

Quantikine[®] ELISA

Human Amyloid β (aa1-40) Immunoassay

Catalog Number DAB140B

For the quantitative determination of human Amyloid beta (amino acids 1-40) concentrations in cell culture supernates, tissue lysates, and cerebrospinal fluid.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Amyloid beta-peptides ($A\beta$) are proteolytic fragments of the Amyloid Precursor Protein (APP), and their formation into fibrils plays an integral role in Alzheimer's disease pathology. $A\beta$ fibrils constitute the major protein component of senile plaques that accumulate in the brain of Alzheimer's patients. Amyloid plaque accumulation is increased in the presence of hypoxia, traumatic brain injury, and chronic inflammation (1, 2). $A\beta$ also directly exert multiple effects on neurons (1, 3). APP is a multifunctional transmembrane protein that consists of a 682 amino acid (aa) extracellular domain (ECD), a 24 aa transmembrane segment, and a 47 aa cytoplasmic domain. Its ECD contains two heparin binding domains, one copper binding domain, a BPTI/ Kunitz inhibitor domain, and a collagen binding domain. Alternative splicing generates multiple isoforms including the most prevalent APP695, APP751, and APP770 which contain various deletions or substitutions in the ECD. The $A\beta$ peptide sequences extend from the extracellular juxtamembrane region into the transmembrane segment.

APP is subject to proteolytic cleavage at multiple sites that may or may not result in the generation of $A\beta$. In the non-amyloidogenic pathway, the APP ECD is shed by β -secretase cleavage (ADAM10) within the $A\beta$ sequence, thereby preventing the formation of $A\beta$ peptides (4). In the amyloidogenic pathway, APP is cleaved by BACE (β -secretase activity) just N-terminal to the $A\beta$ sequence (5, 6). This releases a slightly shorter ECD fragment known as APPbeta. Following amyloidogenic ECD shedding, intramembrane cleavage of the remaining portion by γ -secretase/Presenilin protein complexes generates the $A\beta$ peptides 1-40 or 1-42 (7-9). These peptides are 40 and 42 aa in length, respectively, and share Asp672 as their N-terminal residue. $A\beta$ 1-40 is relatively abundant in the CSF, while $A\beta$ 1-42 is more hydrophobic, more prone to aggregation, and more closely associated with Alzheimer's pathology (1, 10). Human $A\beta$ 1-40 shares 93% amino acid identity with mouse and rat $A\beta$ 1-40. γ -secretase cleavage also releases the intracellular domain (AICD) which translocates to the nucleus and regulates the transcription of a wide variety of genes (11). Soluble fragments of the APP ECD are released into the cerebrospinal fluid (CSF), interstitial fluid in brain, plasma, and urine (12-15). They can be further cleaved to generate a 35 kDa fragment which binds the receptor DR6 and triggers axonal pruning and neurodegeneration (16).

$A\beta$ peptides can associate intracellularly into dimers, higher order oligomers, and insoluble fibrils (17-19). They enhance the hyperphosphorylation of Tau and the formation of neurofibrillary tangles, another hallmark of Alzheimer's disease (20). $A\beta$ also form fibrils with ApoE and bind to the prion protein PrP(C) (21, 22). Of the various forms of $A\beta$, oligomers are the most potent at impairing cognitive function, inhibiting long term potentiation (LTP) in hippocampal neurons, and inducing neuronal oxidative stress (17, 23, 24). In contrast, monomeric $A\beta$ can be protective against excitotoxic neuronal cell death (25). $A\beta$ can directly disrupt the integrity of neuronal membranes (26). The neurotoxicity of $A\beta$ is increased by its *in vivo* glycation or pyroglutamylation (27, 28). $A\beta$ interaction with RAGE induces neuronal mitochondrial dysfunction, oxidative stress, inflammatory activation of astrocytes and microglia, and transport of circulating $A\beta$ across the blood brain barrier into the brain (29-31). $A\beta$ also induces neuroinflammation through interactions with TLR2 (32). Extracellular $A\beta$ can be degraded by Neprilysin or Insulysin/IDE, and $A\beta$ clearance is impaired in Alzheimer's (33-36).

INTRODUCTION *CONTINUED*

A β peptides exert direct actions on several neurotransmitter receptors, and APP metabolism is regulated by the activity of these receptors. A β bind and activate the nicotinic acetylcholine alpha 7 receptor, leading to neuroprotection, enhanced synaptic plasticity and memory formation, and the upregulation of acetylcholinesterase (37-39). A β activation of NMDA and AMPA receptors, however, leads to mitochondrial dysfunction and neuronal apoptosis (40). A β inhibit the release of dopamine and GABA in response to muscarinic receptor activation (41). The non-amyloidogenic processing of APP is induced by multiple triggers including Substance P or the activation of particular subtypes of muscarinic, nicotinic, purinergic, serotonin, NMDA, or metabotropic glutamate receptors (42-48).

