biosensis® ApoE/β-Amyloid (ApoE/Aβ) Complex ELISA Kit

Catalogue Number: BEK-2224-1P/2P

For the detection of human ApoE/Aβ complexes in human CSF, brain tissue extracts and human transgenic mouse tissue samples only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application. Some minor changes have been made to earlier versions of this kit to enhance performance and are detailed in the text of this protocol. Reagents from previous lots should not be used.

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

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1. Intended Use

The purpose of this kit is the detection of human ApoE/Aβ complexes in human CSF, brain tissue extracts and human transgenic mouse tissue samples only if used as directed. Please refer to the Sample Preparation Section for specific use instructions for each substrate application. This kit has not been tested for other sample applications. Biosensis does not assume responsibility if this kit is used for unintended purposes. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Apolipoprotein E (ApoE) is a 35-kDa secretory protein involved in plasma cholesterol transport and clearance. However, ApoE also has functions in the central nervous system with the brain being second only to the liver in ApoE mRNA abundance. While the complete role of ApoE in the nervous system is still being examined, research has shown that ApoE mRNA levels change in response neuronal health. For instance in both the central and peripheral nervous systems, ApoE mRNA levels rise in response to injury and research has demonstrated a potential link between ApoE and the pathogenesis of Alzheimer's disease (AD). In particular, certain alleles of ApoE, have been genetically linked to the incidence of AD. ApoE is present in the extracellular senile plaques and intracellular neurofibrillary tangles associated with AD, and ApoE mRNA is increased in the brains of AD patients. Recent research indicates that ApoE seems to contribute to Alzheimer's through two distinct pathways, one of which is amyloid-dependent [Spinney, L. (2014) *Nature* **510**:26–28; Kim, J., Basak, J. M. & Holtzman, D. M. *Neuron* **63**:287–303 (2009)]. As soluble Aβ is considered a major neurotoxin in AD, the apparent correlation between ApoE and increased levels of particularly soluble oAβ suggests that understanding the underlying pathway(s) that mediates this effect may be critical to understanding the ApoE-induced risk for AD, and in turn the fundamental biology and potential therapeutic and treatment strategies.

Biosensis is proud to offer the first commercially available ApoE/β-amyloid (ApoE/Aβ) complex ELISA kit. As a result of extensive collaboration with Dr. LaDu's laboratory at UIC and validation by Biosensis, this ELISA can be used to accurately and consistently measure the extent of ApoE/Aβ complex in tissue extracts and other samples. The Biosensis ApoE/Aβ complex ELISA kit is a sandwich ELISA that allows the preferential measurement of ApoE/Aβ complexes. This kit consists of a pre-coated mouse monoclonal anti-Aβ capture antibody, a highly validated ApoE/Aβ complex standard that is pre-formed, lyophilized and ready for reconstitution, a biotinylated ApoE detection antibody, and horseradish peroxidase (HRP)-conjugated streptavidin and detection reagent. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the level of ApoE/Aβ complex present in samples and protein standards.
Importantly, a well-characterized and unique ApoE/Aβ complex is included as a standard*. This complex is pre-formed and lyophilized, requiring only reconstitution with assay diluent prior to use.

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

**Note: while the concentration of ApoE and monomeric Aβ peptide used to generate the standard is accurately determined prior to complex formation, the precise characteristics, size and number of ApoE/Aβ complexes cannot be quantified by any known method. Consequently, results should be presented as a percentage rather than an absolute concentration as published by Tai et al. (2013, 2014).*

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
MOAB-2 antibody coated 96 well microplate	96 wells	192 wells
Control antibody coated 96 well microplate	96 wells	192 wells
Assay diluent D (2x)	2 x 25 mL	4 x 25 mL
Lyophilized ApoE/Aβ complex standard	2 vials	4 vials
ApoE/Aβ complex detection antibody	1 x 220 µL	2 x 220 µL
Streptavidin-HRP (100x)	1 x 220 µL	2 x 220 µL
Wash buffer (20x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 22 mL	2 x 22 mL
TMB stop solution (1x)	1 x 22 mL	2 x 22 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard	Use on same day; aliquot unused standard to prevent multiple freeze-thaw cycles and store at -20°C for maximum of 2 weeks
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer and assay diluent (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use assay diluents from other ELISA kits
- The ApoE/Aβ complex standard was prepared by complexing 4.2 µg/mL monomeric Aβ42 peptide with 5 µg/mL ApoE as published by Tai *et al.* (2013, 2014) and then diluted to "100%" and freeze-dried from a propriety buffer to stabilize the complex
- Once reconstituted, ApoE/Aβ complex standards should be used within 24 hours to ensure the stability of the complex

4. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 1-1000 µL volumes
- Laminar flow hood (preferable, but not essential)
- Plastic and glassware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450nm

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online at: https://www.youtube.com/watch?v=7EOuc9qYL0E
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- 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
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

6. Sample Preparation

Preparation of Assay Buffer

To prepare assay buffer (1x), dilute Assay Diluent D (2x) two-fold with ultrapure water and mix thoroughly.

For unknown amounts of ApoE/Aβ complexes in samples, it is important to perform several dilutions of the sample to allow the ApoE/Aβ complex concentration to fall within the range of the ApoE/Aβ complex standard curve (6.25% – 0.1%).

It is also suggested that for the sample preparation protocols for mouse brain samples, human synaptosomes and human CSF samples as discussed by Tai. L *et al.* (2013) [PMID: 23293020] are thoroughly reviewed prior to attempting the ELISA. The following enclosed protocols have been adapted from Tai *et al.* and are placed here for researcher ease of use and completeness of instruction; they are to be used only as guide. *Optimization tailored to the particular sample, laboratory skill, and equipment available is highly recommended.*

5a. ApoE/Aβ complexes isolated from transgenic mouse brains

Briefly, 6-month-old male EFAD mice were anesthetized with sodium pentobarbital (50 mg/kg) and transcardially perfused with a solution of tissue culture PBS plus protease inhibitors (AEBSF 0.5 mM, Aprotinin 0.4 uM, Bestatin 0.25 mM, E-64 0.0075 mM, Leupeptin 0.01 mM, Pepstatin A 0.005 mM final concentrations) and brains were removed and dissected at the midline. Right hemi-brains were dissected on ice into cortex, hippocampus and cerebellum, immediately snap-frozen in liquid nitrogen, and stored at −80 °C until use. The

dissected tissue was homogenized in 15 volumes (w/v) of TBS; samples were centrifuged (100,000 x *g*, 1 h at 4 °C), and the TBS (soluble) fraction was divided into aliquots prior to freezing in liquid nitrogen and stored at −80 °C.

When ready to use, it is suggested to dilute the TBS extracted samples 4-fold with assay buffer for measurement in the ApoE/Aβ ELISA. It is recommended to normalize the data obtained to the protein concentration in each sample. Also in the original work of Tai *et al.*, there was no detectable soluble complex levels in cerebellum.

5b. ApoE/Aβ detection in human synaptosomes

Brain samples of parietal cortex (A7, A39, and A40,~0.3–5 g each) were immediately minced in 0.32 M sucrose with protease inhibitors (2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 1 mM sodium pyrophosphate, 5 mM NaF, 10 mM Tris) and then stored at −70 °C until homogenization. The P-2 (crude synaptosome; synaptosome-enriched fraction) is prepared as described in Munson GW *et al*. (2000) [PMID: 11123940]. Briefly, tissue was homogenized in ice-cold buffer [0.32 M sucrose, 10 mM Tris, pH 7.5, plus protease inhibitors: pepstatin (4 mg/mL), aprotinin (5 mg/mL), trypsin inhibitor (20 mg/mL), EDTA (2 mM), EGTA (2 mM), PMSF (0.2 mM), leupeptin (4 mg/mL)]. Then the homogenate was first centrifuged at 1000 x *g* for 10 min; the resulting supernatant was centrifuged at 10,000 x *g* for 20 min to obtain the crude synaptosomal pellet. Aliquots of P-2 were routinely cryopreserved in 0.32 M sucrose and banked at −70 °C until the day of the experiment.

On the day of the experiment, cryopreserved human P-2 aliquots were defrosted at 37 °C, resuspended in PBS with protease inhibitors, sonicated, and centrifuged for 4 min at 6000 rpm. Supernatant was collected, and total protein concentration was defined using BCA protein assay. It is suggested to dilute the samples using a 5-fold sample dilution with assay buffer prior to measurement on the ApoE/Aβ ELISA. It is recommended to normalize the data obtained to the protein concentration in each sample.

