3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

SIGMA®

ProductInformation

b-Amyloid 1-40 ELISA, Human

hAβ40 ELISA

Catalog Number **BE0100**
Storage Temperature 2-8 °C

Product Description

β-Amyloid 1-40 ELISA, Human is used for *in vitro* quantitative determination of human β-Amyloid 1-40 (hAβ40) protein in cell culture supernatants, tissue homogenate, cerebrospinal fluid (CSF), and other biological samples. The assay recognizes both natural and synthetic forms of hAβ40. The Anti-Human Aβ40 used in this kit is capable of selectively detecting Aβ40 and not Aβ42/Aβ43.[1,2]

The β-Amyloid 1-40 detection assay is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for the NH_2 terminus of hAβ has been coated onto the wells of the microtiter strips provided. Standards of known hAß40 content, control specimens and unknown samples are pipetted into these wells and co-incubated with a rabbit antibody specific for the 1-40 sequence of human Aß. During the first incubation, hAß antigen binds to the immobilized (capture) antibody and the detection antibody binds to the bound hAß40 protein. Bound rabbit antibody is detected by the use of Anti-Rabbit IgG-Horseradish Peroxidase. After a second incubation and washing to remove all the excess Anti-Rabbit IgG-HRP, a substrate solution is added. It interacts with the bound enzyme and results in a color reaction. The intensity of this colored product is directly proportional to the concentration of hAß40 present in the original specimen.

This assay has been calibrated against the mass determination of highly purified native Aβ, where mass was corrected for peptide content by amino acid analysis. Researchers may also use β Amyloid 1-42 ELISA, Catalog Number BE0200, to detect hAβ42.

Alzheimer's Disease (AD) is characterized by the presence of extracellular plaques and intracellular neurofibrillary tangles (NFTs) in the brain.[1-5] The major protein component of these plaques is β amyloid peptide (Aβ), a 40 to 43 amino acid peptide cleaved from amyloid precursor protein by β-secretase (e.g., BACE) and a putative ?-secretase. Increased release of the 'longer forms' of Aβ peptide, Aβ42 or Aβ43, which have a greater tendency to aggregate than Aβ40, occurs in individuals expressing certain genetic mutations or certain ApoE alleles, or may involve other, still undiscovered, factors. Many researchers theorize that it is this increased release of Aβ42/Aβ43 that leads to the abnormal deposition of Aβ and to the associated neurotoxicity in the brains of affected individuals. In patients with AD, reduced levels of Aβ42 in CSF have been described as predictive of AD.[6]

Reagents

- **b-Amyloid 1-40 Standard, Human, 1 vial, Catalog Number A1478** – Lyophilized synthetic peptide. Refer to vial label for quantity and reconstitution volume, and to **Preparation Instructions.**
- **Standard/Sample Diluent**, **60 mL**, **Catalog Number S5443** - contains BSA and red dye. Ready to use.
- **Monoclonal Anti- Human Ab-coated 96 well plate, 1 plate, Catalog Number A1103** - A plate using break-apart strips coated with monoclonal antibody specific for NH_2 Terminus of hAβ.
- **Anti-Human b-Amyloid 1-40, 6 mL, Catalog Number A1228** – A detection antibody, produced in rabbit, which recognizes human Aβ 1-40. Contains sodium azide, BSA and blue dye. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate (100x), 1 vial, 0.125 mL, Catalog Number I5033** – contains 3.3 mM thymol and 50% glycerol, viscous. See **Preparation Instructions** for handling, dilution and storage instructions.

- **HRP Diluent, 25 mL, Catalog Number H8912** – contains 3.3 mM thymol, BSA and yellow dye. Ready to use.
- **Wash Buffer Concentrate 25x, 100 mL, Catalog Number W2639** - See **Preparation Instructions** for handling, dilution and storage instructions.
- **Stabilized Chromogen (TMB), 25 mL, Catalog Number S3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Catalog Number S2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Catalog Number P4870**

Reagents and Equipment required but not provided

- Standard Reconstitution Buffer - 55 mM $NaHCO_3$, ultrapure grade, Catalog Number S6297, pH 9.0.
- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µl and 1,000 µl.
- Reagents reservoirs for dispensing standards, antibodies and substrate.
- 4-(2-aminoethy)-benzenesulfonyl fluoride (AEBSF), Catalog Number A8456 or protease inhibitor cocktail containing AEBSF, Catalog Number . P8340.
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Disposable polypropylene 12 x 75 mm tubes for diluting reagents, standards and samples.
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer
- Shaking platform or mini-orbital shaker

