ELISA PRODUCT INFORMATION & MANUAL

APP

NBP2-61301

Enzyme-linked Immunosorbent Assay for quantitative detection of Human APP.
For research use only. Not for diagnostic or therapeutic procedures.

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Several Novus Biologicals products and product applications are covered by US and foreign patents and patents pending.

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NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Reagents require separate storage conditions.

Please read entire booklet before proceeding with the assay.

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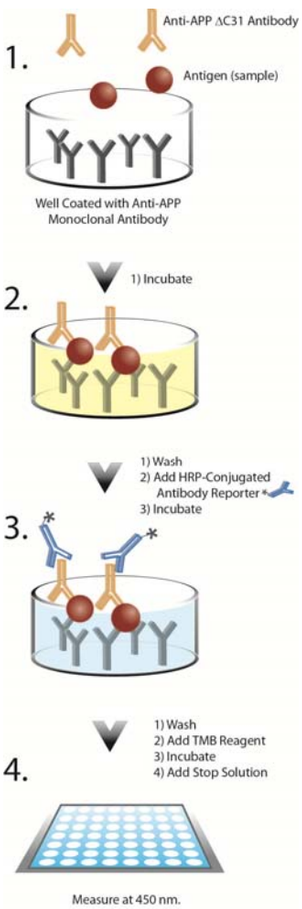
BACKGROUND

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the senile plaques, neurofibrillary tangles and loss of synapses and neurons. AD has been largely viewed as a disease of toxicity being mediated by the accumulation of the amyloid beta (Aβ) peptide as plaques within the brain resulting in damage to brain cells from the binding of damaging metals, reactive oxygen species production and direct damage to cellular membranes. Recent research has suggested that the Aβ peptide is a multifunctional peptide with non-pathological effects[1] and that its association with AD is in conjunction with its roles in combination with other proteins such as the amyloid precursor protein (APP) resulting in the imbalance between the processes of memory formation and normal forgetting. It is through the interactions of the Aβ peptide with APP that the Aβ peptide itself can affect normal modulation and signaling of APP resulting in its indicated role in the pathogenesis of AD via signaling effects rather than chemical or physical effects.

There are three major APP isoforms (APP_{695}, APP_{751} and APP_{770}) that are formed through alternative splicing of precursor mRNA. APP_{770} represents the canonical sequence. The APP_{695} isoform is preferentially expressed in the central nervous system, while APP_{770} and APP_{751} are more highly expressed in peripheral tissues. It has been demonstrated that the full length APP_{695} can be cleaved via caspase at an intracellular site (Asp664) resulting in the release of a 31 amino acid C-terminal peptide (C31) from the remaining larger neo-APP fragment (APP ΔC31) with both of these entities being pro-apoptotic[2]. Immunohistochemical analysis of human brain tissue demonstrated that this cytoplasmic cleavage occurs 4-fold greater in patients with AD versus normal patients and that these cleavage products are localized to plaques and tangles in key areas of the brain affected by the disease[3]. A single genetic mutation of aspartic acid residue 664 to alanine of APP_{695} led to the complete blockage of the C-terminal cleavage *in vivo* reversing many characteristics of the AD phenotype in a transgenic mouse model[4]. Additionally, in cell culture it has been suggested that the neurotoxicity of Aβ is dependent on the cleavage of APP at Asp664[5] and the resulting Aβ-facilitated APP multimerization[6].

Combined, this research describes the importance of this cleavage event and the associated proteins in the understanding of AD progression and affords a target for

therapeutic development. The APPΔC31 ELISA kit is a complete, immunometric immunoassay kit for the quantitative determination of APPΔC31 in cell lysate and cerebral spinal fluid samples with results in just 2 hours. The kit provides a simple and easy-to-use kit for the specific measurement of the neo-APP fragment from the APP ΔC31 cleavage event which when combined with measurements of other AD associated proteins (Aβ40/42, sAPPα and tau/p-tau) could prove a useful biomarker for the diagnosis and monitoring of AD progression.



