP19 + reductase can be used in this assay provided the performance criteria described in this test guideline can be met. Microsomes should be stored at -70°C but should not be stored longer than 12 months. Human recombinant microsomes are commercially available from Gentest™ (Woburn, MA; www.gentest.com). If a commercial source of microsomes is used, supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity, found on the data sheet accompanying each shipment, should be reported for each aromatase assay performed.
- (ii) **Human Recombinant Microsome Preparation.** To minimize freeze-thaw cycles, aliquot the human recombinant microsomes into individual vials based on the protein content of each batch. Quickly thaw the microsomes in a 37 ± 1°C water bath, place them

in an ice bath, and aliquot into individual vials in an amount sufficient for use in a single assay (typically, 0.4 mg will be sufficient for an 80-90 tube assay). The assay uses approximately 0.004 mg/ml (final concentration) of microsomal protein/assay tube. After aliquoting the microsomes into individual vials, flash freeze the vials in liquid nitrogen and then returned to the -70°C freezer for storage for no longer than 6 months.

Note: Aliquot and flash freeze microsomes that were thawed and kept on ice as quickly as possible to maintain enzyme activity as best as possible.

(7) **Other Assay Components.**

- (i) **Buffer.** The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (e.g., JT Baker, cat # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (e.g., JT Baker, cat # 4062-01, 141.96 g/mol) are used in the preparation of the buffer. Solutions of each reagent at 0.1 M are prepared in distilled, deionized water and then the solutions are combined in equal volumes, resulting in a 0.1M solution with a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (2-8°C).
- (ii) **Propylene Glycol.** Propylene glycol (e.g., JT Baker, cat # 9402-01, 76.1 g/mol) is added to the assay directly as described below. Propylene glycol is necessary for optimal performance of this assay.
- (iii) **NADPH.** NADPH (β -nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, e.g., Sigma, cat # 1630, 833.4 g/mol) is the required co-factor for aromatase (CYP19). The final concentration in the assay is 0.3 mM. Typically, a 6 mM stock solution is prepared in assay buffer and then 100 μ L of the stock is added to the 2 mL assay volume. Prepare NADPH fresh each day and keep on ice.

(f) **Determine Suitability of Microsome Preparations.**

- (1) **Protein Assay.** Determine the protein concentration of the microsome preparation on each day of use in the aromatase assay. A 6-point standard curve is prepared using bovine serum albumin (BSA), ranging from 0.13 to 1.5 mg protein/mL. Protein concentration is determined by using a standard protein assay kit such as the DC Protein Assay kit (Bio-Rad, Hercules, CA) or BCA Protein Assay (Pierce, Rockford, IL) as directed in the manufacturer's instructions.

- (2) **Cytochrome P450 (CYP19) Aromatase Activity.** Measure aromatase activity in each new lot of recombinant microsomes to demonstrate sufficient activity for use with the test chemicals. The minimum acceptable aromatase activity in human recombinant microsomes is 0.1 nmol/mg-protein/min. If the aromatase activity for the microsomal preparation is below the minimum level, it cannot be used in the assay. In this test, tubes to determine full activity control and background activity will be run in triplicate using the optimized conditions presented in Table 2.

Table 2. Tubes Needed for Determination of CYP19 Aromatase Activity.

Sample Type	Repetitions (tubes)	Description
Full Activity Control	3	All test components ^a plus solvent vehicle
Background Activity Control	3	Same as full activity control, but no NADPH

^aThe complete assay ("all test components") contains buffer, propylene glycol, microsomal protein, [³H]ASDN, and NADPH.

- (g) **Assay Controls.** A run is defined as an independent experiment. Each run contains quadruple tubes (as shown in Table 5) for the full activity control and background activity control and a full concentration curve run in triplicate (i.e., 3 replicates for each concentration) for the positive control.

(1) **Definitions of Controls.**

- (i) **Full Enzyme Activity Control.** The full enzyme activity control is used to determine the maximum aromatase activity as measured by the production of ³H₂O during the conversion of the substrate ([³H]ASDN) to the product (estrone). Assay tubes for the full enzyme activity control contain the following components: buffer, propylene glycol, microsomal protein, [³H]ASDN, NADPH, and the vehicle solvent that will be used for any test chemicals.

