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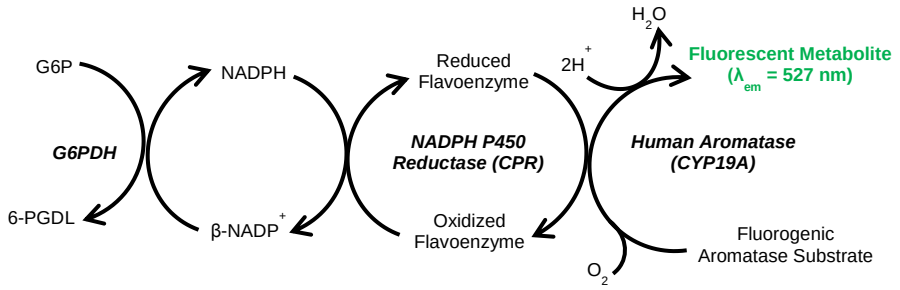
Aromatase (CYP19A) Inhibitor Screening Kit (Fluorometric)

rev 06/19

(Catalog # K984-100; 100 Reactions; Store at -20°C)

I. Introduction:

Aromatase (CYP19A, EC 1.14.14.14) is a member of the cytochrome P450 monooxidase (CYP) family of microsomal xenobiotic metabolism enzymes. Aromatase plays a critical role in steroidogenesis, catalyzing the conversion of androgenic hormones into estrogens. The enzyme is expressed in high levels in reproductive tissues, placenta, brain and adipose tissue and is responsible for mammalian sexual dimorphism and development of secondary sexual characteristics. Inhibitors of aromatase are used to treat estrogen-dependent breast cancer, as estrogens promote the expression of peptide growth factors responsible for tumorigenesis. Aromatase activity and expression can be affected by many organic environmental pollutants such as pesticides and plasticizers. Such compounds, known as endocrine disruptors, are suspected of causing precocious puberty, obesity, infertility and various cancers. BioVision's Aromatase Inhibitor Screening Kit enables rapid screening of drugs and other small molecules for compound-Aromatase interaction in a reliable, high-throughput fluorescence-based assay. The assay utilizes a fluorogenic substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 488/527 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96-well plate format.



II. Applications:

- Rapid, high-throughput screening and characterization of drugs and novel ligands for interaction with human aromatase.
- Development of structure-activity relationship (SAR) models to predict aromatase inhibition liability of novel compounds and analogues.
- Prediction of aromatase-dependent endocrine disruption potential for novel compounds.

III. Kit Contents:

Components	K984-100	Cap Code	Part Number
Aromatase Assay Buffer	100 ml	NM	K984-100-1
Fluorescence Standard (1 mM)	50 µl	Yellow	K984-100-2
Aromatase Inhibitor (Letrozole)	1 vial	Blue	K984-100-3
NADPH Generating System (100X)	1 vial	Green	K984-100-4
β-NADP$^+$ Stock (100X)	1 vial	Amber	K984-100-5
Aromatase Substrate	1 vial	Red	K984-100-6
Recombinant Human Aromatase	2 vials	Violet	K984-100-7

IV. User Supplied Reagents and Equipment:

- Multi-well fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) acetonitrile and DMSO
- White 96-well plates with flat bottom

V. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the Aromatase Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Fluorescence Standard (1 mM):** Provided as a 1 mM stock solution in DMSO. Prior to use, warm to room temperature and vortex until fully dissolved. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **Aromatase Inhibitor (Letrozole):** Reconstitute in 55 µl of acetonitrile and vortex until fully dissolved to yield a 1 mM stock solution. To obtain a 5 µM working solution of letrozole (5X final concentration), add 5 µl of the 1 mM stock solution to 995 µl of Aromatase Assay Buffer. The 5 µM working solution should be stored at -20°C and is stable for 2 freeze/thaw cycles. The stock solution is stable for 2 months at -20°C.
- **NADPH Generating System (100X):** Reconstitute with 220 µl Aromatase Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.
- **β-NADP$^+$ Stock (100X):** Dissolve in 110 µl Aromatase Assay Buffer and vortex thoroughly to yield a 100X stock solution of NADP$^+$. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **Aromatase Substrate:** Reconstitute with 55 µl anhydrous reagent-grade acetonitrile and vortex until fully dissolved to obtain a 1 mM stock solution. Store at -20°C. Allow the vial to warm to room temperature before opening and promptly retighten cap after use to avoid absorption of airborne moisture.

