biosensis® Oligomeric Amyloid-β ELISA Kit (2 Plates)

Catalogue Number: BEK-2215-2P

For the detection of human β-amyloid oligomer complexes in human CSF, brain tissue extracts and human transgenic mouse samples.

For research use only, not for use in clinical and diagnostic procedures.

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1. Introduction

The oligomeric form of Amyloid Beta peptide (Aβ, 1-42) has been closely linked to Alzheimer's Disease. Several ELISAs targeting Aβ have been developed; however, these ELISAs are known to cross-react with Amyloid Beta precursor protein (APP) and are poorly characterized against monomeric and oligomeric forms of the peptide. The Biosensis MOAB-2 antibody, developed by LaDu and co-workers (Youmans K. *et al.*, 2012), has been shown to specifically detect Aβ, but not the precursor molecule APP. When utilized in ELISAs, the oligomeric form of Aβ peptide (o-Aβ) can be assayed independently of the other forms of the molecule when assayed with the MOAB-2 monoclonal antibody.

As a result of extensive validation by Biosensis and LaDu's laboratory, this ELISA can be used to accurately determine the level of the oligomeric form of Aβ peptides in tissue extracts and other samples. In addition, the inclusion of a highly validated oligomeric standard results in a unique, ready-to-use ELISA kit.

The Biosensis oligomeric Aβ ELISA kit is a sandwich ELISA that allows the preferential quantification of oligomeric Aβ peptides. This kit consists of a pre-coated mouse monoclonal anti-Aβ capture antibody (MOAB-2), a biotinylated MOAB-2 detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of o-Aβ present in samples and protein standards. This MOAB-2 ELISA kit employs an HFIP-treated peptide standard that becomes oligomeric upon treatment as described in the protocol, as shown by Atomic Force Microscopy (AFM, see data in Section 11).

The purpose of this kit is the *in vitro* qualitative measurement of oligomeric Aβ complexes in brain extracts and CSF samples from both transgenic mice and humans relative to a known o-Aβ standard. This kit has not been tested for other sample applications.

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2. Materials Provided and Storage Conditions

Reagent	Quantity
Capture antibody coated 96 well microplate	2 plates
Assay diluent (1x)	4 x 25 mL
HFIP-treated synthetic Aβ42 standard	4 x 45 µg
Aβ42 standard reconstituting buffer	1 x 100 µL
F-12K media	1 x 1 mL
Aβ detection antibody (100x)	2 x 110 µL
Streptavidin-HRP (100x)	2 x 110 µL
Wash buffer (10x)	2 x 33 mL
TMB substrate (1x)	2 x 11 mL
TMB stop solution (1x)	2 x 11 mL
Plate sealer	2

- The ELISA kit may be stored unopened for up to 12 months at 2-8°C from the date of shipment
- The detection antibody may be aliquoted and stored at -20°C for extended storage
- Do not freeze the streptavidin-HRP conjugate!
- The Aβ standard has been HFIP treated and lyophilized
- The Aβ standard has to be oligomerized prior to the ELISA assay according to the instructions provided within the sample preparation section
- The oligomerization protocol has been developed and published by Professor M.-J. LaDu's (Youmans K. *et al.*, 2012)
- Once reconstituted and oligomerized, o-Aβ standards should be used as soon as possible and within 24 hours to ensure the stability of the oligomers

3. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 1 -1000 µL volumes
- Laminar flow hood
- Plastic and glass ware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm

4. Before You Start….

- Read the entire protocol before starting
- Preparation of the o-Aβ standard requires a 8-24 hour incubation PRIOR to performing the ELISA. Keep this in mind when planning your experiment
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- Bring the microplate and all reagents and solutions except the F12-K media to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- The stop solution provided with this kit is an acid; wear protective equipment when handling

5. Sample Preparation

For unknown concentrations of o-Aβ in samples, it is important to perform several dilutions of the sample to allow the o-Aβ concentration to fall within the range of the o-Aβ standard curve (10-1000 pg/mL).

Brain Tissue Extracts & CSF

Extraction of o-Aβ complexes in straightforward using simple TBS extraction protocols outlined in Youmans *et al.* (2011 & 2012) for EFAD samples and Tai *et al.* (2013) for human tissue extract and CSF samples. Extraction of o-Aβ is highest in TBS, lower and less stable in TBSX and not recommended in harsher treatments.

6. Preparation of o-Aβ Standard

The HFIP-treated Aβ standard supplied with this kit is used to prepare an oligomeric Aβ standard.

