User Guide

High Sensitivity Human Amyloid β40

96-Well Plate

EZHS40

Intended Use

Amyloid beta peptides have been implicated in the etiology of Alzheimer's disease. Amyloid beta 40 is the most prominent peptide and Amyloid beta 42 is the neurotoxic form. The Amyloid beta 42/40-ratio (AB ratio) has been reported as a better indicator of the Alzheimer pathology. High Sensitivity Human Amyloid β40 ELISA kit is used for the measurement of Amyloid beta 40 in cerebrospinal fluid, cell culture supernatants, primary neurons and plasma in a 96-well format.

For research use only. Not for use in diagnostic Procedures.

Principles of Procedure

With this Amyloid β test-kit, the principle of a solid phase enzyme immunoassay, an Enzyme-Linked-Immunosorbent-Assay (ELISA), for the quantitative analysis of hAβ40 is applied. The antigen hAβ40 to be tested, is detected by selective monoclonal anti-Aβ-antibodies at two different binding sites (Epitopes), forming a "Sandwich-Complex". The polystyrene surface of the microtiter plate is layered with an antibody (capture antibody) which selectively recognizes the C-terminal end of the antigen. During the test procedure, a selective anti-Aβ-antibody conjugate (detection antibody) is incubated together with the standard/sample and forms an antibody-Amyloid-antibody-complex. This complex is indirectly linked with a Biotin-Streptavidin bridge to an enzyme in the next step. In a follow-up reaction the enzyme catalyzes the conversion of a substrate (Chromogen) into a colored product, and the color intensity is measured by a spectrophotometer. The Amyloid β40 concentration in unknown samples are calculated from the standard curve.

Characteristics of the Aß40 ELISA

- Highly sensitive, selective quantitative analysis of human Amyloid β40
- Test range from 16 to 500 pg/mL
- High reproducibility and accurate linearity of the standard curve
- Precoated strips (12x8) for flexible usage of samples according to individual customer requirements
- Low sample volumes (50 µL or less)

Reagents Supplied

Each kit is sufficient to run one 96-well plate and contains the following reagents:
Note: Store all reagents at 2-8 °C.

Reagents Supplied	Volume	Quantity	Cat. No.
Human Amyloid β 1-40 ELISA Plate with 2 plate sealers **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C	-	1 plate 2 sealers	3TS
Synthetic Amyloid β1-40 Standard	250 µL/vial upon hydration (1000 ng/mL Lyophilized)	1 bottle	0STM
Amyloid β1-40 Quality Controls 1 and 2	250 µL/vial	2 vials	0QC
Standard & Sample Diluent	25 mL	1 bottle	SD
Antibody Conjugate (100X)	100 µL	1 bottle	HSAC
Antibody Conjugate Diluent	8 mL	1 bottle	HSAD
Enzyme Conjugate	150 µL	1 bottle	0EC
Enzyme Conjugate Diluent	13 mL	1 bottle	0ED
Washing Solution (20X)	25 mL	2 bottles	WS
Stop Solution	13 mL	1 bottle	ET-TMB
Substrate Solution 0.3 M HCl (**Caution:** Corrosive Solution)	12 mL	1 bottle	ES

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Technical Guidelines

- The hAmyloid β40-ELISA (HS) is for *in vitro* use only.
- Carefully read and follow the test-instructions in this user guide included in every test-kit. Test performance and data calculation should always be done by qualified staff.
- Do not mix reagents from different test-kits.
- Some of the test components are concentrated solutions. After dilution the working solution should be used within 14 days (2 to 8 °C). Standard dilutions must be diluted always just before the test starts.
- To calibrate the test-system (standard), the dilutions should be made according to the description in the test procedure. The resulting internal standard curve is a fixed component of each measurement. A transfer of the absorbance data from one test plate to another is not suitable.
- To avoid a cross contamination and carryover of reagents, the use of clean pipet tips for each sample pipetting is necessary.
- The pipetting of reagents and samples starts / stops kinetic reactions. To obtain a high precision for the test, be sure to treat each well of the microtiter plate in an identical manner.
- The washing solution must be tapped out of the wells after the last washing step to assure the removal of buffer residues from the wells completely.