The Quantikine Human Amyloid β (aa1-40) Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Amyloid β (aa1-40) in cell culture supernates, tissue lysates, and cerebrospinal fluid. It contains synthetic human Amyloid β (aa1-40) and antibodies raised against the synthetic peptide. Results obtained for naturally occurring human Amyloid β (aa1-40) showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Amyloid β (aa1-40).

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Amyloid β (aa1-40) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Amyloid β (aa1-40) present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Amyloid β (aa1-40) is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Amyloid β (aa1-40) bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Diluent RD2-7 and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Amyloid β (aa1-40) Microplate	894651	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Amyloid β (aa1-40).	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Amyloid β (aa1-40) Standard	894653	2 vials of Synthetic human Amyloid β (aa1-40) peptide in a buffered protein base with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	Discard after use. Use a new standard for each assay.
Human Amyloid β (aa1-40) Conjugate	894652	21 mL of monoclonal antibody specific for human Amyloid β (aa1-40) conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Diluent RD2-7	896015	2 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Amyloid β Controls (optional; R&D Systems, Catalog # QC216).

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- RIPA Buffer with protease inhibitors.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Note: *Thaw samples on ice to prevent aggregation. Avoid repeated freeze-thaw cycles.*

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C.

Tissue Lysates - Lysates were prepared as described in the Sample Value section.

Cerebrospinal Fluid - Freeze sample within one hour of collection to avoid aggregation. Store samples at ≤ -70 °C.

Note: **Amyloid β is labile in cerebrospinal fluid and can become undetectable over time.**

SAMPLE PREPARATION

Use polypropylene tubes.

Note: *Thaw and handle samples on ice.*

Tissue lysate samples require a 40-fold dilution due to high endogenous levels. A suggested 40-fold dilution is 20 μ L of sample + 780 μ L of Diluent RD2-7.

Cerebrospinal fluid samples require a 20-fold dilution due to high endogenous levels. A suggested 20-fold dilution is 30 μ L of sample + 570 μ L of Diluent RD2-7.

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REAGENT PREPARATION

The Human Amyloid β (aa1-40) Conjugate must remain at 2-8 °C during use. Bring all other reagents to room temperature before use.

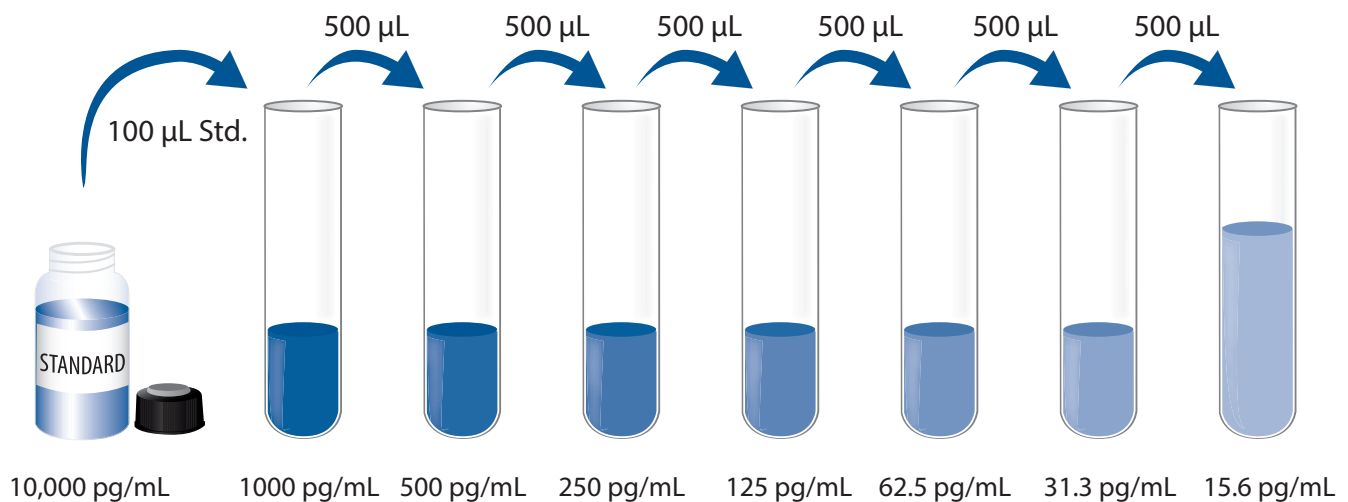
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Human Amyloid β (aa1-40) Standard - Refer to the vial label for reconstitution volume.