PRINCIPLE

1. A yellow solution of polyclonal antibody developed against the neo epitope of APP ΔC31 is added to the wells of a microtiter plate coated with a monoclonal antibody specific for the N-terminal portion of APP. Next, samples or standards are introduced to wells and the plate incubated.
2. Assay wells are washed and a blue solution of horseradish peroxidase (HRP) conjugate is added to wells and the plate incubated once again.
3. After washing, a TMB substrate solution is introduced to the plate wells where an HRP-catalyzed reaction generates a blue color during the final incubation step.
4. Stop solution is added to the wells and the resulting intensity of the yellow color is read at 450nm. The amount of signal is directly proportional to the level of APP ΔC31 in the sample

MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. **APP ΔC31 Assay Buffer:**
   28 mL
   Phosphate buffered saline containing proteins and detergents
2. **APP ΔC31 Standard, 50x:**
   25 µL
   One vial containing 75nM of recombinant APP ΔC31 standard
3. **APP ΔC31 Microtiter Plate:**
   One plate of 96 wells
   A clear plate of break-apart strips coated with a monoclonal antibody specific for APP
4. **APP ΔC31 Antibody:**
   5 mL
   1x yellow solution of rabbit polyclonal antibody specific for APP ΔC31
5. **APP ΔC31 Conjugate:**
   10mL
   1x blue solution of donkey anti-rabbit IgG conjugated to horseradish peroxidase
6. **Wash Buffer Concentrate:**
   27 mL
   20X Tris buffered saline containing detergents
7. **TMB Substrate:**
   10 mL
   A solution of 3,3'5,5' tetramethylbenzidine (TMB) and hydrogen peroxide
8. **Stop Solution 2:**
   10 mL
   A 1N solution of hydrochloric acid in water
9. **APP ΔC31 Assay Layout Sheet:**
   1 each
10. **Plate Sealer:**
    2 each

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Reagents require separate storage conditions.

STORAGE

All kit components should be stored at 4ºC **except the APP ∆C31 Standard**, upon receipt. The Standards must be stored at or below -20ºC. Shipping conditions may not reflect storage conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 50 µL and 100 µL
4. Disposable polypropylene tubes for dilution of samples and standards
5. Disposable beakers or graduated cylinders for diluting buffer concentrates
6. A microplate shaker
7. Adsorbent paper for blotting
8. Microplate reader capable of reading a 450 nm
9. Software (such as AssayBlaster™ catalog number ADI-28-0002) for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

SAMPLE HANDLING

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If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

The Novus Biologicals APP ΔC31 ELISA kit is compatible with human cerebrospinal fluid (CSF) samples and various cell lysates (with indications for human serum and plasma) for the determination of APP ΔC31 protein concentration. The amino acid sequences recognized by the ELISA antibodies are conserved between humans and rodents. Detection of mouse and rat APP ΔC31 is predicted.

Prior to testing, frozen samples should be completely thawed and mixed to ensure homogeneity and if necessary, clarified via centrifugation to isolate residual debris.

For testing and validation of the APP ΔC31 ELISA, cell lysis utilizing RIPA buffer with the following critical components was found to not interfere with the assay performance:

Component	Assay tolerance	
	(%)	Concentration
Triton X-100	≤ 1	≤ 15mM
Sodium Deoxycholate	≤ 0.5	≤ 8.3mM
SDS	≤ 0.1	≤ 3.3mM
EDTA	-----	≤ 10mM
Protease inhibitor cocktail (Roche Complete)	-----	1x

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Sample handling procedures should be completed prior to reagent preparation.