Reagent Precautions

Sodium Azide

Sodium azide or ProClin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and ProClin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

Note: See Full Labels of Hazardous Components on next page.

Ingredient	Cat. No.	Full Label	
Stop Solution	ET-TMB		**Danger.** May be corrosive to metals. Causes severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF exposed or concerned: immediately call a POISON CENTER or doctor/ physician.
Antibody Conjugate	HSAC		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Enzyme Conjugate Diluent	0ED		**Warning.** May cause an allergic skin reaction. Avoid breathing dust/fume/gas/ mist/vapours/spray. Contaminated work clothing should not be allowed out of the workplace. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. If skin irritation or rash occurs: Get medical advice/ attention. Wash contaminated clothing before reuse.
Antibody Conjugate Diluent	HSAD		**Warning.** May cause an allergic skin reaction. Avoid breathing dust/fume/gas/ mist/vapours/spray. Contaminated work clothing should not be allowed out of the workplace. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. If skin irritation or rash occurs: Get medical advice/ attention. Wash contaminated clothing before reuse.
Standard and Sample Diluent	SD		**Warning.** May cause an allergic skin reaction. Avoid breathing dust/fume/gas/ mist/vapours/spray. Contaminated work clothing should not be allowed out of the workplace. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. If skin irritation or rash occurs: Get medical advice/ attention. Wash contaminated clothing before reuse.

Materials Required

(Not Provided)

- Multi-channel Pipettes and pipette tips: 10 µL-1000 µL
- Timer
- Buffer and Reagent Reservoirs
- Vortex Mixer
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Microtiter Plater Washer
- Orbital Microtiter Plate Shaker
- Ice and ice container for sample preparation

Note: We recommend the use of multi-channel pipettes and automated plate-washers to achieve parallel working steps and simultaneous incubation times for the best reproducibility.

Sample Collection and Storage

- We recommend diluting the working solutions only for the intended use. The test plate is subdivided in strips of 8 wells for flexible sample handling. The stability of Amyloid ß is critical, because the peptides tend to aggregate in samples. For this reason, the preanalytic sample preparation is a major influencing parameter within the analysis of Amyloid peptides. Samples should be collected according to clinical approved standard procedures and immediately stored at −20 °C. During the handling of thawed samples, it is important to keep these chilled (for example working on ice). For the preparation of the samples polypropylene vials are recommended to avoid interaction with sample materials during storage. Avoid repeated thawing and freezing of samples and standards. For research use only. Plasma samples do not require dilution for the assay. CSF samples should be diluted 1:20 using Standard & Sample Diluent (#SD). Cell culture supernatants should be diluted 1:5 – 1:10 using Standard and Sample Diluent (#SD).

Reagent Preparation

Preparation of Human Amyloid β1-40 Standard (Day 1)

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Amyloid β 1-40 Standard with 0.25 mL distilled or deionized water to give a concentration of 1000 ng/mL. Invert and mix gently, let sit for 5 minutes then vortex gently.

2. Label eight tubes as Stock (S), 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 16 pg/mL, and Blank. Using the chart below, add appropriate volumes of Standard and Sample Diluent to each of the eight tubes. Prepare dilutions according to the chart below and mix well.

   Note: Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of reconstituted standard should be stored at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration
0.25 mL	0	1000 ng/mL

Standard	Concentration of Amyloid β1-40	Volume of Standard and Sample Diluent to Add	Volume of Standard to Add
Stock (S)	25,000 pg/mL	780 µL	20 µL of reconstituted std
Std 1	500 pg/mL	1470 µL	30 µL of Stock (S)
Std 2	250 pg/mL	150 µL	150 µL Std 1
Std 3	125 pg/mL	150 µL	150 µL Std 2
Std 4	62.5 pg/mL	150 µL	150 µL Std 3
Std 5	31.25 pg/mL	150 µL	150 µL Std 4
Std 6	16 pg/mL	150 µL	150 µL Std 5
Blank	0 pg/mL	150 µL	0

Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Amyloid β1-40 Quality Control 1 and Quality Control 2 with 0.25 mL distilled or de-ionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at −20 °C. Avoid further freeze/thaw cycles.