Note: These tubes do not contain any test chemical or positive control chemical.

- (ii) **Background Activity Control.** The background activity control is used to determine non-specific radioactivity when aromatase is not activated by the co-factor NADPH. Assay tubes for the background activity control consists of buffer, propylene glycol, microsomal protein, [³H]ASDN, and vehicle solvent that will be used for any test chemicals.

Note: These tubes do not contain NADPH, test chemicals or the positive control chemical. In a properly functioning assay system the average DPMs for the background activity control are generally no greater than 10-15% of those observed for the full enzyme activity control.

- (iii) **Positive Control.** The positive control is used to demonstrate that the assay is being conducted properly for each run, by detecting a known inhibitor of aromatase activity, 4-hydroxy-androstenedione (4-OH ASDN), and producing a sigmoidal inhibition curve from 0.1 – 10,000 nM. Performance criteria (Table 3) are included for the positive control. The assay components used in these assay tubes consist of: buffer, propylene glycol, microsomal protein, [³H]ASDN, NADPH, and varying concentrations of 4-OH ASDN.

Table 3. Positive Control Study Design.

Sample Type	Repetitions (tubes)	Description	4-OH ASDN(M)
Full Activity Control	4	All test components ^a plus solvent vehicle	N/A
Background Activity Control	4	Same as full activity control, but no NADPH	N/A
4-OH ASDN Conc 1	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻⁵
4-OH ASDN Conc 2	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻⁶
4-OH ASDN Conc 3	3	All test components plus 4-OH ASDN (positive control)	1X10 ^{-6.5}
4-OH ASDN Conc 4	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻⁷
4-OH ASDN Conc 5	3	All test components plus 4-OH ASDN (positive control)	1X10 ^{-7.5}
4-OH ASDN Conc 6	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻⁸
4-OH ASDN Conc 7	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻⁹
4-OH ASDN Conc 8	3	All test components plus 4-OH ASDN (positive control)	1X10 ⁻¹⁰

^aThe complete assay ("all test components") contains buffer, propylene glycol, microsomal protein, [³H]ASDN, and NADPH.

- (h) **Aromatase Assay Method.** The aromatase assay is performed using 13x100 mm test tubes maintained at 37 ±1°C in a shaking water bath. Each set of test chemical tubes is run in triplicate using the optimized conditions (Table 2) with a final assay volume of 2mL/tube.

Follow the steps below to set up the assay:

- Label each test tube.
- Pipet the following assay components into each test tube (total volume, 1 mL):
 - 100 uL propylene glycol

- 100 μL ^3H -ASDN (200 nM)
 - 100 μL NADPH (Note: substitute assay buffer in tubes designated as background control)
 - 20 μL test chemical (Note: use vehicle solvent for full activity and background controls)
 - 680 μL buffer (0.1 M sodium phosphate, pH 7.4)
- Place test tubes in water bath ($37 \pm 1^\circ\text{C}$) for 5 min. prior to initiation of assay.
 - Prepare microsomal protein for the assay by diluting in buffer to 0.008 mg/mL and warming in water bath ($37 \pm 1^\circ\text{C}$) for 5 min. prior to initiation of assay.
 - Initiate the assay by adding 1 mL recombinant microsomal preparation to each assay tube and vortex gently using a multi-tube vortex.
 - Incubate all test tubes for 15 min. in water bath ($37 \pm 1^\circ\text{C}$).
 - Stop the assay by the adding methylene chloride (2 mL).
 - Vortex-mix the tubes for ca. 5 sec. and placed on ice for 5 min.
 - Vortex-mixed the tubes an additional 20-25 sec.
 - Centrifuged the tubes at 200 x g for 10 min., 4°C .
 - Remove the methylene chloride (**bottom layer**) with Pasteur pipet and discard.
 - Extract the aqueous layers again with methylene chloride (2 mL), centrifuge for 10 min. at 1000 rpm, and again discard the methylene chloride layer (**bottom**).
 - Perform this extraction procedure one additional time, again discarding the bottom methylene chloride layer.
 - Transfer and separate the aqueous layer from each tube into two 20 mL liquid scintillation counting vials as duplicate aliquots (0.5 mL each).
 - Add liquid scintillation cocktail (10 mL) to each counting vial, cap, and vortex. The radiochemical content of each aliquot will be determined as described below.

