γ-Secretase Activity Kit

Catalog Number: FP003
100 Tests

Reagents Provided

EDANS/DABCYL Substrate: 500 µL of APP peptide GVVIATVIV using EDANS/DABCYL as the reporter system. *See Technical Notes.*

Extraction Buffer: 100 mL of 1X γ-Secretase Cell Extraction Buffer

Reaction Buffer: 10 mL of 2X γ-Secretase Reaction Buffer

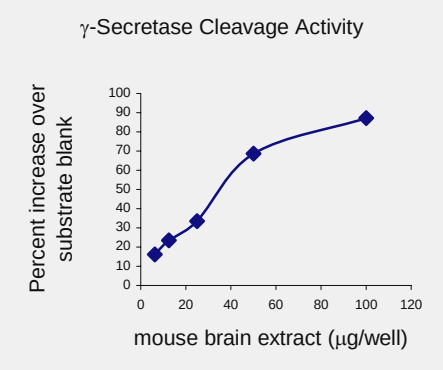
96 well Microplate: Nunc F16 Black MaxiSorp™ polystyrene microplate (6 strips of 16 wells)

Storage

Upon arrival, store the kit at 2 - 8° C.

References

1. Haass, C. *et al.* (1992) Nature **359**:322.
2. Seubert, P. *et al.* (1992) Nature **359**:325.
3. Gravina, S.A. *et al.* (1995) J. Biol. Chem. **270**:7013.
4. Selkoe, D.J. (1996) J. Biol. Chem. **271**:18295.
5. Sinha, S. and I. Lieberburg (1999) Proc. Natl. Acad. Sci. USA **96**:11049.
6. Esler, W.P. and M.S. Wolfe (2001) Science **293**:1449.
7. Mullan, M. *et al.* (1992) Nature Gen. **1**:345.
8. Citron, M. *et al.* (1992) Nature **360**:672.
9. Goate, A. *et al.* (1991) Nature **349**:704.
10. Suzuki, N. *et al.* (1994) Science **264**:1336.
11. Haass, C. and D.J. Selkoe (1993) Cell **75**:1039.
12. Hardy, J. and A. Israel (1999) Nature **398**:466.
13. Murphy, M.P. *et al.* (1999) J. Biol. Chem. **274**:11914.
14. Haass, C. and B. De Strooper (1999) Science **286**:916.



Intended Use

To detect enzymatic activity of the γ-secretase class of proteases associated with the cleavage of amyloid precursor protein (APP) from cell lysates using a fluorometric reaction.

Background Information

Proteolytic cleavage of the APP results in the generation of Aβ peptides of various lengths (1, 2). In Alzheimer's disease, the deposition of insoluble fibrillar plaques is facilitated by an increase and accumulation of $A\beta_{42}$ peptides (3, 4). Three proteases, α, β and γ-secretase, appear to be involved in APP cleavage (4 - 6).

Generation of the plaque forming Aβ peptides begins with β-secretase mediated cleavage between the Met_{671} and Asp_{672} residues of APP (5, 6). A described mutation in APP results in the substitution of Asn and Leu for amino acids Lys_{670} and Met_{671}, respectively. The resulting "Swedish mutation" is associated with increased β-secretase-mediated cleavage of APP resulting in the early onset of Alzheimer's disease (7, 8).

Subsequent to the beta-cleavage, the 12 kD C-terminal fragment can then be further cleaved by γ-secretase within the hydrophobic transmembrane domain at either Val_{711} or Ile_{713} (5, 6). This second cleavage event releases the $A\beta_{40}$ or $A\beta_{42}$ peptide. Another mutation in APP, referred to as the "London" mutation, substitutes Ile for Val_{718}. This substitution results in an increase in Aβ peptide formation that has been attributed to an increase in γ-secretase activity. Patients expressing the London mutation also have familial early-onset Alzheimer's disease (9, 10).

At the cell surface, APP undergoes proteolysis by an α-secretase that cleaves between Lys_{687} and Leu_{688} releasing a large, soluble ectodomain (4 - 6). The α-secretase cleavage site lies within the Aβ peptide associated with Alzheimer's plaques. Thus, increases in α-secretase activity have been associated with a decrease in Aβ formation (11).

Identification and characterization of the three secretases are important foci in Alzheimer's disease research. Despite numerous studies linking the presenilins and γ-secretase activity, a definitive γ-secretase has yet to be identified (12 - 14).

