Cystathionine β Synthase Inhibitor Screening Kit (Fluorometric) (#BN00919)

(Catalog # BN00919; 100 assays; Store at -20°C)

I. Introduction:

Cystathionine β Synthase (EC 4.2.1.22, CβS) is a Pyridoxal 5-Phosphate-dependent enzyme that uses cysteine and homocysteine as substrates to catalyze the formation of H_2S and cystathionine. CβS is well-known for its role in human sulfur metabolism. The overexpression of CβS has been implicated in Down Syndrome, and also results in homocystinuria; or low levels of homocysteine in blood. Homocystinuria leads to a mutli-systemic disorder of the connective tissue, muscles, and central nervous system (CNS). For that reason, pharmacological inhibitors of human CβS present attractive therapeutic potential in order to restore baseline CβS activity and blood homocysteine levels. It has been shown that aminooxyacetic acid (AOAA) is an irreversible inhibitor that targets the PLP-binding site of CβS. That interaction inhibits the transaminase reaction that would yield cystathionine and H_2S in wildtype conditions. Assay Genie's Cystathionine β Synthase Inhibitor Screening Kit is the first available kit that enables customers to screen inhibitors of CβS and quantify their therapeutic potential. The kit has a simple, easy-to-follow protocol and is high-throughput adaptable. In this kit, the product hydrogen sulfide reacts with the non-fluorescent azido-functional group to yield a fluorescent amino group (Ex/Em = 368/460 nm). In the presence of AOAA, CβS activity is inhibited, which subsequently abolishes the production of H_2S and thus the fluorescent signal is reduced.

Cysteine + Homocysteine $\xrightarrow{\text{CβS}}$ Cystathionine + H_2S $\xrightarrow{\text{CβS Probe}}$ Fluorescent detection (Ex/Em= 368/460 nm)

Cysteine + Homocysteine $\xrightarrow{\text{CβS + AOAA}}$ Cystathionine + H_2S $\xrightarrow{\text{CβS Probe}}$ No Fluorescence/Low Fluorescence

II. Applications:

- Screening and characterization of inhibitors of Cystathionine β Synthase (CβS)

III. Kit Contents:

Components	BN00919	Cap Code	Part Number
CβS Assay Buffer	25 ml	WM	BN00919-1
CβS Probe in DMSO	0.5 ml	Purple	BN00919-2
CβS Substrate	4.0 ml	NM	BN00919-3
Cofactor 1	0.5 ml	Amber	BN00919-4
Cofactor 2	0.5 ml	Blue	BN00919-5
Reducing Agent	1 vial	Yellow	BN00919-6
CβS	0.5 ml	Green	BN00919-7
AOAA (100 mM in DMSO)	100 µl	Orange	BN00919-8

IV. User Supplied Reagents and Equipment:

- Multi-well fluorescence microplate reader
- 96-well white microtiter plates with flat bottom

V. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge vials prior to opening. Read entire protocol before performing the assay.

- **CβS Assay Buffer:** Equilibrate to room temperature before running the assay. Store at 4°C.
- **CβS Probe in DMSO and CβS Substrate:** Light sensitive. Aliquot and store at -20°C. Allow reagents to equilibrate to RT before use.
- **Cofactor 1 and Cofactor 2:** Aliquot and store at -20°C, stable for at least 4 freeze/thaw cycles.
- **Reducing Agent:** Reconstitute with 250 µl CβS Assay Buffer. Store at 4°C. Keep on ice during use. Stable for 4 freeze/thaw cycles.
- **CβS:** Aliquot and store at -20°C. Keep on ice while in use. Use within two months. Store CβS in -80°C to preserve activity for long-term use.
- **AOAA (100 mM in DMSO):** Aliquot and store at -20°C. Avoid repeated freeze/thaw. Use within six months.

VI. CβS Inhibitor Screening Protocol:

1. **Screen Compounds (Co), Inhibitor Control (IC), and Enzyme Control Preparation (EC):** Dissolve screen compound into a proper solvent at 100X the desired working concentration. Dilute Co to 20X desired test concentration with CβS Assay Buffer. Add 10 µl each screen compound (Co) into a designated well of a white 96-well microplate.