Table 4. Optimized Conditions for the Aromatase Assay.

Assay factor (units)	Final Concentration
Microsomal Protein (mg/mL) ^a	0.004 ^b
NADPH (mM) ^a	0.3
[^3H]ASDN (nM) ^a	100
Propylene glycol	5%
Incubation Time (min)	15

^aThe complete assay contains buffer, propylene glycol, microsomal protein, [^3H] ASDN, and NADPH.

^bThe concentration of microsomal protein has been optimized when using microsomes that produce approximately 1200 pmol product/(min x mg protein), 5 pmol product/pmol P450/min.

The amount of ³H₂O in the aqueous fraction is quantified for each assay tube using liquid scintillation spectrometry (LSS), and each mole of ³H₂O represents the conversion of one mole of substrate ([³H]- ASDN) to estrone. Aromatase activity is reported as nmol ³H₂O/ mg microsomal protein/min and is calculated as described below. Note: The assay may be conducted using smaller volumes as long as the relative concentrations of each component of the assay remain the same as indicated in Table 4, and all the performance criteria for the assay are met (Table 5).

(i) **Demonstration of Proficiency.**

(1) **Positive Control Chemical.** Prior to conducting the assay for evaluation of test chemicals, it is recommended that each technician conduct at least one single run of the positive control experiment as outlined in Table 3 to demonstrate assay proficiency. Evaluate these data against the following criteria:

- The suggested mean for aromatase activity (see above) in the absence of an inhibitor is at least 0.1 nmol/mg-protein/min.
- The recommended mean background control activity is ≤ 10% of the full activity control.
- The suggested coefficient of variation (CV) for replicates within each sample type and concentration of 4-OH ASDN is less than 15%.
- Performance criteria are provided (Table 5) to serve as guidance in identifying runs that provide parameters in the preferred ranges. When data are analyzed as described later in this protocol, preferential concentration response curve(s) generated for 4-OH ASDN, can be identified by comparison with these performance criteria listed (Table 5).

Table 5. Performance Criteria for the Positive Control.

	Parameter	Lower limit	Upper Limit
Positive Control	Slope	-1.2	-0.8
	Top (%)	90	110
	Bottom (%)	-5	+6
	Log IC ₅₀	-7.3	-7.0

(2) **Proficiency Chemicals.** After successfully conducting the positive control run in below, each new technician is expected to conduct full scale tests (3 runs) with the chemicals in Table 6. These chemicals were selected to span the range of responses expected in the assay based on the validation studies conducted by participating laboratories and are described in the Integrated Summary Report for Aromatase (**Ref. 3**).

Table 6. List of Proficiency Chemicals.

Compound	CAS No.	Class
Econazole	24169-02-6	Inhibitor
Fenarimol	60168-88-9	Inhibitor
Nitrofen	1836-75-5	Inhibitor
Atrazine	1912-24-9	Non-inhibitor

(j) **Determination of the Response of Aromatase Activity to TEST Chemicals.**

A run is an independent experiment. Each run contains replicate tubes for full activity control, background activity control, positive control, and test chemical as shown in Table 7 and described below.

Each run tests the response of aromatase activity to the presence of eight concentrations of a test chemical run in triplicate (*i.e.*, there are three tubes of each test chemical concentration per run of the assay). Test each chemical in three independent runs. Conduct each run for a given test chemical entirely independently of the other runs for that test chemical. There are three (triplicate) repetitions for each concentration of a test chemical. A single run of a given test chemical is described in Table 7.

Three types of control samples will be included for each run. These include:

1. Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes);
2. Background activity controls (all components that are in the full aromatase activity controls, except NADPH); and
3. Positive controls (4-OH ASDN run at eight concentrations in the same manner as test chemicals).