- **Recombinant Human Aromatase:** The Recombinant Human Aromatase should be reconstituted immediately before use as directed in Section VI.2 below. Each vial is sufficient for preparation of 50 reactions in a 96-well plate format.

VI. Aromatase (CYP19A) Inhibitor Screening Protocol:

1. Standard Curve Preparation:

a. Dilute the Fluorescence Standard by adding 10 µl of the 1 mM stock to 990 µl Aromatase Assay Buffer to yield a 10 µM solution. Mix 50 µl of the 10 µM solution with 950 µl Aromatase Assay Buffer to generate the final 0.5 pmole/µl (0.5 µM) Fluorescence Standard. Add 0, 2, 4, 6, 8, 12, 16 and 20 µl of the 0.5 pmole/µl Standard into a series of wells in a white 96-well plate, yielding 0, 1, 2, 3, 4, 6, 8 and 10 pmole/well Fluorescence Standard. Adjust the volume of each well to 100 µl with Aromatase Assay Buffer.

b. Measure fluorescence at Ex/Em = 488/527 nm. Subtract the zero standard (0 pmole/well) reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve.

2. Test Compound and Aromatase Enzyme Preparation:

a. Dissolve test compounds into proper solvent to produce stock solutions (see note regarding solvent effects below). For each test compound, prepare a 5X solution of each desired test concentration by diluting in Aromatase Assay Buffer. To determine IC_{50} values for test compounds, 5X test compound solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve. It is also possible to perform a cursory initial screen of a large number of test compounds by observing the percent inhibition at a single fixed concentration of each test compound.

b. Prepare the Recombinant Human Aromatase stock (2X) by reconstituting with 1 ml of Aromatase Assay Buffer. Mix contents thoroughly by vortexing to obtain a homogeneous solution (the solution will have a slightly opaque, milky appearance) and transfer the solution to a 15 ml conical tube. Bring the volume up to 2450 µl with Aromatase Assay Buffer and add 50 µl of the NADPH Generating System (100X) for a final total volume of 2.5 ml. The Aromatase stock is stable for up to 4 hours at room temperature or one day if kept on ice. In order to minimize enzyme instability, we do not recommend long term storage of the reconstituted enzyme system mix.

Note: Many commonly-used organic solvents can severely impact aromatase activity. Importantly, DMSO causes significant inhibition of aromatase at final concentrations of ≥0.25% (v/v). We recommend using acetonitrile to dissolve test ligands, which has been shown to have the least impact on CYP activity (at a final concentration ≤1%). We recommend preparing a parallel solvent control (SC) well with the same final concentration of solvent used to solubilize test ligands (particularly if using a solvent other than acetonitrile) and use this well to determine 100% activity if significantly different from No Inhibitor well(s) in 3.a. below.

3. Reaction Preparation:

a. Prepare reaction wells containing test compounds and corresponding no inhibitor controls (which may also serve as a solvent control), as well as a background control (containing no fluorogenic Aromatase Substrate) and (if desired) a positive inhibition control using 5 µM letrozole (5X solution, 1 µM final concentration). For solvent control, prepare a small aliquot of Aromatase Assay Buffer containing the organic solvent used to dissolve the test compounds at 5X final concentration.