Note: Experimentally observed concentrations of APP ΔC31 protein in cell lysates may vary due to cell culture/treatment conditions and/or alterations of the lysis procedure. Variations may be caused by, but are not limited to one or more of the following: cell type/species, frequency of media changes, concentration of chemical treatment, treatment duration, media supplements and cell confluency. Therefore, it is very important for each end user to optimize sample handling/dilution for their unique samples in order to obtain the best possible results for their experiment, and interpretation of experimental data should include considerations of these sources of variability.

Linearity

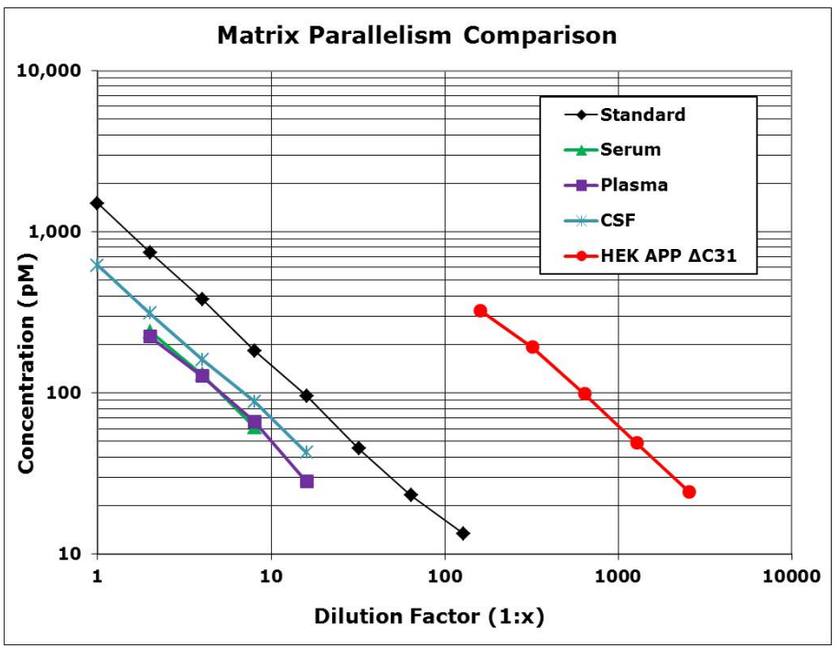
Various biological matrices and a cell lysate were spiked with recombinant APP ΔC31 (recAPP ΔC31) and serially diluted in assay buffer and compared to the standard prepared in assay buffer and a serially diluted cell lysate prepared from transfected HEK cells overexpressing the human APP ΔC31 protein. The minimum required dilution for each matrix was determined by identifying the dilution at which observed linearity began.

Dilution	CSF	Serum	Plasma	Cell lysate (HEK APP_{695})
Neat	96	----	----	----
1:2	97	92	88	----
1:4	100	100	100	101
1:8	109	94	105	93
1:16	106	----	89	100

Data reported are percentages of expected value at the given dilution tested.

Parallelism

Parallelism experiments were carried out to determine if the recAPP ΔC31 standard accurately mimicked native APP ΔC31 in biological matrices. Human CSF, serum and plasma all spiked with the recAPP ΔC31 standard were diluted with assay buffer and compared to the APP ΔC31 standard curve and cell lysates from APP ΔC31 expressing HEK cells. The parallel response indicates the standard effectively mimics the native molecule with no matrix interference at the dilutions tested.



Spike and Recovery

Recombinant APP ΔC31 was spiked at multiple concentrations into lysates from cells transfected to over express the APP_{695} protein and CSF samples. Matrix background was subtracted from the spiked values and the recovery was compared to the recovery of identical spikes in assay buffer. The average percent recovery for each matrix is indicated below.