Four test tubes of the full enzyme activity control and background activity controls are included with each run. The full enzyme and background activity controls sets are split so that two tubes (of each control type) are run at the beginning and two at the end of each run. The positive control is tested at eight concentrations in each run as indicated in Table 7. All controls are treated the same as the other samples.

The aromatase assay is conducted as described in Section (h).

After completion of the first run, the data are reviewed and, if necessary, the concentration of test chemical used in the second and third runs is adjusted. The decision on how to adjust test chemical concentration is based on the results from the first run with the following guidelines in mind:

- ❑ If insolubility (cloudiness or a precipitate) is observed at the highest concentration (10^{-3} M), then set the highest concentration for the second and third runs at the highest concentration that appeared to be soluble using mid-log concentrations; i.e., try $10^{-3.5}$ M if the test chemical is insoluble at 10^{-3} M as it is important to define the lower portion of the curve. Do not use a concentration lower than 10^{-5} M for the highest concentration tested.
- ❑ If the highest concentration to be tested is lowered to 10^{-4} or 10^{-5} M, then add mid-log concentration(s) near the lower end of the curve (higher concentrations) and around the estimated IC_{50} based on the results of the first run in order to keep eight concentrations in the test set.
- ❑ The lowest concentration to be tested is generally 10^{-10} M, but lower concentrations may be required to obtain the “top of the curve”. That is, obtain the full enzymatic activity at the two lowest concentrations of the test chemical in order to define the top of the concentration-response curve.

Table 7. Test Chemical Study Design.

Sample Type	Repetitions (tubes)	Description	Reference or Chemical (M)
Full Activity Control	4	All test components ^a plus solvent vehicle+	N/A
Background Activity Control	4	Same as full activity control, but no NADPH	N/A
4-OH ASDN Conc 1	2	All test components plus 4-OH ASDN	1×10^{-5}
4-OH ASDN Conc 2	2	All test components plus 4-OH ASDN	1×10^{-6}
4-OH ASDN Conc 3	2	All test components plus 4-OH ASDN	$1 \times 10^{-6.5}$
4-OH ASDN Conc 4	2	All test components plus 4-OH ASDN	1×10^{-7}
4-OH ASDN Conc 5	2	All test components plus 4-OH ASDN	$1 \times 10^{-7.5}$
4-OH ASDN Conc 6	2	All test components plus 4-OH ASDN	1×10^{-8}
4-OH ASDN Conc 7	2	All test components plus 4-OH ASDN	1×10^{-9}
4-OH ASDN Conc 8	2	All test components plus 4-OH ASDN	1×10^{-10}
Test Chem. Conc 1	3	All test components ^a plus test chemical	1×10^{-3}
Test Chem. Conc 2	3	All test components ^a plus test chemical	1×10^{-4}

Sample Type	Repetitions (tubes)	Description	Reference or Chemical (M)
Test Chem. Conc 3	3	All test components ^a plus test chemical	1X10 ⁻⁵
Test Chem. Conc 4	3	All test components ^a plus test chemical	1X10 ⁻⁶
Test Chem. Conc 5	3	All test components ^a plus test chemical	1X10 ⁻⁷
Test Chem. Conc 6	3	All test components ^a plus test chemical	1X10 ⁻⁸
Test Chem. Conc 7	3	All test components ^a plus test chemical	1X10 ⁻⁹
Test Chem. Conc 8	3	All test components ^a plus test chemical	1X10 ⁻¹⁰

^a The complete assay ("all test components") contains buffer, propylene glycol, microsomal protein, [³H]ASDN and NADPH.

(k) **Data Analysis.**

(1) **Aromatase Activity and Percent of Control Calculations.** Convert raw data to aromatase activity (nmol/mg protein/min) and percent control. Report data in electronic format (spreadsheet or comma-separate values), being sure to provide all formatting information that is necessary to read the data. The Agency intends to provide a suggested template on the Agency's Web site (**Ref. 1**). The following data are requested. Submit all raw data and calculated endpoints with the report including:

- DPM/mL for each aliquot of extracted aqueous incubation mixture.
- Average DPM/mL for each aqueous portion (after extraction).
- Total DPM for each aqueous portion (after extraction).
- The total DPM present in the assay tube at initiation. The volume (mL) of substrate solution added to the incubation multiplied by the substrate solution's specific activity (DPM/mL) yields the total DPM present in the assay tube at initiation.
- Percent of substrate converted to product. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product.
- Total DPM after extraction correct for background. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background tubes. This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol).

