

A Colorimetric ELISA for Alzheimer's Disease Research Enabling Quantification of APP Δ C31 in Cell Lysates and Cerebrospinal Fluid

APP Δ C31 ELISA kit (ADI-900-227)

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ABSTRACT

The amyloid precursor protein (APP) undergoes a variety of modifications through enzymatic cleavage leading to the formation of pro-Alzheimer's disease (AD) and anti-AD biologically active peptides. One such cleavage product, APP Δ C31 results from the caspase cleavage of the full length APP protein (APP₆₉₅) at amino acid Asp664 as well as the concomitant formation of the 31 amino acid C-terminal peptide (C31). The APP Δ C31 truncated protein has been shown to accumulate in brain tissue of AD patients as well as in brain tissue from AD model mice and is suggested to play a role in the etiology of the disease. To enable the quantitative measurement of APP Δ C31 formation in cells and tissues we have developed a colorimetric ELISA assay. The assay is highly specific to APP Δ C31 exhibiting no cross-reactivity to APP. The assay has a sensitivity of 1 pM with an assay range of 12 to 1500 pM. The ELISA has been validated for use in cell lysates and cerebral spinal fluid and is indicated for use in serum and plasma. The applicability of the assay to drug screening studies in cell culture was demonstrated through simvastatin induction of APP Δ C31 production in the presence of increasing concentrations of a pan caspase inhibitor. The amount of APP Δ C31 in cell lysates decreased with increased concentration of caspase inhibitor. Establishing the ability of the assay to function in a wide variety of matrices provides the opportunity to evaluate APP Δ C31 as a biomarker for diagnosing and monitoring Alzheimer's disease.

BACKGROUND

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by 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 amyloid beta (A β) 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 A β peptide is a multifunctional peptide with non-pathological effects¹ 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 A β peptide with APP that the A β peptide itself can affect normal modulation and signaling of APP resulting in its indicated role in the pathogenesis of AD.

It has been demonstrated that APP₆₉₅ 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 (APP₁₋₆₆₄) with both of

these entities being pro-apoptotic². Immunohistochemical analysis of human brain tissue demonstrated that this cytoplasmic cleavage occurs 4-fold greater in patients with AD versus normal patients and these cleavage products are localized to plaques and tangles in key areas of the brain affected by the disease³. A single genetic mutation of aspartic acid residue 664 to alanine of APP₆₉₅ led to the complete blockage of the C-terminal cleavage *in vivo*, reversing many characteristics of the AD phenotype in a transgenic mouse model⁴. Additionally, in cell culture it has been suggested that the neurotoxicity of A β is dependent on the cleavage of APP at Asp664⁵ and the resulting A β -facilitated APP multimerization⁶.

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 APP Δ C31 ELISA provides an easy-to-use kit for the specific measurement of the product from this cleavage event which when combined with measurements of other AD associated proteins (A β 40/42, sAPP α and tau/p-tau) could prove a useful biomarker for the diagnosis and monitoring of AD progression.

ASSAY VALIDATION

Principle

A yellow solution of polyclonal antibody developed against the neo epitope of APP Δ C31 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 for one hour. The wells are washed and a blue solution of horseradish peroxidase (HRP) conjugate is added to wells and the plate incubated for 30 minutes. After washing, a TMB substrate solution is introduced to the plate wells where an HRP-catalyzed reaction generates a blue color during the final 30 minute incubation step. 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 Δ C31 in the sample.

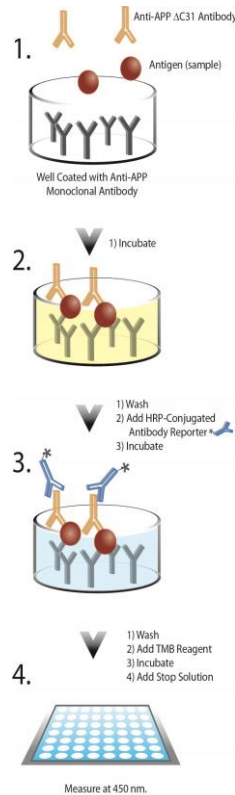


Figure 1. Assay Protocol

Standard Curve

To determine a typical standard curve, recombinant APP Δ C31 (rAPP Δ C31) was serially diluted in assay buffer and run in the assay. The standard curve was plotted with optical density as a function of antigen concentration (pM) (Figure 2). Sensitivity of the assay, defined as the concentration of analyte measured at 2 standard deviations above background, was determined to be 1 pM with a standard range of 12 to 1500 pM. The ELISA sensitivity is approximately 600 fold greater than obtained in corresponding Western blots using the APP Δ C31 specific antibody (Figure 2 inset).

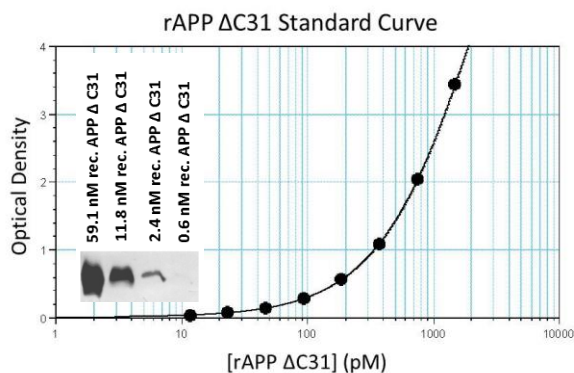


Figure 2. Typical standard curve produced using the rAPP Δ C31 ELISA kit standard.

