ELISA Kit
Catalog #KHB0051

Human

APP

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INTRODUCTION

Amyloid Precursor Protein (APP) is a large transmembrane protein encoded by a single gene located on human chromosome 21. APP is found as an immature form, a protein with M_r=95 kDa and also as the APP holoprotein, a post-translationally modified protein with M_r=100-120 kDa. APP localizes to the Golgi apparatus, endosome, and cell membrane. Within the Golgi apparatus, APP is oriented such that its N-terminus is within the lumen while the C-terminus extends into the cytoplasm. Within the cell membrane, APP contains a large ectodomain which corresponds to the N-terminus, a transmembrane domain, and a short cytoplasmic tail which corresponds to the C-terminus.

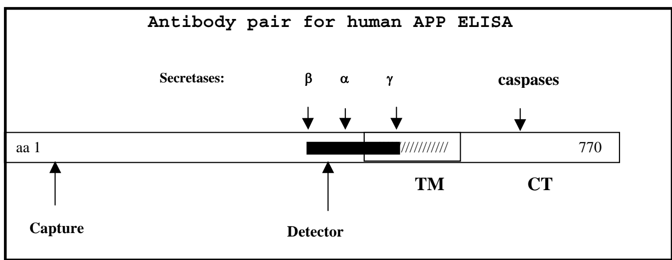
APP is proteolytically cleaved through the action of several proteases. β-secretase, an enzyme activity recently attributed to BACE, cleaves APP to generate the APP N-terminal fragment (sAPPβ) with M_r=100 kDa and a C-terminal fragment (C99) with M_r=12 kDa. The smaller 12 kDa fragment can be further cleaved by presenilin-dependent γ-secretase. This second cleavage produces the insoluble β-amyloid (A β), a peptide of ~4 kDa comprised of 40-43 amino acid residues. Alternatively, APP can be cleaved by α-secretase to create a large soluble fragment known as sAPPα and a smaller fragment comprised of 83 amino acid residues, known as C83 (M_r=10 kDa), which is retained in the membrane. Cleavage of C83 by γ-secretase generates a small fragment known as p3. Thus cleavage of APP by α-secretase precludes the formation of β-amyloid.

The function of APP and the roles of the various fragments resulting from the proteolytic cleavage of APP in behavior, cognition, and learning are under investigation. APP expression levels are upregulated by nerve growth factor in a mouse dorsal root ganglion model. APP may protect cells against apoptosis when faced with growth factor withdrawal. Secreted sAPPα is observed to be neuroprotective, neurotropic, regulating cell excitability and synaptic plasticity.

While APP and sAPPα are observed to exert protective effects on neurons, β-amyloid, the product of APP cleavage by β-secretase and presenilin-dependent γ-secretase, is observed to be detrimental to neuronal function and cognition.

The Invitrogen Human APP ELISA is designed to specifically detect and quantify the levels of the APP in human cerebral spinal fluid samples (CSF) and human cell lysates. The assay will recognize both natural and recombinant human APP, and may also detect Mouse APP. The capture antibody for this assay binds to the N-terminal part of human APP and the detection antibody recognizes the N-terminal part of amyloid β peptide (Figure 1). Therefore, this ELISA kit will detect the isoforms of APP_{770}, APP_{751} (soluble APP), APP_{733} and APP_{695}. This kit detects soluble APPα which is cleaved by α-secretase, but not soluble APPβ which is cleaved by β-secretase.

Figure 1



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Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen Human APP kit (Hu APP) is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Hu APP has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu APP content, control specimens, and unknowns, are pipetted into these wells.

During the first incubation, the Hu APP antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Hu APP is added. During the second incubation, this antibody binds to the immobilized Hu APP captured during the first incubation.

After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu APP present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
Hu APP Standard, recombinant Hu APP expressed in *E. coli*; lyophilized. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 25 mL per bottle.	1 bottle
Antibody Coated Wells, 12x8 Well Strips.	1 plate
Hu APP Biotin Conjugate (Biotin-labeled anti-APP). Contains 0.1% sodium azide; 11 mL per bottle.	1 bottle
Streptavidin HRP, (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin HRP Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
Stabilized Chromogen, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic CSF or cell lysate. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.

6. It is recommended that all standards, controls and samples be run in duplicate.
7. Samples that are >50 ng/mL should be diluted with *Standard Diluent Buffer*.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots.**
11. Read absorbances within 2 hours of assay completion.
12. Do not use reagents after the kit expiration date.
13. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS

A. Recommended Formulation of Cell Extraction Buffer:

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)
Protease inhibitor cocktail (ex., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.