Reconstitute the Human Amyloid β (aa1-40) Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μ L of Diluent RD2-7 into the 1000 pg/mL tube. Pipette 500 μ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Diluent RD2-7 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

The Amyloid β (aa1-40) Conjugate must remain at 2-8 °C during use. Bring all other reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 200 μ L of Standard, control, or sample* per well. Cover with the adhesive strip provided. **Incubate for 2 hours at 2-8 °C.** A plate layout is provided to record the standards and samples assayed.
4. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 200 μ L of **cold** Human Amyloid β (aa1-40) Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at 2-8 °C.**
6. Repeat the aspiration/wash as in step 4.
7. Add 200 μ L of Substrate Solution to each well. Incubate for 30 minutes **at room temperature** on the benchtop. **Protect from light.**
8. Add 50 μ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Sample may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

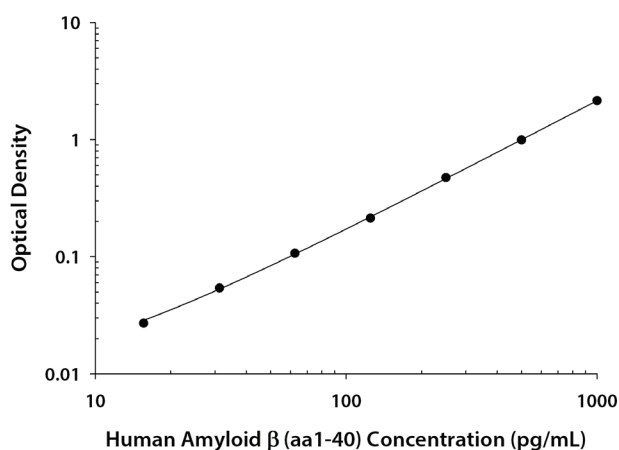
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Amyloid β (aa1-40) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.027 0.031	0.029	—
15.6	0.052 0.059	0.056	0.027
31.3	0.082 0.084	0.083	0.054
62.5	0.131 0.140	0.136	0.107
125	0.236 0.247	0.242	0.213
250	0.493 0.511	0.502	0.473
500	1.016 1.025	1.021	0.992
1000	2.166 2.194	2.180	2.151

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	58.9	264	556	56.3	247	518
Standard deviation	4.23	5.76	11.3	4.31	22.4	30.5
CV (%)	7.2	2.2	2.0	7.7	9.1	5.9

RECOVERY

The recovery of human Amyloid β (aa1-40) spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	95-105%
Cell Lysis Buffer (n=2)	99	91-110%
Cerebrospinal fluid* (n=4)	104	96-113%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human Amyloid β (aa1-40) were diluted with Diluent RD2-7 to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysis buffer (n=2)	Cerebrospinal fluid* (n=4)
1:2	Average % of Expected	98	99	102
	Range (%)	95-100	98-101	97-110
1:4	Average % of Expected	95	98	104
	Range (%)	93-98	93-104	99-114
1:8	Average % of Expected	96	101	105
	Range (%)	92-100	94-108	97-118
1:16	Average % of Expected	95	97	100
	Range (%)	87-101	88-106	85-110

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-six assays were evaluated and the minimum detectable dose (MDD) of human Amyloid β (aa1-40) ranged from 1.31-8.17 pg/mL. The mean MDD was 3.97 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against synthetic human Amyloid β (aa1-40).

SAMPLE VALUES

Note: All samples were handled on ice.

Cerebrospinal Fluid - Purchased samples were evaluated for the presence of human Amyloid β (aa1-40) in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Cerebrospinal fluid (n=60)	5875	95	ND-15,708

ND=Non-detectable

Cell culture supernates:

Peripheral blood leukocytes (1×10^6 cells) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were cultured unstimulated or stimulated with 10 μ g/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for human Amyloid β (aa1-40).

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	42.2	ND
Stimulated	39.6	31.3

ND=Non-detectable

IMR-32 human neuroblastoma cells were cultured in serum-free MEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human Amyloid β (aa1-40), and measured 102 pg/mL.

Tissue Lysates - Tissues were rinsed with PBS, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. An equal volume of RIPA buffer containing protease inhibitors was added and tissues were lysed on ice for 30 minutes with gentle agitation. Debris was then removed by centrifugation. Aliquots of the lysates were removed and assayed for human Amyloid β (aa1-40).

Tissue	pg/mL
Human hippocampus	363
Human inner cortex	513
Human motor cortex	49.9
Human outer cortex	3980

SPECIFICITY

This assay recognizes natural human Amyloid β (aa1-40).

The factors listed below were prepared in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors in a mid-range recombinant human Amyloid β control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Amyloid Precursor Protein

Apolipoprotein E2

Apolipoprotein E3

Apolipoprotein E4

Synthetic proteins:

human Amyloid β (aa4-40)

human Amyloid β (aa17-40)

human Amyloid β (aa22-40)

mouse/rat Amyloid β (aa1-40)

mouse/rat Amyloid β (aa1-42)

Synthetic human Amyloid β (aa2-40) cross-reacts approximately 11%.

Synthetic human Amyloid β (aa3-40) cross-reacts approximately 1.5%.

Synthetic human Amyloid β (aa1-42), (aa1-43), (aa1-44), and Amyloid β [Pyr3] (aa3-40) cross-react $\leq 0.2\%$.

Synthetic human Amyloid β [Pyr11] (aa11-40) does not cross-react in this assay but does interfere at concentrations > 20 ng/mL.

Synthetic human Amyloid β (aa11-40) does not cross-react but does interfere at concentrations > 12.5 ng/mL.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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5								
4								
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	A	B	C	D	E	F	G	H

NOTES