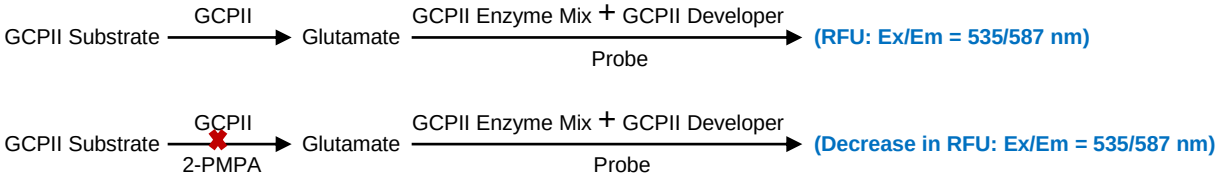
Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

Glutamate Carboxypeptidase II Inhibitor Screening Kit (Fluorometric)

(Catalog # K440-100; 100 assays; Store at -20°C)

I. Introduction:

Glutamate Carboxypeptidase II [GCPII, EC 3.4.17.21; *N*-acetylated-alpha-linked acidic dipeptidase (NAALADase), prostate-specific membrane antigen (PSMA) or folate hydrolase (FOLH1)] is a multifunctional zinc-metallopeptidase. Within the last decade, GCPII has been studied as a promising pharmacological target for the treatment of various pathological disorders and diseases and tremendous efforts have been made in order to discover potent GCPII inhibitors. NAALADase, the brain isozyme, plays an important role in Glutamate biosynthesis and could be used for treating neuronal damage caused by excess glutamate in brain. For example, inhibition of GCPII has shown neuroprotective characteristics in animal models suffering several acute and chronic conditions (stroke, neuropathic and inflammatory pain, schizophrenia, depression or amyotrophic lateral sclerosis). In males, PSMA is overexpressed in patients suffering prostate cancer and thus, it is used as a diagnostic marker for this disease. BioVision's Glutamate Carboxypeptidase II Inhibitor Screening Kit is an *in vitro* tool in order to screen potential human GCPII inhibitors. The provided substrate is transaminated in the presence of GCPII producing glutamate. The detection system is based on an enzymatic reaction in which a fluorogenic probe is reduced generating a stable signal. In the presence of 2-PMPA, a potent GCPII Inhibitor, the enzymatic activity is arrested, thus generating lower fluorometric signal. The assay kit is a simple plate-based format and provides a rapid and reliable test for high-throughput screening of GCPII inhibitors.



II. Applications:

- Screening/characterizing Glutamate Carboxypeptidase II inhibitors

III. Kit Contents:

Components	K440-100	Cap Code	Part Number
GCPII Assay Buffer	35 ml	NM	K440-100-1
PicoProbe™ (in DMSO)	0.4 ml	Blue	K440-100-2
GCPII Substrate	35 µl	Orange	K440-100-3
Human GCPII	1 vial	Purple	K440-100-4
GCPII Enzyme Mix	1 vial	Green	K440-100-5
GCPII Developer	1 vial	Red	K440-100-6
2-PMPA (10 mM)	20 µl	Brown	K440-100-7

IV. User Supplied Reagents and Equipment:

- Multi-well spectrophotometer (Fluorescent plate reader)
- 96-well white plate with flat bottom

V. Storage Conditions and Reagent Preparation:

Store kit at -20 °C, protect from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay. Upon opening, use within two months.

- **GCPII Assay Buffer:** Store at 4 °C or -20 °C. Bring to room temperature before use.
- **PicoProbe™:** Ready to use as supplied. Warm to room temperature before use. Store at -20 °C.
- **GCPII Substrate:** Ready to use as supplied. Store at -20 °C.
- **Human GCPII:** Reconstitute with 55 µl of GCPII Assay Buffer. Pipette up and down to mix well. Aliquot and store at -20 °C. Keep on ice while in use. Use within two months.
- **GCPII Enzyme Mix & GCPII Developer:** Reconstitute each vial with 220 µl of GCPII Assay Buffer. Mix thoroughly. Aliquot and store at -20 °C. Keep on ice while in use.
- **2-PMPA:** Ready to use as supplied. Store at -20 °C.

VI. Glutamate Carboxypeptidase II Inhibitor Screening Protocol:

1. Screening Compounds, Inhibitor Control, Enzyme Control & Background Control Preparations:

Test compound [S]: Dissolve test sample(s) into an appropriate solvent. Prepare a 10X working solution by diluting the stock solution in GCPII Assay Buffer. Add 10 µl diluted test sample(s) (10X) into wells of a 96-well white plate.
Inhibitor Control [IC]: Prepare a 100-fold dilution of 10 mM 2-PMPA to 0.1 mM by diluting 2 µl of 10 mM 2-PMPA with 198 µl of GCPII Assay Buffer, mix well; Further prepare a 100-fold dilution of 0.1 mM 2-PMPA to 1 µM by diluting 2 µl of 0.1 mM 2-PMPA with 198 µl of GCPII Assay Buffer, mix well. Add 10 µl of 1 µM of 2-PMPA into well(s).

Enzyme Control [EC] (no inhibitor) and Background Control [BC]: add 10 µl GCPII Assay Buffer into desired wells, respectively.
Solvent Control [SC][b,c]: add 10 µl of 10X solvent in Assay Buffer into assigned wells.
For all wells: Bring volumes of [S], [IC], [EC], [BC] and [SC] wells to 40 µl using GCPII Assay Buffer.