This buffer is stable for 2-3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the Cell Extraction Buffer should be thawed on ice. Important: add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

B. Protocol for Cell Extraction

This protocol has been applied to several cell lines with the Cell Extraction Buffer above. Researchers may optimize the cell extraction procedures that work best in their hands.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80°C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of APP. For example, 10^8 HeLa cells can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in *Standard Diluent Buffer* (See **Assay Method**) is sufficient for the detection of APP.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80°C. Avoid multiple freeze-thaws.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Human APP Standard

The Hu APP standard was prepared using purified soluable APPα expressed in *E. coli*. It was calibrated against the mass of a highly purified recombinant Hu APP.

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 50 ng/mL with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
2. Add 0.600 mL of the reconstituted standard to a tube. Label as 50 ng/mL Hu APP. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 25, 12.5, 6.25, 3.13, 1.56, and 0.78 ng/mL Hu APP.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Human APP Standard

Standard:	Add:	Into:
50 ng/mL	Prepare as described in Step 2.	
25 ng/mL	0.300 mL of the 50 ng/mL std.	0.300 mL of the Diluent Buffer
12.5 ng/mL	0.300 mL of the 25 ng/mL std.	0.300 mL of the Diluent Buffer
6.25 ng/mL	0.300 mL of the 12.5 ng/mL std.	0.300 mL of the Diluent Buffer
3.13 ng/mL	0.300 mL of the 6.25 ng/mL std.	0.300 mL of the Diluent Buffer
1.56 ng/mL	0.300 mL of the 3.13 ng/mL std.	0.300 mL of the Diluent Buffer
0.78 ng/mL	0.300 mL of the 1.56 ng/mL std.	0.300 mL of the Diluent Buffer
0 ng/mL	0.300 mL of the Diluent Buffer	An empty tube

Remaining reconstituted standard should be discarded or frozen in aliquots at -80°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

C. Storage and Final Dilution of Streptavidin-HRP (100X)

Please Note: The *Streptavidin-HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100X concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP 100X	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards and samples (CSF, cell lysate, and controls), to the appropriate microtiter wells. Samples prepared in Cell Extraction Buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample into 90 µL buffer). While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:50 or 1:100 may be optimal. The dilution

chosen should be optimized for each experimental system. Tap gently on side of plate to mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)

4. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
6. Pipette 100 µL of biotinylated *Hu APP Biotin Conjugate* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or**

metalized mylar. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

14. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
16. Plot on graph paper the absorbance of the standards against the standard concentration. (The background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
17. Read the Hu APP concentrations for unknown samples and controls from the standard curve plotted in step 16. (Samples producing signals greater than that of the highest standard (50 ng/mL) should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 50 ng/mL Hu APP.

Standard Hu APP (ng/mL)	Optical Density (450 nm)
0	0.120
0.78	0.225
1.56	0.306
3.13	0.428
6.25	0.705
12.5	1.292
25	2.099
50	3.342

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 50 ng/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >50 ng/mL with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of biological fluids other than CSF has not been thoroughly investigated. The rate of degradation of native APP in various matrices has not been investigated. Although APP degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

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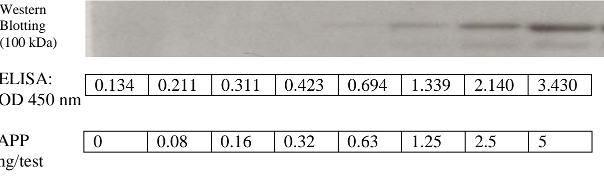
PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <0.4 ng/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

The sensitivity of this ELISA was compared to Western blotting using known quantities of APP. Human APP-transfected CAD cells were extracted using the procedures described previously. Equal amounts of APP protein were analyzed by Western blotting and ELISA. The data presented in Figure 2 show that the sensitivity of the ELISA is approximately 8x greater than that of Western blotting. The bands shown in the Western blot data were developed using mouse anti-human APP from Invitrogen (Cat. #44-352), an alkaline phosphatase conjugated anti-mouse IgG followed by chemiluminescent substrate and autoradiography.

