ELISA Kit
Catalog # KMB3481

Mouse
Aβ40

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PURPOSE

The Invitrogen Mouse β Amyloid 1-40 (Ms Aβ40) ELISA is to be used for the quantitative determination of mouse and rat Aβ40 in samples (e.g., tissue culture supernatant, tissue homogenate, cerebrospinal fluid [CSF]). The assay will recognize both natural and synthetic forms of mouse and rat Aβ40. The anti-mouse Aβ40 antibody used in this kit is capable of selectively detecting Aβ40 and not Aβ42/Aβ43.

INTRODUCTION

Alzheimer's Disease (AD) is characterized by the presence of extracellular plaques and intracellular neurofibrillary tangles (NFTs) in the brain. The major protein component of these plaques is beta amyloid peptide (Aβ), a 40 to 43 amino acid peptide cleaved from amyloid precursor protein by β-secretase (e.g., BACE) and a putative γ (gamma) 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, expressing certain ApoE alleles, or may involve other, still undiscovered, factors. Many researchers theorize that it is this increased release of Aβ42/Aβ43 which leads to the abnormal deposition of Aβ and the associated neurotoxicity in the brains of affected individuals.

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen Ms Aβ40 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody to the NH_2-terminus of Ms Aβ has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Ms Aβ40 content, control specimens and unknowns, are pipetted into these wells. The Aβ antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for the COOH-terminus of the 1-40 Aβ sequence is added to the wells. 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 Ms Aβ40 present in the original specimen.

REAGENTS PROVIDED

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

Reagent	***96 Test Kit***
Ms Aβ40 Standard. Lyophilized synthetic peptide. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	1 vial
Standard Diluent Buffer. Contains 0.1% sodium azide; red dye*; 60 mL per bottle.	1 bottle
Antibody Coated Wells, 12x8 Well Strips. Plate pre-coated with mAb to NH_2 terminus of Aβ.	1 plate
MS Aβ40 Detection Antibody. Contains 15 0.1% sodium azide; blue dye*; 11 mL per bottle.	1 bottle
Anti-Rabbit IgG HRP (100X). Contains 3.3 mM thymol; 0.125 mL 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.

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. Disposable 12 x 75 mm polypropylene tubes for diluting standards and samples.
8. 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or protease inhibitor cocktail containing AEBSF.
9. Absorbent paper towels.
10. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.

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. It is recommended that all standards and samples be run in duplicate. We recommend diluting samples 1:2 to 1:10 for Aβ40.
7. Samples that are >500 pg/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. **Do not mix or interchange different reagent lots from various kit lots**.
10. Do not use reagents after the kit expiration date.
11. Read absorbances within 2 hours of assay completion.
12. We recommend covering plate with adhesive plate covers during incubation steps.
13. 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.
14. 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 the *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 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.

REAGENT PREPARATION AND STORAGE

This assay has been calibrated against the mass determination of highly purified native Ms 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 Ms Aβ40 Standard

Note: Polypropylene tubes may be used for standard dilutions.

1. Remove the *Ms Aβ40 Standard* vial from storage and let equilibrate to room temperature (RT). Reconstitute the *Ms Aβ40 Standard* to 60 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. Add 0.025 mL of the reconstituted standard to a tube containing 2.975 mL *Standard Diluent Buffer* or as otherwise dictated by the treatment of experimental samples. Label as 500 pg/mL Ms Aβ40. Mix.
3. 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 Ms Aβ40.

4. 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 0.025 mL of 60,000 pg/mL standard to 2.975 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 Ms Aβ40 standard may be stored in aliquots at -80ºC 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 (page 23) for procedure for homogenization of transgenic mouse brains.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have dissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* 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 IgG HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette *Anti-Rabbit IgG 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 *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-Rabbit IgG HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Anti-Rabbit IgG HRP (100X)	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 IgG HRP (100X)* at 2 to 8°C.