Sample	Recommended Dilution	Recovery of Spike
Cell lysate (in RIPA buffer)	1mg/mL total protein	101.1%
Cerebrospinal Fluid	Neat	93.6%

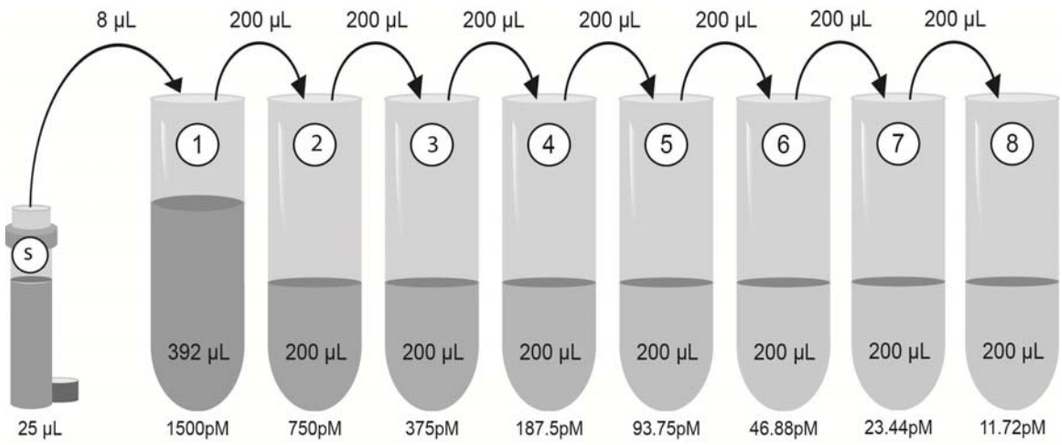
REAGENT PREPARATION

1. **Wash Buffer**

   Prepare Wash buffer by diluting 25 mL of the supplied Wash Buffer concentrate with 475 mL of deionized water. Store the diluted wash buffer at room temperature.

2. **Recombinant APP ΔC31 Standard**

   Label eight disposable 12 x 75mm (or similar) polypropylene tubes #1 through #8. Pipet 392 µL assay buffer into tube #1 and 200 µL assay buffer into tubes #2 through #8. Pipet 8 µL of recombinant standard into tube 1. Vortex gently. Serially dilute 200 µL of tube #1 standard to tubes #2 through #8 gently vortexing after each dilutional transfer.



Do not store diluted standard.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

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Bring all reagents to room temperature for at least 30 minutes prior to opening.

1. Pipet 50 µL of yellow APP ΔC31 Antibody solution into each well, except the Blank.
2. Pipet 50 µL of assay buffer into the S0 (0 pM standard) wells.
3. Pipet 50 µL of APP ΔC31 Standards #1 through #8 into the appropriate wells.
4. Pipet 50 µL of the Samples into the appropriate wells.
5. Seal the plate. Incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
6. Empty the contents of the wells and wash by adding 300 µL of wash solution to each well. Empty or aspirate the wells and repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
7. Add 100 µL of blue APP ΔC31 Conjugate solution to each well, except the Blank.
8. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
9. Wash as above (Step 5). Pipet 100 µL of TMB Substrate Solution into each well.
10. Seal the plate. Incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
11. Pipet 100 µL Stop Solution to each well.
12. Zero the plate reader against the Blank wells, read the optical density at 450 nm. If the plate reader is not able to automatically subtract blank well values from each well, manually subtract the mean optical density of the Blank wells from all the readings.

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All standards and samples should be run in duplicate.

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Pipet the reagents to the sides of the wells to avoid possible contamination.

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Prior to the addition of the substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of APP ΔC31 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program. Assay Blaster! Data analysis software (Prod. no. ADI-28-0002) is an easy-to-use and cost effective program that provides the options of point-to-point, 4PL and 5PL curve fitting options.