Principle of the Test

Cells of interest are first lysed to collect their intracellular contents. The cell lysate is then tested for secretase activity by the addition of a secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL. In the uncleaved form the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction.

Exposure to mouse brain extract (*i.e.* a tissue known to contain γ-secretase activity) results in cleavage activity for the γ-substrate sequence.The data presented in the Figure were obtained using a SPECTRAmax® Gemini XS fluorimeter with SOFTmax® PRO software from Molecular Devices. The plate was read in endpoint mode at an excitation wavelength of 355 nm and emission at 510 nm with a 495 nm cutoff. The assay was set up in duplicate wells of a Nunc F16 Black MaxiSorp 96 strip well plate.

R&D Systems, Inc.
1-800-343-7475

Assay Procedure

1) Collect cells of interest by centrifugation in a conical tube at 250 x g for 10 minutes (**Note:** *We recommend counting the cells before pelleting*). The supernatant is gently removed and discarded while the cell pellet is lysed by the addition of cold 1X Cell Extraction Buffer. The amount of Cell Extraction Buffer to be added to the pellet is determined by the number of cells present (this can be estimated from the number of cells initially cultured). Add 1 mL of cold Cell Extraction Buffer per 25 - 50 x 10^6 cells. Lyse adherent cells by pouring off the cell culture media and adding 5 - 10 mL of 1X Cell Extraction Buffer to the flask. If extraction is to be performed on whole tissue (*e.g.* brain), homogenize the tissue in cold 1X Cell Extraction Buffer to yield a final protein concentration of roughly 0.5 - 2.0 mg/mL. See Technical Notes section below.
2) Incubate the cell lysate on ice for at least 10 minutes and centrifuge at 10,000 x g for 1 minute. Transfer the supernatant to a new tube and keep on ice. This should yield a cell lysate with an approximate protein concentration of 2 - 4 mg/mL. The protein content of the cell lysate must be estimated using a protein determination assay that is compatible with detergents present in the Cell Extraction Buffer, *e.g.* BCA Protein Assay, Pierce Chemical Co., Catalog # 23225.
3) The enzymatic reaction for secretase activity should be carried out in the microplate provided and can be read with a microplate fluorimeter.
4) Add 50 μL of cell lysate (*i.e.* derived from approximately 2 - 5 x 10^6 cells or 25 - 200 μg of total protein) to each well. If there is a need to dilute the cell lysate, this should be done using 1X Cell Extraction Buffer. If larger volumes of cell lysates are necessary to meet the above requirements, the total reaction volume may be scaled up (*i.e.* the volume of <u>each</u> reagent added should be proportionally increased). We recommend the assay be set-up in duplicates or triplicates for greater accuracy.
5) Add 50 μL of 2X Reaction Buffer to each well.
6) Add 5 μL of Substrate to each well. The substrate is provided in DMSO and therefore must be brought to room temperature to liquefy before use.
7) Cover the plate, tap gently to mix, and incubate in the dark at 37° C for 1 - 2 hours.
8) Read the plate on a fluorescent microplate reader using light filters that allow for EDANS excitation between 335 - 355 nm wavelength. Collect emitted light between 495 - 510 nm.
9) This assay should include the following negative controls: a) no cell lysate and; b) no substrate.

This protocol may require modification, depending upon tissue source, cell number and secretase activity of the tissue.

Technical Notes

- Cell lysates should be generated so as to contain the maximum protein concentration possible. This may require a minimum utilization of 1X Cell Extraction buffer and longer incubation times at 2 - 8° C to allow for complete cell lysis and release of the enzyme into the lysate. The investigator may wish to keep the membrane fraction of the cell preparation (store at ≤ -20° C) for future use. Dilutions of the cell lysate can be made after a dose response is performed to optimize the results obtained using the Secretase Kit. Data suggest that 1 mg/mL total protein obtained from normal mouse brain (50 μg/50 μL in assay) results in an increase in fluorescence over background of typically 30% - 100%.
- The results are best expressed as the percent of increase in fluorescence over that of background controls (reactions where no cell lysate is added or where no substrate is added). It is recommended that these values be subtracted from the experimental results prior to calculating the percent of increase.
- Use of black microplates reduces stray light contamination of nearby wells. We recommend using the microplate provided.
- The substrate sequence provided with the kit corresponds to the amino acid sequence associated with γ-secretase cleavage of amyloid precursor protein (aa 710 - 718) and includes the London mutation. Actual cleavage of the substrate may occur at one or more sites within the peptide.