Preparation of Antibody Conjugate Solution (Day 1)

Dilute Antibody Conjugate (100x) 1:100 with Antibody Conjugate Diluent.

Example: 60 µL Antibody Conjugate (100x) + 5940 µL Antibody Conjugate Diluent = 6000 µL

Preparation of Washing Solution (Day 2)

Dilute Washing Solution (20x) 1:20 with deionized water

Example: 50 mL Washing Solution (20x) + 950 mL deionized water = 1000 mL

Preparation of Enzyme Conjugate Solution (Day 2)

Dilute Enzyme Conjugate (100x) 1:100 with Enzyme Conjugate Diluent

Example: 110 µL Enzyme Conjugate (100x) + 10890 µL Enzyme Conjugate Diluent = 11000 µL

Assay Procedure

Day 1

- The following kit components are required for day 1:
    - 8 well test strips
    - Standard & Sample Diluent
    - Synthetic Aß1-40 Standard
    - Aß 1-40 Quality Controls 1,2
    - Antibody Conjugate Diluent
    - Antibody Conjugate (100x)
- We recommend diluting the test reagents just before each application. The samples shall be chilled (at < 4 °C, working on ice) during the complete test procedure to achieve high stability and optimal data results.
- All standards, quality controls or samples should be mixed gently just before pipetting. Accurate mixing and pipetting of the standard solutions are essential to the precision of the assay.

1. Add 50 µL Antibody Conjugate Solution into all wells.

3. Add 50 µL of Standard and Sample Diluent to the background (0 pg/mL) wells.

4. Add in duplicate 50 µL standard and quality controls in order of ascending concentration to the appropriate wells. Add sequentially 50 µL of samples in duplicate to the remaining wells. **For best results all additions should be completed within 30 minutes**.

5. Cover the plate with a plate sealer and thoroughly mix the contents of the wells for a period of 5 minutes on an orbital plate shaker (500-600 rpm/min). Incubate without shaking overnight (16-20 hours) at 2 to 8 °C.

   Note: The test reagents needed on the following day can be taken out of the refrigerator to allow them to reach room temperature overnight.

Day 2

- The following kit components are required for day 2:
    - Washing Solution (20x)
    - Enzyme Conjugate (100x)
    - Enzyme Conjugate Diluent
    - Substrate Solution
    - Stop Solution
- **Caution:** All reagents must be at room temperature before use.

1. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

6. Wash test plate 5 times with 300 µL Washing Solution per well, remove the remaining fluid by tapping the plate on an absorbing paper.

7. Add 100 µL Enzyme Conjugate Solution to each well. Cover the plate with a plate sealer and incubate for 30 minutes at room temperature on an orbital shaker (800 rpm/min).

8. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

9. Wash test plate 5 times with 300 µL Washing Solution per well, remove the remaining fluid by tapping the plate on an absorbing paper.

10. Add 100 µL Substrate Solution to each well. Cover plate with sealer and shake on the plate shaker for 5-30 minutes (A longer development time may be needed if using a plate washer). Blue color should be formed in wells of standards with intensity proportional to increasing concentrations of Amyloid β1-40.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

11. Remove sealer and add 100 µL Stop Solution (**Caution:** Corrosive solution) and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference in absorbance units. The absorbance of the highest Amyloid β1-40 standard should be approximately 2.0–3.2, or not to exceed the capability of the plate reader used.