The concentration of APP ΔC31 can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

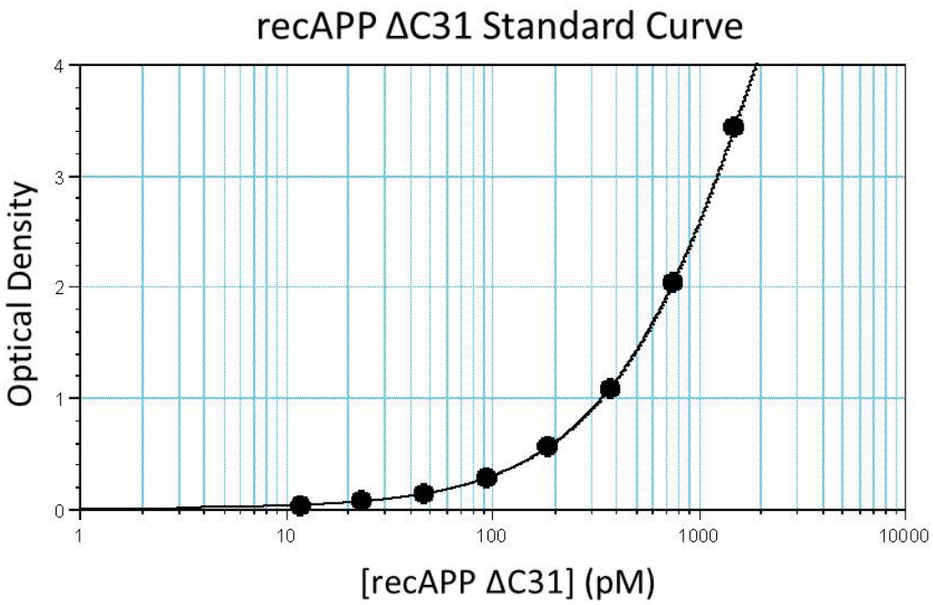
2. Plot the Net OD versus concentration of recAPP ΔC31 for the standards. Approximate a line through the points. The concentration of APP ΔC31 in the unknown samples can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustrative purposes only and should not be used to calculate results from another assay.

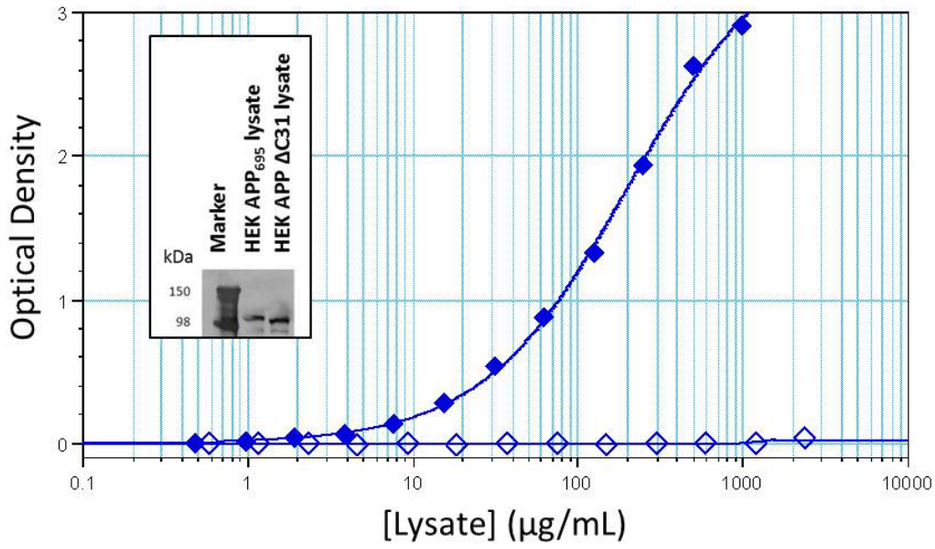
Sample	Average Net OD	APP ΔC31 (pM)
Blank	0.002	---
S0	0.002	0
S1	3.433	1500
S2	2.032	750
S3	1.087	375
S4	0.559	187.5
S5	0.287	93.75
S6	0.142	46.88
S7	0.071	23.44
S8	0.035	11.72



PERFORMANCE CHARACTERISTICS

Specificity

To assess the specificity of the assay, cell lysates from HEK cells transfected to express either APP ΔC31 or APP_{695} were tested in both the ELISA and western blot (with monoclonal antibody 3E9, which recognizes both forms of APP). The western blot indicated similar amounts of the two APP forms were present in the cell lysates. The ELISA demonstrates the sensitive detection of APP ΔC31 with no recognition of APP_{695}.