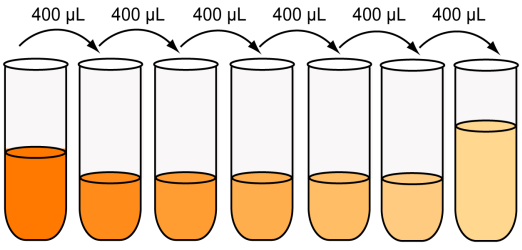
Within the laminar flow hood:

- Add 2 µL of Aβ reconstitution buffer to one vial of 45 µg of HFIP-treated Aβ peptide
- To ensure that all Aβ is dissolved, the side of the vial should be repeatedly scraped with a pipette tip (2-10 µL tip) and then spun for 5 minutes
- Add 98 µL of cold F-12K media
- Vortex for 1 min and leave at 2-8°C for 24 hours (protected from light)
- Final concentration of o-Aβ is 450 µg/mL

We recommend 1000 pg/mL of o-Aβ standard as the highest concentration of the standard curve, followed by a 1:2 serial dilution down to 15.6 pg/mL. The volumes used for the dilution series depends on the number of repeats per o-Aβ peptide standard. For triplicate measurement (100 µL per well) of each o-Aβ standard concentration, you may want to follow this procedure:

1. Label 8 tubes with 0.45 µg/mL, 1000 pg/mL 500 pg/mL, 250 pg/mL, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml and 15.6 pg/ml, respectively
2. Aliquot 400 µL of the assay diluent into each tube except the tubes labeled “0.45 µg/mL” and “1000 pg/mL”
3. Dilute the 450 µg/mL o-Aβ stock solution 1000-fold (1 µL of o-Aβ stock solution added to 999 µL assay diluent); the concentration of o-Aβ is 0.45 µg/mL
4. Add 2 µL of the 0.45 µg/mL o-Aβ standard to 898 µL of assay diluent; the concentration of o-Aβ is 1000 pg/mL
5. Take 400 µL from the “1000 pg/mL” tube and transfer to the tube labeled as “500 pg/mL”
6. Repeat step 5 for each consecutive concentration until the last tube “15.6 pg/mL” is prepared

Note: Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex.



o-Aβ (pg/mL)	900	500	250	125	62.5	31.3	15.6
Final volume in vial (µL)	500	400	400	400	400	400	800

7. Other Reagents and Buffer Preparation

- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with the assay diluent; prepare enough volume to add 100 µL per well.
- Wash buffer (10x): dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

8. Assay Procedure

1. Add 100 µL of diluted o-Aβ standards, samples and blank (assay diluent only) to the pre-coated microplate wells
2. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate overnight at 2-8°C
3. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
4. Add 100 µL of the detection antibody (1x) into each well
5. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm) for 30 minutes at room temperature (20-25°C, 70-75°F)
6. Discard the solution inside the wells and wash as described in step 3
7. Add 100 µL of the 1x streptavidin-HRP conjugate into each well
8. Seal the plate (eg., with plate sealer supplied or parafilm) and incubate the plate on a shaker (140 rpm) for 30 minutes at room temperature
9. Discard the solution inside the wells and wash as described in step 3
10. Add 100 µL of TMB solution into each well and incubate plate at room temperature for 10-15 minutes without shaking in the dark
11. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence we recommend plate be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
12. See Appendix A for a troubleshooting guide when unexpected difficulties are encountered

9. Technical Hints

1. Do not perform dilutions within the wells of the microplate
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to an o-Aβ concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
   a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
   b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
   c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b)
   d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after 15 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm in our laboratories when we perform incubations at room temperature (20-25˚C, 70-75˚F). Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.

10. Calculation of Results

1. Average the readings for each o-Aβ standard concentration, blank and sample
2. Plot a standard curve with the o-Aβ standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the o-Aβ standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit
5. Perform a regression analysis to calculate the concentration of o-Aβ in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well).

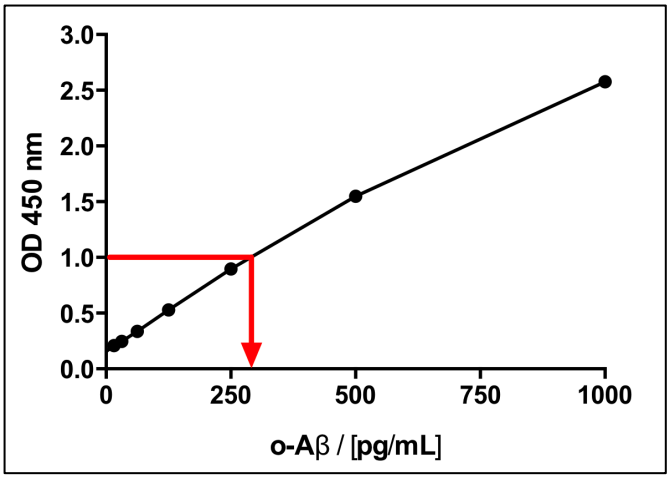
1. The **standard curve** can be plotted as the relative OD_{450} of each o-Aβ standard solution (Y-axis) vs. the respective known concentration of the o-Aβ standard solution (X-axis)
2. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}
3. **Determine unknown concentration of o-Aβ in your sample.** The o-Aβ concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of o-Aβ in the sample. In the exemplary standard curve on the right, a sample with OD_{450} = 1 reads as 290 pg/mL o-Aβ (red line)
4. If the samples measured were diluted, multiply the concentrations from interpolation (see step 3) with the dilution factor to obtain the actual o-Aβ concentration in the sample

11. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each o-Aβ ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.