Assay Procedure I for Human Amyloid β40 ELISA Kit

	Day 1				Day 2						
	Step 1	**Step 2**	**Step 3**	**Step 4**	**Step 1-2**	**Step 3**	**Step 4-5**	**Step 6**		**Step 7**	
Well #	Ab. Conj. Solution	Standard & Sample Diluent	Standards/ QCs & Samples	Seal, agitate for 5 minutes, then incubate overnight at 2 to 8 °C.	Wash test plate 5 times with 300 µL Washing Solution per well, remove the remaining fluid by tapping the plate on an absorbing paper.	Enzyme Conj Solution	Seal, agitate, and incubate at room temperature for 30 minutes. Remove residual buffer by tapping smartly on absorbent towels. Wash 5X with 300 µL Wash Buffer Remove the remaining fluid by tapping the plate on an absorbing paper.	Substrate	Seal, agitate and incubate 5 to 30 min at room temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1	50 µL	50 µL	-			100 µL		100 µL		100 µL	
C1, D1	50 µL	-	50 µL of 16 pg/mL Standard								
E1, F1	50 µL	-	50 µL of 31.25 pg/mL Standard								
G1, H1	50 µL	-	50 µL of 62.5 pg/mL Standard								
A2, B2	50 µL	-	50 µL of 125 pg/mL Standard								
C2, D2	50 µL	-	50 µL of 250 pg/mL Standard								
E2, F2	50 µL	-	50 µL of 500 pg/mL Standard								
G2, H2	50 µL	-	50 µL of Quality Control 1								
A3, B3	50 µL	-	50 µL of Quality Control 2								
C3, D3	50 µL	-	50 µL of Sample								
E3, F3	50 µL	-	50 µL of Sample								
G3, H3.	50 µL	-	50 µL of Sample								

Microtiter Plate Arrangement

Human Amyloid β1-40 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	(Blank) 0 pg/mL	125 pg/mL	QC 2	Etc.								
B	(Blank) 0 pg/mL	125 pg/mL	QC 2									
C	16 pg/mL	250 pg/mL	Sample 1									
D	16 pg/mL	250 pg/mL	Sample 1									
E	31.25 pg/mL	500 pg/mL	Sample 2									
F	31.25 pg/mL	500 pg/mL	Sample 2									
G	62.5 pg/mL	QC 1	Sample 3									
H	62.5 pg/mL	QC 1	Sample 3									

Interpretation

Analysis of the measured absorbance data (mean, standard deviation) for the standards and for the samples is performed with the help of a microtiter plate reader software.

The blank (zero standards) is not integrated into the calculation of the standard curve. The blank is taken only as a control for a non-specific binding of the antibody; the mean absorbance of the blank shall be below 0.2.

Construct a standard curve by plotting the mean absorbance of standard 1-6 on the vertical axis versus the corresponding Aβ1-40 concentration on the horizontal axis. The data can be calculated by linear fit (linear regression) or by a point-to-point fit (cubic spline). The test results are not valid if the standard 1 (500 pg/mL) shows an absorbance below 0.6 in magnitude. Please control your test handling (For more information, see Troubleshooting Guide).

The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QCs fall outside of the control range, review results with a supervisor.

The resulting Aβ40-concentrations of the samples can be calculated with this standard curve. Only samples that are in the measured range of the standard curve can be calculated.

If the Aβ-concentration value of a sample exceeds 500 pg/mL, the test sample must be measured again by using a higher sample dilution (with appropriate amount of Standard & Sample Diluent).

Assay Characteristics

Sensitivity

The lowest level of Amyloid β1-40 standard used in this assay is 4.0 pg/mL (50 µL sample size).

Specificity

The Amyloid β40 ELISA (HS) uses monoclonal anti-Aβ antibodies with high selectivity for human Aβ. The capture antibody recognizes the C-terminal end of Amyloid β1-40, which causes a high selectivity for Aβ40. The cross-reactivity of the used antibodies to other Amyloid peptides was tested by ELISA and BIACORE and shows no significant cross-reactivity to Aβ1-38, Aβ1-39, Aβ1-42, Aβ1-43 and Aβ1-44.

