BioVision
BioVision Incorporated

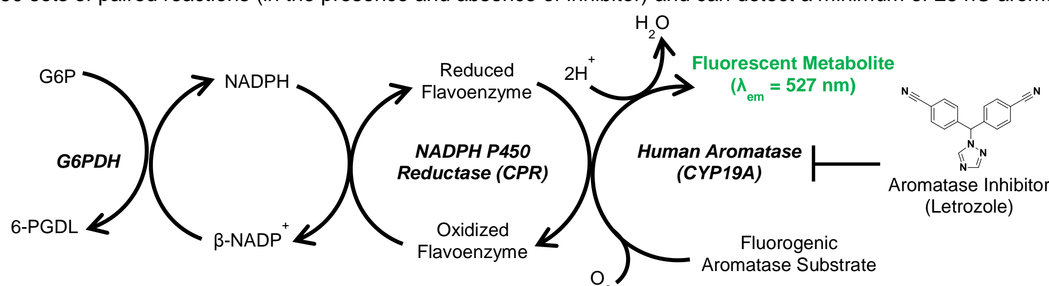
Aromatase (CYP19A) Activity Assay Kit (Fluorometric)

rev 06/19

(Catalog # K983-100; 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 Activity Assay Kit enables rapid measurement of native or recombinant aromatase activity in biological samples such as placental microsomes. 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. A highly selective aromatase inhibitor is provided for determination of aromatase activity in heterogeneous biological samples, where other CYP isozymes may contribute to substrate metabolism. The inhibitor displays greater than 100-fold selectivity for aromatase over other enzymes, ensuring targeted inhibition. Aromatase specific activity is calculated by running parallel reactions in the presence and absence of the inhibitor and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for 50 sets of paired reactions (in the presence and absence of inhibitor) and can detect a minimum of 25 nU aromatase activity.



II. Applications:

- Rapid assessment of native/recombinant aromatase activity in fractions prepared from tissues and cells.
- Characterizing drugs and potential endocrine disruptors based upon interaction with native/recombinant aromatase.

III. Sample Type:

- Human and animal tissue microsomes (*e.g.* human placental and rat ovarian microsomes) and S9 fractions
- Lysates of tissues and cultured cells, primary cells
- Heterologously expressed recombinant aromatase preparations

IV. Kit Contents:

Components	K983-100	Cap Code	Part Number
Aromatase Assay Buffer	100 ml	NM	K983-100-1
Fluorescence Standard (1 mM)	50 µl	Yellow	K983-100-2
Aromatase Inhibitor (Letrozole)	1 vial	Blue	K983-100-3
NADPH Generating System (100X)	1 vial	Green	K983-100-4
β-NADP$^+$ Stock (100X)	1 vial	Amber	K983-100-5
Aromatase Substrate	1 vial	Red	K983-100-6
Recombinant Human Aromatase	1 vial	Violet	K983-100-7

V. User Supplied Reagents and Equipment:

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

VI. 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:** Do not reconstitute until ready to use. Reconstitute with 230 µl Aromatase Assay Buffer and add 20 µl of NADPH Generating System (100X). Mix thoroughly to ensure a homogenous solution (the solution will have a slightly opaque, milky appearance), aliquot and store at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month (*the Recombinant Human Aromatase will lose approximately 10% activity per week when stored at -80°C*). Thaw aliquots rapidly at 37°C and place on ice until use (thawed aliquots should be used within 4 hours).

VII. Aromatase (CYP19A) Activity Assay 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. Sample and Test Compound Preparation:

a. Standardized microsomal preparations may be purchased commercially (*e.g.* donor-pooled human placental microsomes) or prepared from tissue or cultured cells using the Microsome Isolation Kit (Cat. #K249). Alternatively, a crude enriched lysate can be prepared: start with ~50 mg tissue or ~5 x 10^6 pelleted, pre-washed cells and homogenize in 500 µl ice-cold Aromatase Assay Buffer with a Dounce homogenizer (Cat. #1998 or equivalent) on ice. Incubate the homogenate on ice for 5 min and then centrifuge at 15,000 x *g* for 15 min in a refrigerated centrifuge at 4°C. Collect the resultant clarified supernatant for the assay in a new pre-chilled microfuge tube and store on ice until use (cell and tissue lysates can also be stored at -80°C in aliquots for future experiments).

b. If desired, sample aromatase activity may be measured in the presence of test ligands. Test ligands should be dissolved into proper solvent to produce stock solutions (see note regarding solvent effects below). For each ligand, prepare a 5X solution by diluting in Aromatase Assay Buffer.

Notes:

- To quantify specific activity in terms of sample protein content, use the Bradford reagent (Cat. #K810) or an equivalent protein assay.
- If measuring aromatase activity in presence of ligands (other than the included Aromatase Inhibitor), run parallel solvent control well(s) to account for additional solvent in the reaction mix. Many commonly-used organic solvents can severely impact aromatase activity. Importantly, DMSO causes significant inhibition of aromatase at final concentrations ≥ 0.25% (v/v). Our assay is designed to use acetonitrile at a final concentration of ≤1% (v/v), which has been shown to have little impact on aromatase activity.