	No Inhibitor	+ Test Compound	Background Control	Positive Inhibition Control
Aromatase Stock (2X)	50 µl	50 µl	50 µl	50 µl
Test Compound Solution (5X)	—	20 µl	—	—
Letrozole 5 µM Solution (5X)	—	—	—	20 µl
Aromatase Assay Buffer (With 5X Solvent)	20 µl	—	50 µl	—

Incubate the plate for at least 10 min at 37°C to allow test ligands to interact with aromatase. The pre-incubation time can be optimized for other test ligands depending on mechanism of action.

b. During the incubation, prepare a Aromatase Substrate/$NADP^+$ mixture (3X) by adding 6 µl of the reconstituted 1 mM Aromatase Substrate stock solution and 50 µl of the reconstituted 10 mM β-$NADP^+$ Stock (100X) to 1444 µl of Aromatase Assay Buffer for a total volume of 1.5 ml. This preparation is sufficient for 50 reactions, but can be scaled depending upon the number of reactions to be performed.

c. Start the reaction by adding 30 µl of the Aromatase Substrate/$NADP^+$ (3X) mixture to each well (aside from the background control) using a multichannel pipette, yielding a final reaction volume of 100 µl/well.

Notes:

- To ensure maximal signal intensity, both the pre-incubation period and the reaction itself should be performed at 37°C.
- During the pre-incubation period, the plate can be pre-read to determine if any test compounds are intrinsically fluorescent.
- The microsomal membranes in the Recombinant Human Aromatase stock may settle at the bottom of the tube over time, so it may be necessary to re-mix to ensure a homogenous solution before dispensing.
- The suggested starting point for the final concentration of Aromatase Substrate is 1.2 µM, which is within 2-fold of the K_m for the recombinant aromatase enzyme. This can be optimized by the user depending on the inhibitory potency of their test compounds and the mechanism of inhibition.

4. Measurement:

Immediately (within 1 min) measure the fluorescence at Ex/Em = 488/527 nm in kinetic mode for 60 min. While the assay can be performed in either endpoint or kinetic mode, we strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the exact reaction temperature and experimental conditions.

Note: Since the reaction starts immediately after the addition of the Aromatase Substrate/$NADP^+$ mix, it is essential to preconfigure the fluorescence microplate reader settings and use a multichannel pipette with a reagent reservoir to minimize lag time among wells.

5. Calculation:

For each reaction well (including background and no inhibitor controls), choose two time points (T_1 and T_2) in the linear phase of the reaction progress curve, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine ΔF

= ($RFU_2 - RFU_1$) and $\Delta T = (T_2 - T_1)$. Calculate the rate of change in fluorescence over time according to the equation below. Subtract the rate of the no substrate/background control (BC) well from the rates of each of the no inhibitor/solvent control (R_{SC}) and test compound (R_{TC}) wells to determine background-corrected reaction rates (denoted by *R*) for each well:

$$R = \frac{\Delta F - \Delta F_{BC}}{\Delta T}$$

Calculate the percent inhibition due to the test ligand or positive inhibition control using the following equation:

$$\text{\% Relative Inhibition} = \frac{R_{SC} - R_{TC}}{R_{SC}} \times 100\%$$

Notes:

- The Aromatase Substrate undergoes rapid photobleaching in aqueous solutions. This photophysical property may give rise to a sharp non-linear phase in first few minutes of the reaction progress curves (a lag phase). When calculating ΔF values, it is important to choose time points that occur *after* this initial lag phase, during the linear range of the reaction. *In our experience, the linear phase begins roughly 5-10 mins after the initiation of the reaction.*
- If the background control (BC) well rate calculation yields a negative value, subtraction of the BC value may be ignored.
- If desired, reaction rate calculations can also be expressed in terms of pmoles of fluorescent metabolite formed per unit time per unit amount of protein by interpolation from the standard curve. Each well will contain a total of 50 µg of protein when the Recombinant Human Aromatase is used at the proportions suggested in the kit protocol.