Notes:

a) To determine IC_{50} values for candidate inhibitors, 10X sample solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve (the amount of organic solvent should be the same for all test concentrations).

b) Organic solvent concentration should be minimized to avoid loss of enzymatic activity (DMSO has little effect on GCPII activity at a final concentration of ≤ 1%).

c) For higher concentrations of solvents other than DMSO, we recommend preparing a solvent control [SC] well with the same final concentration of solvent used to solubilize samples and using this well to define 100% activity if different from [EC] well(s). If [SC] slope is significantly different when compared to [EC], use [SC] values to determine effect of the respective tested compound. (see Step 5)

2. **GCPII Enzyme Preparation:** Prepare 80-fold dilution of Human GCPII Stock Solution (i.e. Dilute of 2 µl of Human GCPII with 158 µl of GCPII Assay Buffer), Mix well. Add 40 µl of diluted Enzyme to test sample(s) [S], Inhibitor Control [IC], Enzyme Control [EC] and Solvent Control [SC] wells, mix well; Add 40 µl of GCPII Assay Buffer to Background Control [BC] well. **Incubate the plate at 37 °C for 20 min.**
3. **Reaction Mix Preparation:** Prepare a 100-fold dilution of GCPII Substrate (Dilute 2 µl of GCPII Substrate to 198 µl GCPII Assay Buffer). Mix enough reagents for the number of assays to be performed**.** For each well, prepare a total 20 µl Reaction Mix :

	Reaction Mix
GCPII Assay Buffer	13 µl
Diluted GCPII Substrate	2 µl
GCPII Enzyme Mix	2 µl
GCPII Developer	2 µl
PicoProbe™	1 µl

After 20 min incubation (Step 2), mix & add 20 µl Reaction Mix to all wells including test sample(s) [S], Inhibitor Control [IC], Enzyme Control [EC], Solvent Control [SC] and Background Control [BC] wells and mix well. ***The total final reaction volume for each well will be 100 µl***.

Note: Prepare Reaction Mix immediately before adding to wells.

4. **Measurement:** Immediately measure fluorescence (Ex/Em = 535/587 nm) in kinetic mode at 37°C for 90 min using a fluorometric microtiter plate reader. Choose two time points (t_1 & t_2) in the linear range of the plot and obtain the corresponding RFU for Sample (R_{S1} and R_{S2}) and Background Control (R_{B1} and R_{B2}). ***Note:*** *It is normal to observe a lag phase in Enzyme Control within the first 30 min. Linear ranges are usually observed after 30 minutes of reaction.*
5. **Calculation:** Calculate the slope for all test samples [S], Enzyme Control [EC], Solvent Control [SC] and Background Controls [BC] by dividing the net ΔRFU (R_{t2}-R_{t1}) values over reaction time Δt (t_2-t_1). Subtract the Slope of Background Control values from [S], [EC] and [SC]. If [SC] slope is significantly different when compared to [EC], use [SC] values to determine effect of tested compound.

$$\% \text{ Relative Inhibition} = \frac{\text{Slope of [EC]} - \text{Slope of [S]}}{\text{Slope of [EC]}} \times 100$$

$$\% \text{ Relative Activity} = \frac{\text{Slope of [S]}}{\text{Slope of [EC]}} \times 100$$

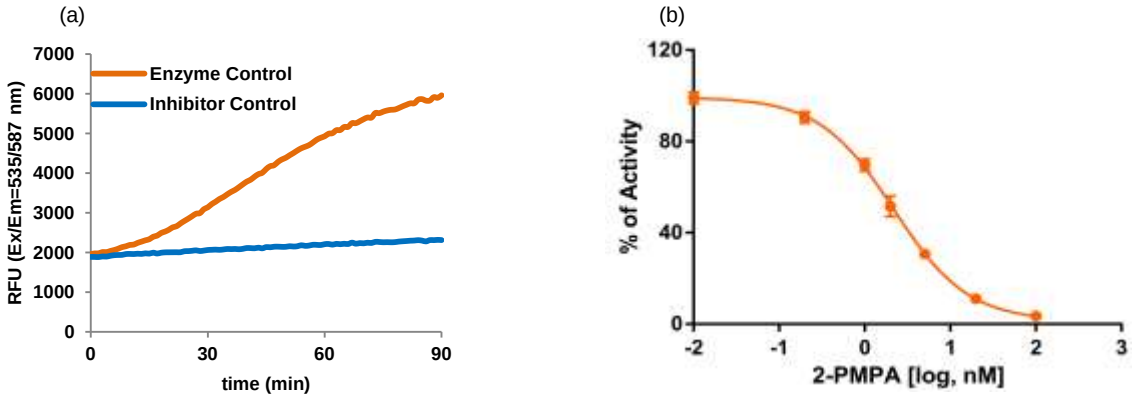


Figure: (a) Progress Curves of Glutamate Carboxypeptidase II Enzyme Control and Inhibitor Control (2-PMPA). (b) Inhibition of Glutamate Carboxypeptidase II activity by 2-PMPA. IC_{50} of 2-PMPA was calculated to be 2.1 ± 0.15 nM. Assay was carried out following the kit protocol.

VII. RELATED PRODUCTS:

PicoProbe™ Glutamate Carboxypeptidase II Activity Assay Kit (K738)
Glutamate Colorimetric Assay Kit (K629)
Human CellExp™ PSMA/FOLH1, Human Recombinant (P1304)
PicoProbe™ Glutamate Assay Kit (Fluorometric) (K413)

FOR RESEARCH USE ONLY! Not to be used on humans.