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – cell culture media and other biological fluids.
- Plasma may be used only when the appropriate procedures are in place to solve the masking proteins issue (see reference 7).
- Samples should be separated from the cells and frozen if not analyzed shortly after collection.
- Dilute samples 1:2 to 1:10 in media or Standard/Sample Diluent.
- Sample matrix has a dramatic impact on Aβ recovery. To ensure accurate quantitation, the standard curves and blanks must be generated in the same diluent as the samples.
- Serine proteases can rapidly degrade Aβ peptides, thus using AEBSF protease inhibitor (water soluble and less toxic than PMSF) at a 1 mM final concentration stabilizes the Aβ. Add AEBSF to all samples before applying to the plate.
- Keep samples on ice until ready to apply to plate.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- When possible avoid use of hemolyzed or lipemic sera.
- Make samples dilutions in polypropylene tubes.
- Avoid use of sodium azide, thymol or thimerosal as sample preservatives.

Reagent Preparation

b-Amyloid 1-40 Standard

Note: Use polypropylene tubes for standard dilutions

1. Prepare Standard Reconstitution Buffer (not supplied). Dissolve 2.31 g of sodium bicarbonate in 500 mL of distilled or deionized water. Add 2N sodium hydroxide until pH is 9.0. Filter buffer through a 0.2 µm filter. The solution is sterile.
2. Reconstitute the hAβ40 Standard to 100 ng/mL with Standard Reconstitution Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow vial to sit for 5 minutes at room temperature.
3. Standard curve of the hAβ40 standards provided in the kit must be generated using the same composition of buffers used for the diluted experimental samples. For example, if brain extracts are diluted 1:10 with Standard/Sample Diluent, then the buffer used to dilute standards should be 90% Standard/Sample Diluent and 10% brain extraction buffer (including AEBSF at a final concentration of 1 mM).

5. Prepare serial standard dilutions as follows:

Tube #	Standard/ Sample Diluent or Sample matrix	Standard from tube #:	Final hAb40- pg/mL
A	Reconstitute Standard according to label instructions		100 ng/mL
B	0.9 mL	0.1 mL (A)	10,000 pg/mL
1	1.9 mL	0.1 mL (B)	500 pg/ mL
2	1 mL	1 mL (1)	250 pg/mL
3	1 mL	1 mL (2)	125 pg/mL
4	1 mL	1 mL (3)	62.5 pg/mL
5	1 mL	1 mL (4)	31.2 pg/mL
6	1 mL	1 mL (5)	15.63 pg/mL
7	1 mL	1 mL (6)	7.81 pg/mL
8	1 mL	0 mL	0 pg/mL

Mix thoroughly between steps.
6. Use within 1 hour of reconstitution.

Anti-Rabbit IgG Horseradish Peroxidase (HRP), 100x Concentrate
Contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:
1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix 10 µl IgG-HRP concentrate +1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed as in the Table below).
   Label as **Anti-Rabbit IgG-HRP Working Solution**
4. Return the unused Anti-Rabbit IgG-HRP concentrate to the refrigerator.

# of 8-well strips	Antibody (100X concentrate) mL	HRP Diluent mL
2	20 µL	2 mL
4	40 µL	4 mL
6	60 µL	6 mL
8	80 µL	8 mL
10	100 µL	10 mL
12	120 µL	12 mL

Wash Buffer
1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25x + 24 volumes of deionized water.
3. Label as **Working Wash Buffer**.
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability
All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for this kit for shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure
Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit.
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Use polypropylene tubes to dilute Standards and samples.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbance within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts in solution.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

hAb 1-40 Assay Summary

1. **Add 50 mL/well Standards and Samples**
   Add 50 mL/well Detection Antibody
   Incubate 3 hours at RT with shaking.
   Optional: Incubate overnight at 4°C without shaking
   Aspirate and wash 4x

2. **Add 100 mL of Anti-Rabbit IgG-HRP and incubate for 30 minutes at RT.**
   Aspirate and wash 4x

3. **Add 100 mL of stabilized Chromogen.**
   Incubate 30 minutes at RT *(in the dark)*.

4. **Add 100 mL of Stop Solution**
   Read at 450 nm.

Total Time 4 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of microtiter strips and return the unused strips to the pouch. Reseal pouch.
- Pre-wash plate 4 times with Working Wash Buffer, then pat dry on a paper towel. Follow Washing Directions.