In the above example graph, o-Aβ standards were run in triplicates following the provided assay protocol. The TMB reaction was stopped after 15 minutes.

Typical optical densities and coefficient of variations are summarized in the following table:

	OD 450 nm		
o-Aβ / [pg/mL]	**Mean**	**SD**	**CV**
1000	2.576	0.040	1.56%
500	1.550	0.058	3.71%
250	0.896	0.054	6.02%
125	0.529	0.043	8.11%
62.5	0.335	0.010	2.99%
31.3	0.246	0.010	4.11%
15.6	0.209	0.019	9.30%
Blank	0.155	0.004	2.42%

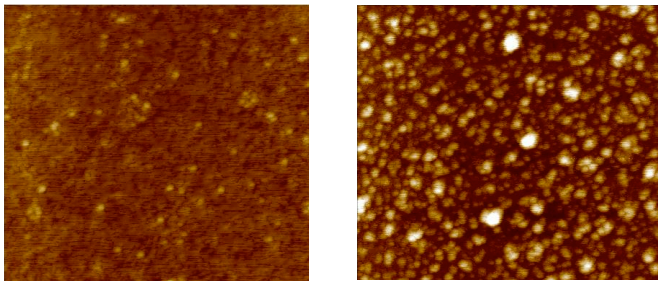
SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Limit of Detection

This o-Abeta ELISA kit typically detects 30-60 pg/mL o-Aβ (defined as 150% of blank value).

AFM Results on Oligomerized Aβ

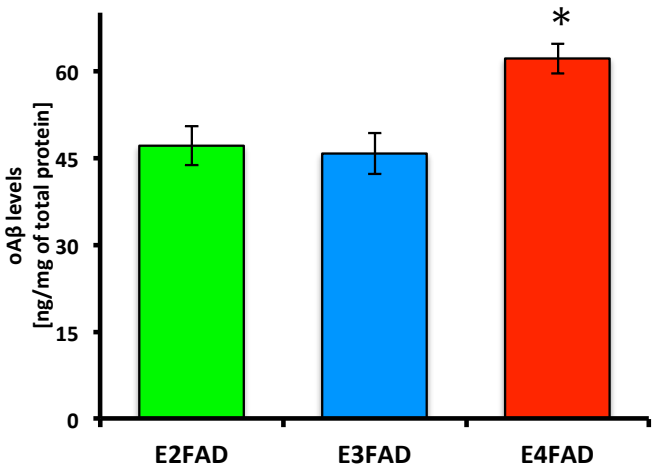
The protocol to prepare the oligomeric Aβ standard has been developed in Professor M.-J. LaDu's laboratory. Atomic Force Microscopy (AFM) demonstrates the oligomeric form of the Biosensis standard o-Aβ (Stine *et al.*, 2011).



Unaggregated (0 hr) | Oligomeric Aβ (24 hr)

Quantification of o-Aβ in Transgenic Mouse (EFAD) Brain Homogenates

FAD mice were crossed with apoE-targeted replacement mice to produce EFAD mice. o-Aβ levels were measured in TBS/soluble homogenates (1:4 dilution) from the cortex of 6-month old E2-, E3- and E4FAD mice (n=4) with the Biosensis o-Aβ ELISA kit.



*p <0.05, E4FAD vs. E3FAD and E2FAD. Mean +/- SEM.

This and other experiments have been performed to validate the o-Aβ ELISA kit.

For more data and applications of the o-Aβ ELISA kit, please refer to Tai *et al.* (2013).

Specificity

The Biosensis o-Aβ Elisa detects Aβ oligomers as validated and described by Youmans *et al.* (2012).

12. References

Youmans KL *et al.* (2011), **Amyloid-β42 Alters Apolipoprotein E Solubility in Brains of Mice with Five Familial AD Mutations.** Journal of Neuroscience Methods, 196:51-59.

Stine WB *et al.* (2011)**, Preparing Synthetic Aβ in Different Aggregation States**. Methods in Molecular Biology, Volume 670, pp. 13-32.

Youmans KL *et al.* (2012)*,* **Intraneuronal Aβ detection in 5xFAD mice by a new Aβ-specific antibody.** Molecular Neurodegeneration, March 7:8.

Tai ML *et al.* (2013), **Levels of Soluble Apolipoprotein E/Amyloid-β (Aβ) Complex Are Reduced and Oligomeric Aβ Increased with APOE4 and Alzheimer Disease in a Transgenic Mouse Model and Human Samples.** The Journal of Biological Chemistry, 288 (8), pp. 5914-5926.

13. Other Information

The MOAB-2 antibody (cat# M-1586-100) and HFIP-treated Aβ standard can be purchased separately from Biosensis (www.biosensis.com)

Appendix A: Troubleshooting Guide

This o-Aβ ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of antigen in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by either spiking a known amount of antigen into your sample or use a sample with known concentration of antigen as positive control
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	Antigen concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
Low coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate