DRG® Human β Amyloid (hAβ40) ELISA (COL-3994)

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1 INTENDED USE

The β Amyloid 1-40 (human) (Hu Aβ -40) ELISA is to be used for determination of human Aβ -40 in samples (e.g., tissue culture medium, tissue homogenate, cerebrospinal fluid (CSF), etc.).

The assay will recognize both natural and synthetic forms of Hu Aβ -40. The anti-human Aβ -40 antibody used in this kit is capable of selectively detecting Aβ -40 and not Aβ 42/Aβ 43 (4-6).

This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.

2 PRINCIPLE OF THE METHOD

The Human Aβ -40 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA).

A monoclonal antibody specific for the NH_2-terminus of Hu Aβ has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu Aβ content, control specimens and unknowns, are pipetted into these wells, and co-incubated with a rabbit antibody specific for the COOH-terminus of the 1-40 Aβ sequence. This COOH-terminal sequence is created upon cleavage of the analyzed precursor. Bound rabbit antibody is detected by the use of a horseradish peroxidase-labeled anti-rabbit antibody. After removal of excess anti-rabbit antibody, 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 Aβ -40 present in the original specimen.

3 REAGENTS PROVIDED

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

Reagent	96 Test Kit
Hu Aβ 40 Standard. Lyophilized synthetic peptide. Refer to vial label for quantity and reconstitution volume.	1 vial
Standard Diluent Buffer. Contains 15 mM sodium azide; red dye*; 60 mL per bottle.	1 bottle
Antibody-Coated Wells, 96 wells per plate. Plate pre-coated with mAb to NH_2 terminus of Aβ.	1 plate
Detection Antibody. Rabbit anti-Hu Aβ 40. Contains 15 mM sodium azide; blue dye*; 6 mL per bottle.	1 bottle
Anti-rabbit Ig's-Peroxidase (HRP), (100x Concentrate). Contains 3.3 mM thymol; 125 µ L per vial.	1 vial
HRP Diluent. Contains 3.3 mM thymol; yellow dye*; 25 mL per bottle.	1 bottle
Stabilized Chromogen. Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	2
* In order to help our customers avoid any mistakes in pipetting the reagents, we provide colored Standard Diluent Buffer, Detection Antibody, and HRP Diluent to help monitor the addition of solution to the reaction well. This does not in any way interfere with the test results.	

Disposal Note:
This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

4 SUPPLIES REQUIRED BUT NOT PROVIDED

1. Standard Reconstitution Buffer [55 mM Sodium Bicarbonate Buffer ($NaHCO_3$, ultrapure grade), pH 9.0].
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips (a manifold multi-channel pipette is desirable for large assays), beakers and graduated cylinders.
3. Reagent reservoirs for dispensing standards, antibody solutions and substrate.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).

5. Distilled or deionized water.
6. Microtiter plate reader capable of measurement at or near 450 nm.
7. Shaking platform (for low to moderate shaking) with plate and tube vortex adapter, or a mini-orbital shaker.
8. Disposable 12 x 75 mm polypropylene tubes for diluting standards and samples.
9. 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or protease inhibitor cocktail containing AEBSF.
10. Absorbent paper towels.
11. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.

5 PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be stored at 2 to 8°C. 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 polypropylene tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely, microfuge and mix well with diluent prior to analysis.
5. When analyzing samples, add a protease inhibitor cocktail with AEBSF (a serine protease inhibitor) and prepare the standard dilutions using the same diluent as used with the biological samples. Serine proteases can rapidly degrade Aβ peptides, thus using AEBSF (water soluble and less toxic than PMSF) at a 1 mM final concentration is very helpful. Keep samples on ice until ready to apply to plate.
6. When possible, avoid use of badly hemolyzed, lipemic or contaminated samples or homogenates. If large amounts of particulate matter are present, centrifuge prior to analysis.
7. Standards can be dispensed into a reservoir and transferred to the plate using a multi-channel pipette. Be sure to aspirate all previous standard solution prior to adding the next standard (in ascending order) to the reservoir.
8. It is recommended that all standards and samples be run in duplicate. Prepare standards and samples in 5 mL polypropylene tubes. We recommend diluting samples 1:2 to 1:10 for Aβ40.
9. Sample matrix has a dramatic impact on Aβ recovery. To ensure accurate quantitation, the standard curves must be generated in the same diluent as the samples.
10. Avoid use of thymol or thimerosal as sample preservatives. These agents inhibit measurement of Aβ peptide.
11. Samples that are > 500 pg/mL should be diluted with Standard Diluent Buffer.
12. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
13. Do not mix or interchange different reagent lots from various kit lots.
14. Do not use reagents after the kit expiration date.
15. Read absorbances within 2 hours of assay completion.
16. We recommend covering plate with adhesive plate covers during incubation steps.
17. 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.
18. Because Stabilized Chromogen is light sensitive, avoid prolonged exposure to light. Also avoid contact between Stabilized Chromogen and metal, or color may develop.

6 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.

7 DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with the Wash Buffer 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 touch 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.

8 REAGENT PREPARATION AND STORAGE

This assay has been calibrated against the mass determination of highly purified native Hu Aβ40 where mass was corrected for peptide content by amino acid analysis.

A. Preparation of Standard Reconstitution Buffer

Dissolve 2.31 grams of sodium bicarbonate in 500 mL of deionized water. Add 2 N sodium hydroxide until pH is 9.0. Filter solution through a 0.2 µm filter unit.

B. Reconstitution and Dilution of Hu Aβ -40 Standard

Note: Polypropylene tubes may be used for standard dilutions.

1. Remove the Hu Aβ -40 Standard vial from storage and let equilibrate to room temperature (RT).
   Reconstitute the Hu Aβ -40 Standard to 100 ng/mL with Standard Reconstitution Buffer (55 mM sodium bicarbonate, pH 9.0). Refer to the standard vial label for instructions.
   Swirl or mix gently and allow vial to sit for 5 minutes at room temperature. Briefly vortex prior to preparing standards.
2. Generation of the standard curve using the Aβ peptide standards provided in the kit must be performed using the same composition of buffers used for the diluted experimental samples.
   For example, if brain extracts are diluted 1:10 with Standard Diluent Buffer, then the buffer used to dilute standards should be 90% Standard Diluent Buffer and 10% brain extraction buffer (including AEBSF at a final concentration of 1 mM).

3. Add 0.1 mL of the reconstituted standard to a tube containing 0.9 mL Standard Diluent Buffer or as otherwise dictated by the treatment of experimental samples.
   Label as 10,000 pg/mL Hu Aβ -40. Mix.
4. Add 0.1 mL of 10,000 pg/mL standard to a tube containing 1.9 mL of Standard Diluent Buffer.
   Label as 500 pg/mL.
5. Add 1.0 mL of Standard Diluent Buffer to each of 6 tubes labeled 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 pg/mL Hu Aβ -40.
6. Make serial dilutions of the standard as described in the dilution table below. Mix thoroughly between steps.

Standard:	Add:	Into:
500 pg/mL	Prepare by adding 0.1 mL of 10,000 pg/mL standard to 1.9 mL of Diluent Buffer.	
250 pg/mL	1.0 mL of the 500 pg/mL std.	1.0 mL of the Diluent Buffer
125 pg/mL	1.0 mL of the 250 pg/mL std.	1.0 mL of the Diluent Buffer
62.5 pg/mL	1.0 mL of the 125 pg/mL std.	1.0 mL of the Diluent Buffer
31.25 pg/mL	1.0 mL of the 62.5 pg/mL std.	1.0 mL of the Diluent Buffer
15.63 pg/mL	1.0 mL of the 31.25 pg/mL std.	1.0 mL of the Diluent Buffer
7.81 pg/mL	1.0 mL of the 15.63 pg/mL std.	1.0 mL of the Diluent Buffer
0 pg/mL	1.0 mL of the Diluent Buffer	An empty tube

Remaining reconstituted Hu Aβ -40 standard may be stored in aliquots at -80°C for up to 4 months. Standard can be frozen and thawed one time only without loss of immunoreactivity.