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. 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, samples or controls to the appropriate microtiter wells. Standards, samples, and controls will have a red color. Tap gently on side of plate to thoroughly 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 form wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING.**
6. Pipette 100 µL of *Ms Aβ40 Detection Antibody* solution into each well except the chromogen blank(s). This solution will have a blue color. 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 form wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING.**
9. Add 100 µL of *Anti-Rabbit IgG HRP* Working Solution to each well except the chromogen blank(s). This solution will have a yellow color. (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover plate with *plate cover* and incubate for **30 minutes at room temperature.**
11. Thoroughly aspirate or decant solution form 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 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.
17. Read the Ms Aβ 1-40 concentrations for unknown samples and controls from the standard curve plotted in step 16. (Samples producing signals greater than 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.)

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 500 pg/mL Ms Aβ40.

Standard Ms Aβ40 (pg/mL)	Optical Density (450 nm)
0	0.066
7.81	0.104
15.63	0.118
31.25	0.191
62.5	0.364
125	0.770
250	1.985
500	3.692

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.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Ms Aβ40 is <5 pg/mL. This was determined by adding two standard deviations to the mean absorbance obtained when the zero standard was assayed 30 times.

PRECISION

1. Intra-Assay Precision

Samples of known Ms Aβ40 concentrations were assayed in replicates of 14 to determine precision within assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	378.3	190.9	91.8
SD	13.3	6.1	7.6
%CV	3.5	3.2	8.3

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	384.6	187.7	90.0
SD	13.6	5.8	6.9
%CV	3.5	3.1	7.7

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Tissue culture supernatant from a Neuro-2a cell culture was serially diluted in *Standard Diluent Buffer* over the range of the assay and measured for mouse Aβ40 content. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Tissue Culture Supernatant			
Dilution	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
Neat	64.9	64.9	100.0
1/2	31.3	32.5	96.4
1/4	15.8	16.2	97.4
1/8	7.8	8.1	95.5

RECOVERY

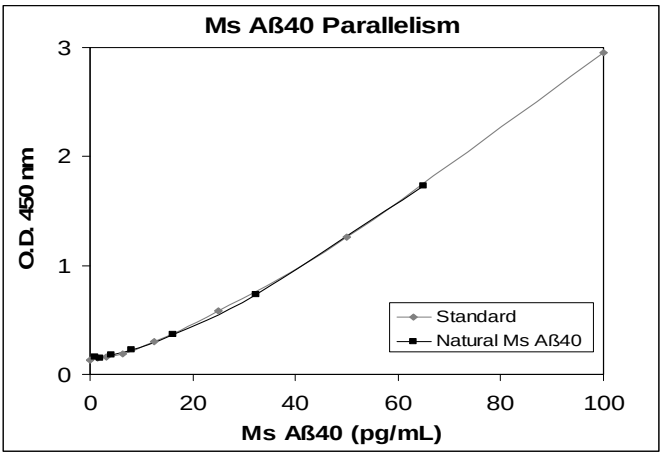
The recovery of Aβ40 added to CSF averaged 83%. The recovery of native Ms Aβ40 added to tissue culture medium containing 10% fetal bovine serum averaged 120%.

SPECIFICITY

A buffered solution of Human Aβ 1-40 at 10 ng/mL was assayed in the Ms Aβ40 kit and found to have no cross-reactivity.

PARALLELISM

Natural Ms Aβ40 was spiked into *Standard Diluent Buffer* and measured against the standard used in this kit. Parallelism demonstrated by the figure below indicated that the standard accurately reflects Aβ40 content in samples.



APPENDIX

β Amyloid Application: Procedure for homogenization of 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 or rat hemibrain (100 mg) 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 Aβ present and on inhibition of the standard curve development due to the presence of guanidine. 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 from Invitrogen .

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 of 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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3. Bancher, C., et al. (1997) Mechanisms of cell death in Alzheimer's disease. *J. Neural Transm. Suppl.* 50:141-152.
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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		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

Mouse β Amyloid 1-40 Assay Summary