1st incubation

a. Add 50 µL/well standards, samples or controls to the appropriate wells.
b. Add 50 µL/well Anti-Human Aβ40 Detection Antibody
c. Tap gently on the plate to mix, cover with Plate Cover and incubate 3 hours, while shaking on the platform at room temperature. Alternatively, plate may be incubated overnight at 2-8 °C without shaking.
d. Wash wells 4 times following washing directions.
e. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

2nd incubation

a. Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b. Cover with Plate Cover and incubate for 30 minutes at room temperature
c. Wash wells for a total of 4 times following washing instructions.
d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a. Add 100 µl of Stabilized Chromogen into all wells. The liquid in the wells will begin to turn blue.
b. Do not cover the plate.
c. Incubate approximately 30 minutes at room temperature in the dark - place plate in a drawer or cabinet

Note: If your microtiter reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.

Stop step

a. Add 100 µl of Stop Solution to each well. This stops the reaction.
b. Tap gently to mix. The solution will turn yellow.

Absorbance reading

a. Any commercially available microplate reader capable of reading at OD 450 nm may be used.
b. Blank the plate reader against the Chromogen Blank wells containing Chromogen and Stop Solution.
c. Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results
The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of hAβ40 may be calculated as follows:

1. Calculate the Average Net OD for each standard dilution and samples as follows:

   Average Net OD = Average Bound OD – Average Chromogen Blank OD

2. On graph paper plot the Average Net OD of the standard dilutions against the concentration (pg/mL) of hAβ40. Draw the best curve through these points to construct the standard curve.
3. The hAβ40 concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by dilution factor of each sample.
5. Samples producing signals higher than the 500 pg/mL standard should be further diluted and assayed.

Product Profile
Typical Results
The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard hAβ40 (pg/mL)	OD 450 nm
0	0.075
7.81	0.101
15.63	0.128
31.25	0.194
62.5	0.386
125	0.946
250	2.434
500	3.887

Limitations:
- Do not extrapolate the standard curve beyond the 500 pg/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated.
- The rate of degradation of native hAβ in various matrices has not been investigated.
- The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics
Specificity
Buffered solutions of a panel of substances of 50 ng/mL were assayed in the hAß40 kit. The following substances were tested and found to have no cross-reactivity: Aß [1-12], Aß [1-20], Aß [12-28], Aß [22-35], Aß [1-42], Aß [1-43], Aß [42-1], a-synuclein (90 ng/mL), APP (20 ng/mL), and Tau (10 ng/mL). Rodent ß Amyloid (1-40) showed approximately 0.5% cross reaction at 50 ng/mL.

Sensitivity
The minimum detectable dose of hAß40 is <6 pg/mL. This was determined by adding two standard deviations to the mean absorbance obtained when the zero standard was assayed 64 times.

Precision
1. Intra-Assay Precision
Samples of known hAβ40 concentrations were assayed in replicates of 14 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	305.7	129.7	49.5
Standard Deviation (SD	7.1	3.3	1.4.
% Coefficient of Variation	2.3	2.5	2.8

2. Inter-Assay Precision
Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	298.2	130.0	50.8
Standard Deviation (SD)	12.1	4.5	2.0
% Coefficient of Variation	4.1	3.5	3.9

Linearity of Dilution

Human CSF containing 195 pg/mL of measured hAß40 was serially diluted in Standard Diluent Buffer over the range of the assay. RPMI containing 10% fetal calf serum was spiked with the native hAß40 from CSF to a level of 212 pg/mL, then 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.

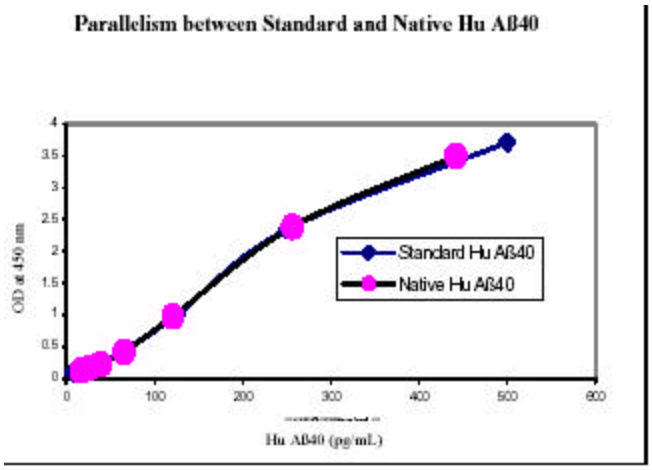
Cerebrospinal Fluid			
Dilution	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
Neat	195	195	-
1:2	81	98	82.7
1:4	43	49	87.7
1:8	24	24	100
Cell Culture Supernatant			
Neat	212	212	-
1:2	100	106	94.3
1:4	49	53	92.5
1:8	28	27	104

Sample Recovery

The recovery of native hAß40 added to human CSF averaged 87%. The recovery of native hAß40 added to tissue culture medium containing 10% fetal calf serum averaged 98%. The recovery of native hAß40 added to 25% tissue homogenate averaged 92%.