- ❑ Aromatase activity. The activity of the enzyme reaction is expressed in $\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$ and is calculated by dividing the amount of $^3\text{H}_2\text{O}$ (nmol) produced by the product of mg microsomal protein used times the incubation time (15 minutes).
- ❑ Average activity in the full activity control tubes.
- ❑ Percent of control activity remaining in the presence of various inhibitor concentrations, including the positive control. This value is calculated by dividing the aromatase activity at a given concentration by the average full activity control and multiplying by 100.

Note: Nominally, one might expect the percent of control activity values for an inhibitor to vary between 0 percent near the high inhibition concentrations and 100 percent near the low inhibition concentrations. However, due to experimental variation, individual observed percent of control values will sometimes extend slightly below 0 percent or above 100 percent as noted in the performance criteria.

- (2) **Model Fitting.** The response curve is fitted by weighted least squares nonlinear regression analysis with weights equal to $1/Y$. Model fits are to be carried out using a non-linear regression program such as Prism software (Version 3 or higher). Concentration response trend curves are fitted to the percent of control activity values within each of the repeat tubes at each test chemical concentration. Concentration is expressed on the log scale. In agreement with past convention, common logarithms (*i.e.*, base 10) are used. The variables in the response curve are defined as follows:

Y \equiv percent of control activity in the inhibitor tube

X \equiv logarithm (base 10) of the concentration

T \equiv average DPMs across the repeat tubes with the same test chemical concentration that define the Top of the curve

B \equiv average DPMs across the repeat tubes with the same test chemical concentration that define the Bottom of the curve

β \equiv slope of the concentration response curve (β will be negative)

μ \equiv $\log_{10} \text{IC}_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

The following concentration-response curve is fitted to relate percent of control activity to logarithm of concentration within each run:

$$Y = B + \frac{(T-B)}{1+10^{(\text{Log IC}_{50} - X)\beta + \log[(T-B/50-B)-1]}}$$

A concentration-response model is fitted for each test run for each test chemical.

- (3) **Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits.** For each run the individual percent of control values are plotted versus logarithm of the test chemical concentration. The fitted concentration response curve is superimposed on the plot. It is suggested that individual plots be prepared for each run.

EPA requests that additional plots be prepared to compare the percent of control activity values across runs through the following steps:

- For each run, plot the average percent of control values versus logarithm of test chemical concentration, and place all of the runs for one chemical on the same graph.
- Use different symbols to distinguish among runs.
- Superimpose the fitted concentration response curves for each run on the graph.
- On a separate graph, plot the average percent of control values for each run versus the logarithm of test chemical concentration.
- Superimpose the average concentration response curve across runs on the same plot.

For each run treat (β, μ) as a random variables with mean (β_{avg}, μ_{avg}) . Let X and Y ($0 < Y < 100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y = B + \frac{(T-B)}{1+10^{(\mu_{avg}-X)\beta_{avg}+\log[(1-B/50-B)-1]}} + \epsilon$$

Compare slope (β) and $\log_{10}IC_{50}$ (μ) across runs based on one-way random effects analysis of variance, treating the runs as random effects. Prepare graphs that display the parameters within each run with associated 95 percent confidence intervals based on the within-run standard error and the average across-run standard error with the associated 95 percent confidence interval incorporating run-to-run variation.

- (4) **Quality Control-Assay Drift Monitored using the Full Enzyme Activity Control and Background Activity Control.** Within each run of each test chemical quadruplicate repetitions are made of the full enzyme activity control and background activity control tubes. Half the repetitions will be carried out at the beginning of the run and half at the end. If the conditions are consistent throughout the test, the control tubes at the beginning will be equivalent to those at the end.