   For IC, dilute the CβS Inhibitor AOAA to 10 mM working solution by adding 10 µl CβS Inhibitor to 90 µl CβS Assay Buffer. Add 10 µl of CβS Inhibitor into desired well(s).

   For EC, add 10 µl CβS Assay Buffer into well designated as Enzyme Control (EC) (no inhibitor).

   Note: Solvents (e.g. DMSO) used to solubilize the inhibitors may affect the enzymatic activity. If that is a concern, prepare a solvent control well (SC) with the same final concentration of the solvent(s) as in the Co or IC sample(s).

2. **Cystathionine β Synthase Enzyme Solution Preparation**: For each well, prepare 50 µl of CβS enzyme solution.

45 µl CβS Assay Buffer
5 µl CβS

Mix well and add 50 µl/well of CβS Enzyme Solution to each well (with Co, IC, EC, or SC) of the 96-well white microtiter plate. Include sufficient number of wells to test each Co, IC, EC, and SC. Incubate at 30°C for 30 minutes.

Note: If the plate reader allows, include a 10 second pulse at the start of the incubation to ensure thorough mixing of enzyme with Co, IC, EC or SC. Also include a 3 second pulse every 1-5 minutes.

3. **Reaction Mix:** During incubation, prepare Reaction Mix (RM). Add RM to each well immediately after incubation. Prepare Working Reducing Agent before use: add 17 µl of Reducing Agent to 483 µl CβS Assay Buffer. Prepare sufficient volume of Reaction Mix (RM) for the number of assays to be performed. Prepare 140 µl RM/well containing:

	Reaction Mix
CβS Assay Buffer	85 µl
CβS Probe (in DMSO)	2 µl
CβS Substrate	40 µl
Cofactor 1	2 µl
Cofactor 2	1 µl
Working Reducing Agent	10 µl

Add 140 µl RM to each well containing Co, IC, EC, or SC.

Note: Do not store the Working Reducing Agent. Always prepare fresh dilution prior to the assay.

4. **Measurement:** Measure fluorescence immediately at Ex/Em= 368/460 nm in kinetic mode for 40-60 min. at 30°C.

Note: The enzymatic product (H_2S) reacts with the CβS probe to yield fluorescence. This may cause a lag phase to appear in the CβS Activity Progress Curves.

5. **Calculation:** For each reaction well (Co, IC, EC, SC), choose two time points (t_1 and t_2) in the linear phase of each reaction progress curve. Obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2). Calculate the slope for all samples, including Enzyme Control (EC), by dividing the net ΔRFU (= RFU_2- RFU_1) values by the time Δt (= t_2-t_1). Calculate % Relative Activity and % Relative Inhibition as follows:

$$\%\ \text{Relative Activity} = \frac{\text{Slope of SC or EC}}{\text{Slope of EC}} \times 100$$

$$\%\ \text{Inhibition} = \frac{\text{Slope of EC-Slope of Co}}{\text{Slope of EC}} \times 100$$

Note: If the values of the Solvent Control(s) (SC) are significantly different from the Enzyme Control, use the SC values instead of EC values.

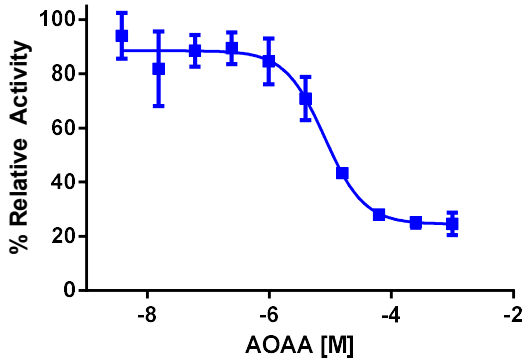

Figure: (a) Inhibition of CβS activity by AOAA Inhibitor Control. IC_{50} was determined to be 8.0 ± 0.11 µM. Assays were performed following the kit protocol.

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