Precision

Analyte	Intra-Assay (% CV)	Inter-Assay (% CV)
Amyloid β 1-40	< 10%	< 10%

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

Problem	Cause	Recommended Solution
No signal	Wrong test reagents used	Ensure that only the reagents for the specific test-lot are used.
	Test reagents damaged	Don't use the test-kit after expiration date.
Weak signal	Test reagents used in a wrong dilution	Control used test dilutions carefully (usually a dilution factor of 100 is used).
	Wrong filter (wavelength)	Check your wavelength in your microtiter plate photometer.
	Incubation time too short / temperature too low	Check the information of incubation times of the lot in the product data sheet. (The incubation time of the enzyme substrate is applied for temperatures from 20 to 28 °C); extend the substrate incubation time, if absorption is below 1.0.
	Reagents not at right temperature	Make sure that the reagents used for day 2 have reached room temperature (20 to 28 °C) before using within the test-kit.

• Problem	• Cause	• Recommended Solution
	• Sodium azide, mercaptoethanol or DTT can interfere with peroxidase activity at high concentrations	• Only use samples which contain no or low contents (< 0.1%) of sodium azide, mercaptoethanol or DTT.
• High signal	• Test reagents used in a wrong dilution	• Check used test dilutions carefully (usually a dilution factor of 100 is used).
	• Incubation time too long / temperature too high	• Check the information of incubation times of the lot in the product data sheet. (The incubation time of the enzyme substrate is applied for temperatures from 20 to 28 °C); shorten the substrate incubation time, if absorption is above 3.0.
• High background (blank)	• Insufficient washing steps	• Wash plate carefully and remove the liquid after each washing carefully.
	• Contamination of the washing solution	• Confirm that the water is not contaminated. Use always double distilled water for the reconstitution and dilution of the washing solution.

• Problem	• Cause	• Recommended Solution
	• Contamination of reagents or vials/tubes from previous experiments	• Avoid pipetting directly out of the reagent vials, if test reagents should be used in further measurements. (Oxidative active contaminants can influence the enzyme substrate by non-specific color development).
	• Test reagents (antibody- and enzyme conjugate) used in wrong dilutions	• Check used test dilutions for antibody- and enzyme conjugate carefully (usually a dilution factor of 100 is used).

• Low precision (= random error)	• Non-homogeneous samples for example cloudy solution, particles in the sample	• Check that the samples are taken, prepared and stored according to a recommended sample procedure (polypropylene tubes, storage of clear samples at −20 °C).
	• Insufficient mixing of samples and standards	• Mix samples and standards before pipetting carefully.
	• Variation in pipetting	• Check your pipettes and calibrate if necessary.
	• Carry over between samples and/or standards	• Change pipet tips after each pipetting.
	• Insufficient mixing of reagents during incubation	• Mix reagents on the test plate after pipetting by moving the test plate carefully; use an orbital microtiter plate shaker on the recommended test steps for optimal mixing of reagents.
	• Insufficient washing	• Check that the automatic microtiter plate washer is working correctly; residues of liquids must be removed

		completely after each washing step.
	• Evaporation of liquids	• Check the contact of the cover seal with the plate during the incubation steps.
• Calculated data are too high or too low (=systematic error, deviation of data from „typical data")	• Calculation of the dilution factor is not correct	• Check the dilution factor used for the sample dilution within the data calculation.
	• Modification of the test procedure	• Follow the instructions in the product data sheet carefully (incubation time, dilution etc.).
	• Incorrect sample treatment	• Check that the samples are taken, prepared and stored according to a recommended sample procedure (polypropylene tubes, storage of clear samples at −20 °C).

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Human Amyloid β 1-40 ELISA Plate	3TS
Synthetic Aβ 1-40 Standard	0STM
Amyloid β 1-40 Quality Control 1,2	0QC
Standard & Sample Diluent	SD
Antibody Conjugate	HSAC
Antibody Conjugate Diluent	HSAD
Enzyme Conjugate	0EC
Enzyme Conjugate Diluent	0ED
Washing Solution	WS
Substrate Solution	ES
Stop Solution	ET-TMB

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