Figure 2: Detection of Human APP by ELISA vs Western Blot:



ELISA: OD 450 nm	0.134	0.211	0.311	0.423	0.694	1.339	2.140	3.430
APP ng/test	0	0.08	0.16	0.32	0.63	1.25	2.5	5

PRECISION

1. Intra-Assay Precision

Samples of known APP concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	21.89	12.68	3.11
SD	0.81	1.01	0.22
%CV	3.72	7.99	7.05

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	22.12	13.38	3.12
SD	1.16	1.18	0.25
%CV	5.24	8.78	8.15

SD = Standard Deviation
CV = Coefficient of Variation

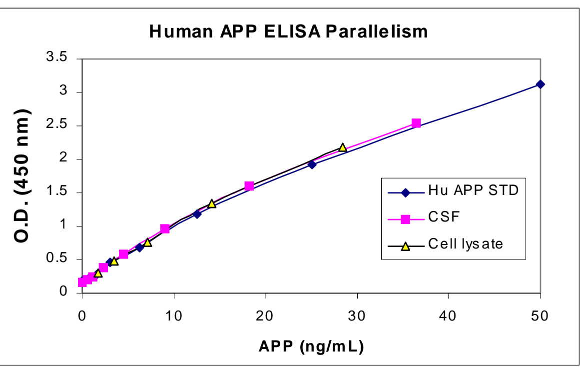
RECOVERY

To evaluate recovery, recombinant APP was spiked into human CSF and then diluted 1:100 with *Standard Diluent Buffer*. The percent recovery over endogenous levels was calculated. On average, 112% recovery was observed.

PARALLELISM

Natural APP from APP-transfected CAD cells and human CSF was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the APP standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects APP content in samples.

Figure 3



LINEARITY OF DILUTION

Human CSF was serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

	Extract Buffer		
CSF Dilution	**Measured (ng/mL)**	**Expected (ng/mL)**	**% Expected**
1/5	35.9	35.9	100
1/10	19.1	18.0	106.4
1/20	9.8	9.0	108.5
1/40	4.8	4.5	105.7
1/80	2.3	2.2	102.9

SPECIFICITY

The Hu APP ELISA kit is specific for measurement of total APP protein. The following proteins were tested at 100 ng/mL and found to have no cross-reactivity: Tau, α-synuclein, β-synuclein, Aβ1-40, and Aβ1-42. This assay may also recognize mouse APP.

To confirm the specificity of this kit, cell extracts from several cell lines at a concentration of 200 μg/mL protein (Figure 4) and human cerebral spinal fluids at 1:16 dilution (Figure 5) were analyzed simultaneously by ELISA and Western blot. The data show that the levels of APP protein detected with this ELISA kit are consistent with results obtained by Western blot analysis (inset in graphs).

Figure 4

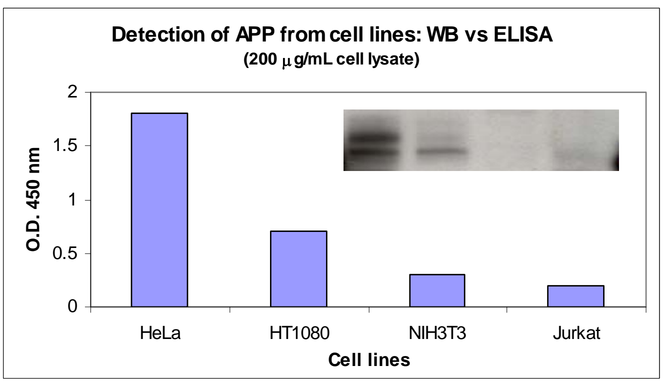
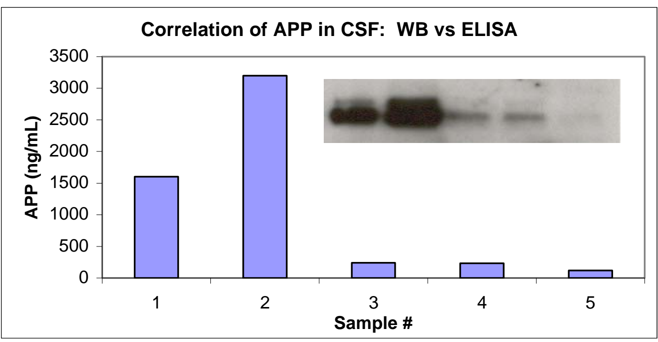


Figure 5



EXPECTED VALUES

20 human CSF samples from chemically normal individuals were evaluated in this assay. The APP values ranged from 245 to 3206 ng/mL (mean 1278 ng/mL).

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
Protect from Light	Protect from light		Consult accompanying documents
i	Directs the user to consult instructions for use (IFU), accompanying the product.		

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NOTES

NOTES

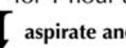
Human APP Assay Summary

Add 100 µL of standards, controls & samples

Incubate for 2 hours at RT

aspirate and wash 4x

Incubate 100 µL of Biotin Conjugate for 1 hour at RT



aspirate and wash 4x

Incubate 100 µL of Streptavidin-HRP Working Solution for 30 minutes at RT

aspirate and wash 4x

Incubate 100 µL of Stabilized Chromogen for 30 minutes at RT

Add 100 µL of Stop Solution and read at 450 nm

Total time: 4 hours