Parallelism

Native Aβ40 was spiked into Standard/Sample Diluent and measured against the standard used in this kit. Parallelism between the two peptides is demonstrated by the figure below:



References

1. Citron, M., et al., β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic Protease BACE. Science, **286**, 735-741 (1999).
2. Savage, M.J., et al. Turnover of β-amyloid protein in mouse brain and acute reduction of its level by phorbol ester. J. Neurosci., **18**, 1743-1752 (1998).
3. Bancher, C., et al., Mechanisms of cell death in Alzheimer's disease. J. Neural Transm. Suppl., **50**,141-152 (1997).
4. Lorenzo, A., et al., Amyloid β interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. Nat. Neurosci., **3**, 460-464 (2000).
5. Sambamurti, K., et al. Advances in the cellular and molecular biology of the β-amyloid protein in Alzheimer's disease. Neuromolecular Med., **1**, 1-31 (2002).
6. Andreasen, N., et al. Cerebrospinal fluid β-amyloid (1-42) in Alzheimer disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease. Arch. Neurol., **56**, 673-680 (1999).
7. Kuo, Y. M., et al., Amyloid β peptides interact with plasma proteins and erythrocytes: implications for their quantitation in plasma. Biochem. Biophys. Res. Communic., **268**, 750-756 (2000).

APPENDIX

ß-Amyloid Application:
Procedure for homogenization of human or transgenic mouse brains

For Tissue Homogenization, Prepare the Following Solutions:

1. 5 M guanidine HCl, 50 mM Tris HCl, pH 8.0

2. Reaction Buffer BSAT-DPBS (Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween®-20, see formulation below) supplemented with 1x Protease Inhibitor Cocktail containing AEBSF, aprotinin, E64, EDTA, and leupeptin).

BSAT-DPBS Formulation
0.2 g/L KCl
0.2 g/L KH_2PO_4
8.0 g/L NaCl
1.150 g/L Na_2HPO_4
5% BSA
0.03% Tween 20
q.s. to 1 L with ultrapure water and adjust the pH to 7.4.

Protocol:

1. Determine the wet mass of the mouse hemibrain (100 mg) or a human brain sample in an Eppendorf tube
2. Add 8x mass of cold 5 M guanidine HCl / 50 mM Tris HCl (Solution "A", above) to the tube by 50 - 100 µL aliquots and grind thoroughly with a hand-held motor after each addition.
   Optional: transfer the homogenate from above to a 1 mL Dounce homogenizer and homogenize thoroughly.
3. Mix the homogenate at room temperature for 3 - 4 hours. The sample is stable and can be freeze-thawed many times at this stage.
4. Dilute the sample with cold Reaction Buffer (Solution "B", above). Centrifuge (microfuge or Sorvall) at 16,000 x g for 20 minutes at 4°C. This dilution factor requires adjustment depending on the quantity of Aß present and on inhibition of the standard curve development due to the presence of guanidine. Initial experiments indicate a dilution factor of 1:200 for human brain and 1:20 to 1:50 for transgenic mouse brains. The optimal dilution factor should be determined for each specific experimental determination. (Note: we have determined that the standard curve can withstand the presence of 0.1 M or less guanidine solution. Inclusion of guanidine at a concentration higher than 0.1 M will result in significant depression of the standard curve.)
5. Carefully decant the supernatant and store on ice until use with the ßA40 ELISA kit.

Alternative Procedure:
Homogenization can be performed with cold 4x volume of PBS supplemented with the 1x protease inhibitor cocktail, followed by the addition of a solution 8.2 M guanidine / 82 mM Tris HCl (pH 8.0) to yield a solution with 5 M final guanidine concentration.

References for Homogenization Procedure:

1. Masliah, E., et al., ß-amyloid peptides enhance á-synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease and Parkinson's disease. Proc. Nat'l. Acad. Sci. **98**, 12245-12250 (2001).
2. Johnson-Wood, K., et al., Amyloid precursor protein processing and Aβ42 deposition in a transgenic mouse model of Alzheimer disease. Proc. Nat'l. Acad. Sci. **94**, 550-1555 (1997).
3. Chishti, M.A., et al., Early-onset amyloid deposition and cognitive defects in transgenic mice expressing a double mutant form of amyloid precursor protein 695. J. Biol. Chem., **276**, 21562-21570 (2001).

AH,PHC 07-05-1