Sensitivity

The sensitivity, defined as 2 standard deviations from the mean signal at zero, was determined from 12 independent standard curves. The standard deviation was determined from 20 zero standard replicates. The sensitivity was found to be 0.92 pM.

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing the recAPP ΔC31 standard in a single assay.

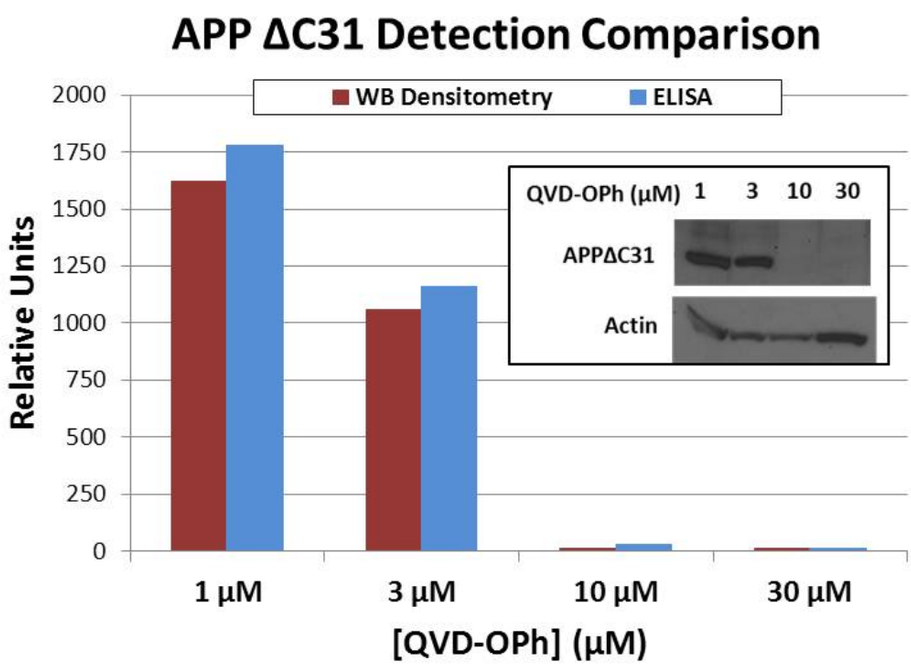
pM	%CV
907.1	3.92
560.9	1.61
208.7	2.02

Inter-assay precision was determined by measuring three buffer controls containing the recAPP ΔC31 standard in multiple assays (n=12) over several days.

pM	%CV
907.4	6.04
556.6	8.26
213.1	3.40

Experimental Evaluation

To demonstrate the use of the ELISA in a drug screening application, APP_{770} transfected 7W CHO cells were treated with 5 µM simvastatin, which has previously been shown to generate APP ΔC31 production via stimulation of intracellular caspase cleavage[7]. Additionally, varying amounts (1-30 µM) of the pan caspase inhibitor (3S)-5-(2,6-Difluorophenoxy)-3-[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl) amino]butyl]amino]-4-oxo-pentanoic acid (QVD-OPh) were administered to the cells in order to arrest the aforementioned cleavage and associated production of APP ΔC31. Cell lysates produced after these treatments were analyzed by both western blot with an APP ΔC31 specific antibody and the APP ΔC31 ELISA. The western blot and ELISA results are in agreement showing treatment with increasing concentrations of caspase inhibitor reduces the production of APP ΔC31.



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NOTES