C. Preparation of Samples

Prepare one or more dilutions of each sample. These dilutions should be made in Standard Diluent Buffer, although the exact dilution must be determined empirically (e.g., 1:2 and 1:10 represent a reasonable range). This dilution must be performed because certain components in samples can interfere with the detection of the Aβ peptides or to bring the levels of Aβ within the range of this assay. AEBSF should be added to the diluted samples and the standards at a final concentration of 1 mM in order to prevent proteolysis of the Aβ peptides.

Refer to "Appendix" for procedure for homogenization of human or transgenic mouse brains.

Please note: Analysis of plasma samples may require pretreatment to disrupt interaction of Aß with masking proteins.

D. Preparation of Wash Buffer

Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have dissolved. Dilute 1 volume of the 25x Wash Buffer Concentrate with 24 volumes of deionized water (e.g., 100 mL may be diluted up to 2.50 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer at 2 to 8°C.

The diluted buffer should be used within 14 days.

E. Preparation of Secondary Antibody Solution

Please Note: The Anti-rabbit Ig's-HRP 100x Concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow Anti-rabbit Ig's-HRP Concentrate to reach room temperature. Gently mix. Pipette Anti-rabbit Ig's-HRP Concentrate 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 HRP Diluent for each 8-well strip used in the assay. Label as Anti-rabbit Ig's-HRP Working Solution. For Example:

# of 8-Well Strips	Volume of Anti-rabbit Ig´s HRP 100x Concentrate	Volume of HRP 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. Store the unused Anti-rabbit Ig's-HRP Concentrate at 2 to 8°C.

9 ASSAY METHOD: PROCEDURE AND CALCULATIONS

Note: The protocol described below has been developed to provide sensitive and reproducible detection of Aβ peptides. Although the assay uses a standard double antibody ELISA approach combined with colorimetric detection, the inherent complexity in handling/detecting Aβ peptides must be recognized.

1. Reconstitute Hu Aβ -40 Standard. *See Reconstitution and Dilution of Hu Aβ -40 Standard.*
2. Prepare samples and standards with appropriate diluents.
3. Add **50 µL/well** of Aβ peptide *standards, controls, and dilutions of samples* to be analyzed.
4. Pour Detection Antibody solution into reservoir; immediately add **50 µL/well** of *Detection Antibody solution* to the plate.
5. **Incubate at room temperature (RT) for 3 hours while shaking.**
6. Wash plate 4x with Working Wash Buffer, then pat plate dry on a paper towel. See DIRECTIONS FOR WASHING.
7. Pour Anti-rabbit Ig's-HRP solution into reservoir; immediately add **100 µL/well** of *Anti-rabbit Ig's-HRP Working Solution* to the plate. Incubate plate at RT for 30 minutes.
8. Wash plate 4x with Working Wash Buffer, pat dry on a paper towel. See DIRECTIONS FOR WASHING.
9. Add **100 µL** of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.

10. 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 15 to 20 minutes is suggested.
11. 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.
12. 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.
13. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, 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.
14. Read the Hu Aß 1-40 concentrations for unknown samples and controls from the standard curve plotted in step 13. **Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution in Section C.** (Samples producing signals greater than that of the highest standard (500 pg/mL) should be further diluted in Standard Diluent Buffer and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

10 TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 500 pg/mL Hu Aβ -40.

Standard Hu Aβ 40 (pg/mL)	Optical Density (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

11 LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 500 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >500 pg/mL with Standard Diluent Buffer; reanalyze these and multiply results by the appropriate dilution factor.

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 Hu Aß 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.

This kit is for research use only.

Not for human therapeutic or diagnostic use.

12 APPENDIX

β -Amyloid Application:

Procedure for homogenization of human or transgenic mouse brains

For Tissue Homogenization, Prepare the Following Solutions:

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

B. 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 (e.g., Calbiochem catalog code 539131; contains 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 (Fisher K749520-0000).
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 (Fisher K749540-0000) 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 β Amyloid 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:

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13 REFERENCES

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