3. Reaction Preparation:

a. Prepare enough reagents for the number of reactions to be performed. For each reaction, prepare a 2X concentrated aromatase reaction mix by combining 2-48 µl of sample and 2 µl of the NADPH Generating System (100X) in a 96-well plate and adjusting the final volume to 50 µl/reaction with Aromatase Assay Buffer. The amount of sample per reaction and the dilution factor required will vary based upon the nature of the sample. For human placental microsomes, we recommend starting with 25-50 µg of microsomal protein per well. For S9 fractions or other cellular lysates, the amount of protein required will be significantly higher. In this case, we recommend starting at 50-100 µg/well. *Due to the large individual variation in aromatase expression level and function, sample protein levels may need to be adjusted.*

b. In addition to the test samples, prepare background control (no enzyme) and positive inhibition control (sample + 1 µM letrozole) wells. If desired, you may also prepare positive control (PC) and PC + inhibitor wells using the Recombinant Human Aromatase and letrozole 5 µM solution (for a 1 µM final concentration). Adjust the volume of test sample, positive inhibition control, background and positive control wells to 70 µl/well with Aromatase Assay Buffer. For measurement of sample aromatase activity in the presence of test ligands, replace the Aromatase Assay Buffer with 5X concentrated solution of test ligand in Aromatase Assay Buffer:

	Test Sample	+ Inhibitor Control	Background	Aromatase PC	PC + Inhibitor
Aromatase Reaction Mix (2X)	50 µl	50 µl	—	—	—
Recombinant Human Aromatase	—	—	—	25 µl	25 µl
Letrozole 5 µM Solution (5X)	—	20 µl	—	—	20 µl
Aromatase Assay Buffer	20 µl	—	70 µl	45 µl	25 µl

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

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

Notes:

- The Recombinant Human Aromatase preparation may settle and should be thoroughly mixed before dispensing.

- The Aromatase Substrate is also metabolized by CYP isoforms 2C8, 2C9 and 2C19, necessitating the use of the selective inhibitor letrozole to determine the contribution of aromatase in biological samples that express aromatase along with other CYPs. The concentration of letrozole used for the positive inhibition control is >100-fold greater than the K_i for recombinant aromatase, but is not high enough to affect other enzymatic targets.

4. **Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 488/527 nm in kinetic mode for 60 min at 37°C. 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 content of active aromatase in the sample.

   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. **Calculations:** For each reaction well (including background and positive inhibition controls), choose two time points (T_1 and T_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$. Subtract the ΔF value of the background control (BC) from those of the test samples (S) and 1 µM letrozole positive inhibition control (I) to determine the background-corrected change in fluorescence intensity for each well.

   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.

   Calculate the specific fluorescence generated by aromatase activity (denoted by C) by subtracting the positive inhibition control from each sample (subtraction of the positive inhibition control should be omitted if the value of ΔF_I is negative):

$$C_S = (\Delta F_S - \Delta F_{BC}) - (\Delta F_I - \Delta F_{BC}) = \Delta F_S - \Delta F_I$$

Aromatase metabolic activity is obtained by applying the C_S values to the fluorescence standard curve to get B pmole of substrate metabolized by aromatase during the reaction time.

$$\textbf{Aromatase (CYP19A) Specific Activity} = \frac{B}{\Delta T \times P} = \textbf{pmole/min/mg} = \textbf{µU/mg}$$

Where: B is the amount of metabolite produced, calculated from the standard curve (in pmole)
ΔT is the linear phase reaction time $T_2 - T_1$ (in minutes)
P is the amount of protein in the well (in mg)

Aromatase Unit Definition: One unit of aromatase activity is the amount of enzyme that generates 1 µmole of fluorescent metabolite per min by hydrolysis of 1 µmole Aromatase Substrate at 37°C and pH 8.

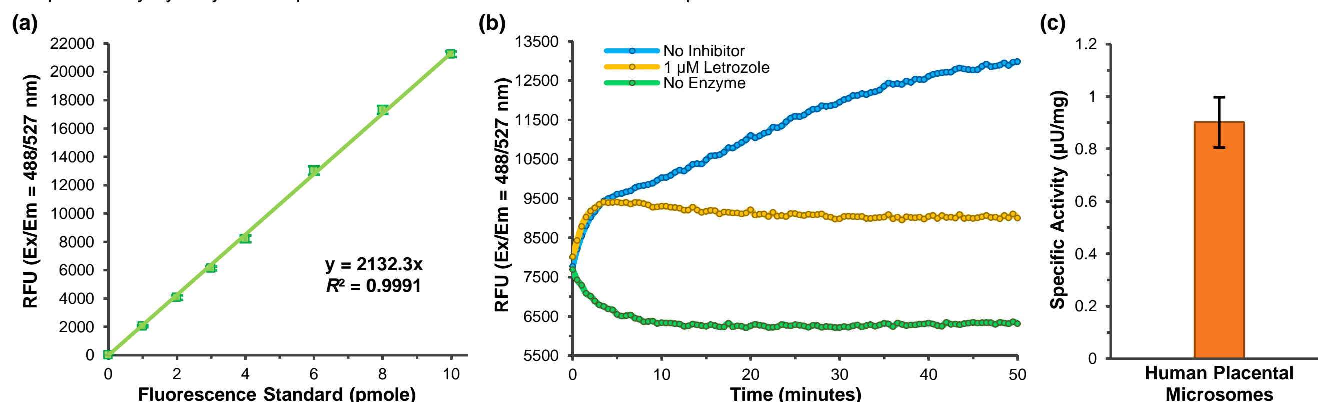


Figure: (a) Standard curve of Aromatase Substrate metabolite fluorescence. One mole of fluorescence standard corresponds to the metabolism of one mole of Aromatase Substrate. **(b)** Reaction kinetics of fluorogenic substrate metabolism in donor-pooled human placental microsomes (0.5 mg/mL) at 37°C in the presence and absence of the competitive aromatase inhibitor letrozole (the no inhibitor condition contained assay buffer with 0.2% acetonitrile as a solvent control). **(c)** Specific activity of aromatase in human placental microsome samples (mean ± SEM of four independent replicates). Assays were performed according to the kit protocol.

VIII. 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)

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