To assess whether this is the case, the control responses are adjusted for background DPMs, divided by the average of the (background adjusted) full enzyme activity control values, and expressed as percent of control. It

is recommended that the average of the four background activity controls within a run fall around 0 percent (with an acceptable range of -5 - +6%) and the average of the four full enzyme activity controls within a run fall around 100 percent (with an acceptable range of 90 - 110%).

- (5) **Data Interpretation.** Data from the assay will be used to classify chemicals according to their ability to inhibit aromatase. To be classed as an inhibitor, the data fit the 4-parameter regression model to yield an inhibition curve and result in greater than 50% inhibition at the highest concentration. Average the value of the inhibition curve at each of three runs at the highest concentration and compared with the following criteria:

Table 8. Data Interpretation Criteria.

Criteria		Classification
Data fit 4-parameter nonlinear regression model	Average curve across runs crosses 50%*	Inhibitor
	Average lowest portion of curves across runs is between 50% and 75% activity **	Equivocal
	Average lowest portion of curves across runs is greater than 75% activity**	Non-Inhibitor
Data do not fit the model	---	

* Ordinarily, an inhibition curve will fall from 90% to 10% over 2 log units with a slope near -1. Unusually steep curves may be a sign that the protein is being denatured or that solubility problems are being encountered. If the slope of the curve is steeper than -2.0 label the result equivocal.

** If the test compound is not soluble above 10^{-6} M and the inhibition curve does not cross 50%, the chemical is judged to be un-testable.

- (6) **Statistical Software.** Concentration response curves will be fitted to the data using the non-linear regression analysis features in a commercial software package such as PRISM statistical analysis package, Version 3 or higher (GraphPad Software, Inc.). Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons can be carried out using PRISM, the SAS statistical analysis system (Version 8 or higher), or other general purpose statistical packages (e.g., SPSS).

- (7) **Retention of Records.** Retain records as required by GLP (**Ref. 2**).

(I) **Data Reporting.**

The data to be reported in the interim data summaries will include (but is not limited to) for example: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test chemical repetition), percent of control activity, IC_{50} , slope, and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the statistical analyses described in Section (k) of this document.

Please include the following information in the test report, as well as any additional relevant information:

(1) **Test Compound(s).**

- Name, chemical structure, and CAS RN (Chemical Abstract Service Registry Number, CAS#), if known.
- Physical nature (solid or liquid), and purity, if known.
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

(2) **Solvent.**

- Justification for choice of solvent/vehicle if other than ethanol.
- Maximum concentration of solvent in assay wells. (Show calculations.)
- Information to demonstrate that the solvent/vehicle, if other than an established solvent, does not bind to, or otherwise affect, the components of the assay (e.g., the aromatase enzyme).

(3) **Radiolabeled Androstenedione ([1 β -³H]- Androst-4-ene-3,17-dione).**

- Name, including number and position of tritium atom(s).
- Supplier, catalogue number, and batch number.
- Specific Activity (SA) and date for which that SA was certified by supplier.
- Concentration as received from supplier.
- Lower SA following addition of unlabeled ASDN.
- Concentration (dpm/ml, nM) used in assay tubes.
- Calculations made to obtain the appropriate concentrations.

(4) **Unlabeled Androstenedione.**

- Supplier, batch, and catalog number.
- CAS number.
- Purity.

(5) **Microsomes of Recombinant Human Aromatase.**

- Type and source of aromatase microsomes. If from a commercial source, identify the supplier.