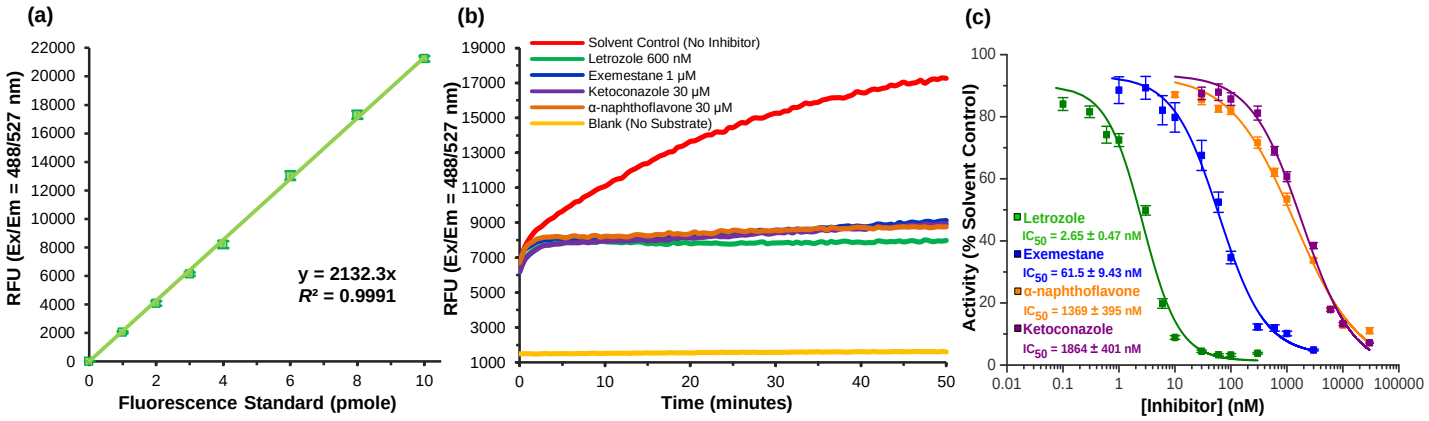


Figure: (a) Standard curve of Aromatase Substrate metabolite fluorescence. One mole of fluorescence standard corresponds to the metabolism of one mole of Aromatase Substrate. **(b)** Reaction kinetics of recombinant human aromatase enzyme at 37°C in the presence and absence of the indicated aromatase inhibitors (the solvent control reaction contained assay buffer with 0.2% acetonitrile). **(c)** Dose-response curves for various aromatase ligands of differing structural and mechanistic classes: the potent competitive inhibitor letrozole, the mechanism-based irreversible inhibitor exemestane, the phytoestrogen-like synthetic flavonoid α-naphthoflavone and the antifungal ketoconazole (a competitive inhibitor of several CYP isoforms). For dose-response curves, percent activity was calculated for each concentration of inhibitor by comparison to activity of reactions containing no inhibitor. For each inhibitor, IC_{50} values were derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least four replicates. Assays were performed according to the kit protocol.

VII. RELATED PRODUCTS:

Microsome Isolation Kit (K249)
Cytochrome P450 Reductase Activity Kit (K700)
Cytochrome P450 3A4 Activity Assay Kit (K701)
Cytochrome P450 3A4 Inhibitor Screening Kit (K702)
Cytochrome P450 2D6 Activity Assay Kit (K703)
Cytochrome P450 2D6 Inhibitor Screening Kit (K704)
Cytochrome P450 2C19 Activity Assay Kit (K848)
Cytochrome P450 2C19 Inhibitor Screening Kit (K849)
Cytochrome P450 1A2 Activity Assay Kit (K893)
Cytochrome P450 1A2 Inhibitor Screening Kit (K894)
Cytochrome P450 2C9 Activity Assay Kit (K895)
Cytochrome P450 2C9 Inhibitor Screening Kit (K896)
Aromatase (Human) ELISA Kit (K3599)
Exemestane (9473)
EZCyp™ Active human Cytochrome P450 3A4 (7872)
EZCyp™ Active human Cytochrome P450 2D6 (7873)
EZCyp™ Active human Cytochrome P450 2C19 (7874)
EZCyp™ Active human Cytochrome P450 2C9 (7875)
EZCyp™ Active human Cytochrome P450 1A2 (7876)
UGT Activity Assay / Ligand Screening Kit (K692)

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