Assay Parallelism & Spiked Recovery in Various Matrices

Various biological matrices were spiked with rAPP Δ C31 and serially diluted in assay buffer and compared to the standard curve. The parallel response (Figure 3) shows the lack of matrix interference at the dilutions reported. The parallelism also indicates the rAPP Δ C31 expressed in bacteria is antigenically identical in the ELISA to the APP Δ C31 expressed in mammalian cells.

Similarly, rAPP Δ C31 was spiked at multiple concentrations into lysates from cells transfected to overexpress the APP₆₉₅ protein and cerebrospinal fluid (CSF) samples. Matrix background was subtracted from the spiked values and the percent recovery determined (Table 1).

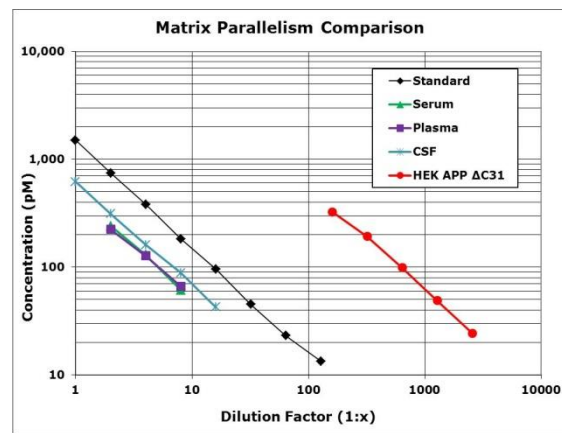


Figure 3. Parallelism between the recombinant assay standard and various matrices spiked with rAPP Δ C31.

Sample	Recommended Dilution	Average Recovery of Spikes
Cell lysate (in RIPA buffer)	1mg/mL total protein	101.1%
Cerebral Spinal Fluid	Neat	93.6%

Table 1. Assay recovery and required dilutions in cell lysate and CSF.

Assay Specificity

To assess the specificity of the assay, cell lysates from HEK-293T cells transfected to express either APP Δ C31 or APP₆₉₅ were tested in both the ELISA and Western blot with monoclonal antibody (3E9) that recognizes both forms of APP (Figure 4 and Figure 4 inset). The Western blot indicated similar amounts of the two APP proteins were present in the cell lysates. The ELISA demonstrates sensitive detection of APP Δ C31 and did not detect APP₆₉₅.

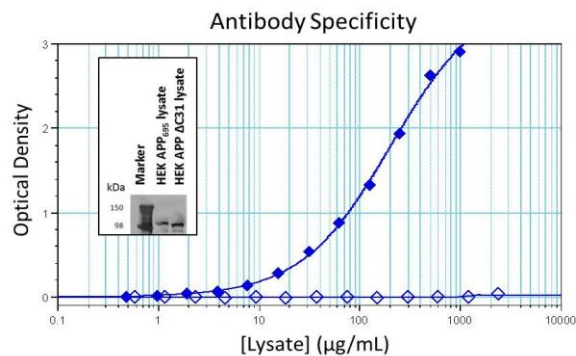


Figure 4. Antibody specificity to APP Δ C31 protein. Open symbols represent lysate containing APP₆₉₅ while closed symbols represent lysate containing APP Δ C31. The inset provides an anti-APP (monoclonal antibody 3E9) Western blot to demonstrate the levels of APP₆₉₅ and APP Δ C31 in the lysates were comparable.

RESULTS

To demonstrate the usefulness of the ELISA in a drug screening application, APP₇₇₀ transfected 7W CHO cells were treated with 5 μ M simvastatin, which has previously been shown to generate APP Δ C31 production via stimulation of intracellular caspase cleavage⁷. Additionally, varying amounts (1-30 μ M) 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 Δ C31. Cell lysates produced after these treatments were analyzed by both Western blot with an APP Δ C31 specific antibody and the APP Δ C31 ELISA (Figure 5 and Figure 5 inset). The Western blot and ELISA results are in agreement showing treatment with increasing concentrations of caspase inhibitor reduces the production of APP Δ C31.

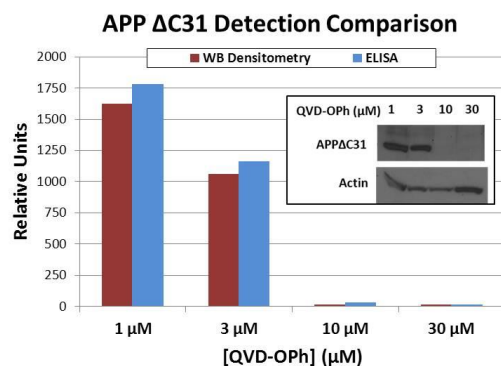


Figure 5. Levels of APP Δ C31 after simvastatin stimulation and concomitant treatment with varying concentrations of caspase inhibitor QVD-OPh.

DISCUSSION

The APP Δ C31 ELISA is a colorimetric immunoassay for the specific measurement of APP Δ C31 in both *in vivo* and *in vitro* studies. The assay is highly specific for the Δ C31 cleaved form of the APP protein exhibiting no cross recognition of APP₆₉₅. The ELISA accurately detects rAPP Δ C31 spiked into CSF and cell lysate matrices. Parallelism in spiked serum and plasma indicates potential for use with these matrices. The APP Δ C31 ELISA has a dynamic range of 12-1500 pM and a sensitivity of 1 pM (69 pg/mL) with results achieved within an assay time of 2 hours. The assay is suited for use in drug screening applications for inducers or inhibitors of caspase mediated cleavage at Asp664 of APP₆₉₅.

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