- Method and conditions of transport and storage.
- (6) **Test Conditions.**
- Demonstration of aromatase activity for each batch of recombinant microsomes used:
 - Raw data for full and background activity controls.
 - Concentration range and spacing of the unlabeled androstenedione positive control.
 - Composition of buffer(s) used.
 - Concentration range and spacing of test compound, with justification if deviating from recommended range and spacing.
 - Volume of vehicle used to dissolve test compound and volume of test compound added.
 - Total volume per assay tube.
 - Incubation time and temperature.
 - Concentration range and spacing of full activity and background activity controls.
 - Notes on any precipitation of test compound while making stock solutions or while adding test dilutions to the assay tubes.
 - Notes on any abnormalities during separation aqueous layer during the methylene chloride extraction.
 - Notes on any problems in analysis of aromatase activity.
 - Notes on reasons for repeating a run, if a repeat was necessary.
 - Methods used to determine IC₅₀ values (software used, formulas, etc.).
 - Statistical methods used, if any.
- (7) **Results.** Report the following for each run. Be sure to include the run number on each product.
- Date of run.
 - Results (e.g., the dpm counts for each tube), inserted into a data worksheet.
 - Report data in electronic format (spreadsheet or comma-separated values), being sure to provide all formatting information that is necessary to read the data.
 - The Agency intends to provide a suggested template with the posting of this guideline on the Agency's Web site (**Ref. 1**).
 - Adjust the data reported as necessary to accommodate the actual concentrations, volumes, etc. used in the assay.
 - Please provide one worksheet per run.

- ❑ Extent of precipitation of test compound (i.e., in stock solution, assay well, or not visible by eye).
- ❑ Values for the Full Activity and Background Activity controls tested to determine aromatase activity of each batch of recombinant microsomes used.
- ❑ Comparison of Full Activity and Background Activity controls positioned at the beginning and at the end of the assay.
- ❑ DPM/ml for each aliquot of extracted aqueous incubation mixture.
- ❑ Average DPM/mL for each aqueous portion (after extraction).
- ❑ Total DPM for each aqueous portion (after extraction).
- ❑ The total DPM present in the assay tube at initiation. The volume (mL) of substrate solution added to the incubation multiplied by the substrate solution's specific activity (DPM/mL) yields the total DPM present in the assay tube at initiation.
- ❑ Percent of substrate converted to product. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product.
- ❑ Total DPM after extraction correct for background. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background tubes. This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol).
- ❑ Enzyme activity. The activity of the enzyme reaction is expressed in nmol (mg product)⁻¹ min⁻¹ and is calculated by dividing the amount of androstenedione converted to ³H₂O (nmol) by the product of mg microsomal protein used times the incubation time (15 minutes).
- ❑ Average activity in the full activity control tubes.
- ❑ Percent of control activity remaining in the presence of various inhibitor concentrations, including the positive control. This value is calculated by dividing the aromatase activity at a given concentration by the average full activity control and multiplying by 100.
- ❑ % Activity data for each replicate at each concentration level for all substances.
- ❑ Plot of each data point, along with the unconstrained curve fitted to the Hill equation, which demonstrates the performance of the test compound in repeat runs.

- Plot the data points and curves for the positive control chemical on the same graph as that of the compound(s) tested in that same run.
- Differentiate data and curves by run (i.e., distinguish the reference chemical and test compound data points and fitted curve from Run A from those of Run B and Run C, and those of Run B from Run C).

- log(IC₅₀) values for androstenedione, and the test compound.
- Keep a record of all protocol deviations or problems encountered and include in the final report.
 - Use this record to improve subsequent runs.

(8) **Discussion.**

- log(IC₅₀) values for positive control, including ranges, means, and standard deviations.
- Reproducibility of the log(IC₅₀) values of the positive control.

(9) **Conclusion.**

- Classification of test compound activity: inhibitor, equivocal, or no inhibition.

(10) **Replicate Studies.**

- Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the IC₅₀ value is not well defined), replicate tests to clarify the results of the initial study would be prudent.

(m) **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.

- (1) Protocol and any Amendments.
- (2) List of any Protocol Deviations.
- (3) List of Standard Operating Procedures.
- (4) QAPP and any Amendments.
- (5) List of any QAPP Deviations.

← Formatted: Bullets and Numbering

← Formatted: Bullets and Numbering

(n) **References.**

1. EPA. OPPTS Harmonized Test Guidelines. Available on-line at: <http://www.epa.gov/oppts> (select “Test Methods & Guidelines” on the left side navigation menu). You may also access the guidelines in

<http://www.regulations.gov> grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

2. EPA. Good Laboratory Practice (GLP) Standards. [40 CFR part 160](#).
3. EPA. Integrated Summary Report on Aromatase. December 11, 2007. Available electronically at: http://www.epa.gov/endo/pubs/aromatase_isr.pdf.