5c. Human CSF sample preparation

Human CSF samples are obtained from licensed sources, divided into aliquots and stored at -80°C until used. Consultation on the collection, storage and use of human sample material is recommended for best results. CSF samples may be diluted at least 2-fold with assay buffer and then measured on the ApoE/Aβ ELISA. Optimization of dilution normalization to total protein per samples is recommended.

Note: Free ApoE protein can bind non-specifically to the microplate and falsely elevate ApoE/Aβ complex levels. For this purpose, this kit contains a microplate pre-coated with control antibody which allows to determine the level of non-specific ApoE binding in your ELISA assay. This is especially important for measurement of complexes in human CSF which contains high levels of ApoE protein.

In order to conduct the control assay, samples are added to the control antibody coated wells the same way they are added to the MOAB-2 coated microplate and the assay procedure is followed as outlined in Section 8. OD values obtained from the control assay can then be subtracted from the signal obtained from the MOAB- 2 coated microplate to assess true ApoE/Aβ complexes levels.

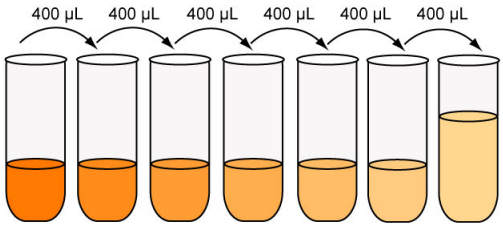
7. Preparation of ApoE/Aβ Complex Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted ApoE/Aβ standard as “100%”; vortex and let stand for 15 minutes
- Dilute the 100% ApoE/Aβ standard 1:16 (eg., 50 µL of 100% standard + 750 µL diluent); label this tube “6.25%”

We recommend 6.25% of ApoE/Aβ complex standard as the highest concentration of the standard curve, followed by a 1:2 serial dilution down to 0.1%. The volumes used for the dilution series depends on the number of repeats per ApoE/Aβ complex standard. For triplicate measurement (100 µL per well) of each ApoE/Aβ complex standard concentration, we suggest the following procedure:

1. Label 6 tubes with 3.13%, 1.56%, 0.78%, 0.39%, 0.2% and 0.1%, respectively
2. Aliquot 400 µL of 1x diluent into each tube
3. Take 400 µL from the “6.25%” tube and transfer to the tube labeled as “3.13%”
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex

5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “0.1%” is prepared and mixed well



ApoE/Aβ complex (in %)	6.25%	3.13%	1.56%	0.78%	0.39%	0.2%	0.1%
Final volume in vial (µL)	400	400	400	400	400	400	800

8. Other Reagents and Buffer Preparation

- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute each vial hundred-fold with 1x Assay Diluent D to yield a 1x working solution; prepare enough volume to add 100 µL per well
- Wash buffer (20x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute twenty-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

1. Add 100 µL of diluted ApoE/Aβ complex standards, samples and blank (sample diluent only) to the pre-coated microplate wells
2. Seal the plate (eg, with plate sealer or Parafilm) and incubate the plate overnight, refrigerated.
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; 0.351 G*) 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; 0.351 G*) 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 8-12 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 B for a troubleshooting guide when unexpected difficulties are encountered.

*RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. 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 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 to reduce 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 3-5 times
   c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in steps a-b)
   d. Repeat this procedure a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, it is important to 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 8-12 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). 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. TMB incubation times can vary between 5 – 15 minutes, and assay sensitivity can be affected if color development is stopped too early. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~1.0 – 1.2 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenize the blue TMB reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each ApoE/Aβ complex standard concentration, blank and sample
2. Plot a standard curve with the ApoE/Aβ complex standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If the control antibody coated microplate was used, subtract OD values from the OD values obtained with the MOAB-2 coated microplate for each sample and dilution
4. If values for the ApoE/Aβ complex standards are adjusted for background absorbance, then also subtract the blank value from the OD_{450} of the samples
5. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression
6. Perform a regression analysis to calculate the concentration of ApoE/Aβ complex 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 the Blank well). The **standard curve** can be plotted as the relative OD_{450} of each ApoE/Aβ complex standard solution (Y-axis) versus the respective known concentration of the ApoE/Aβ complex standard solution (X-axis).

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}

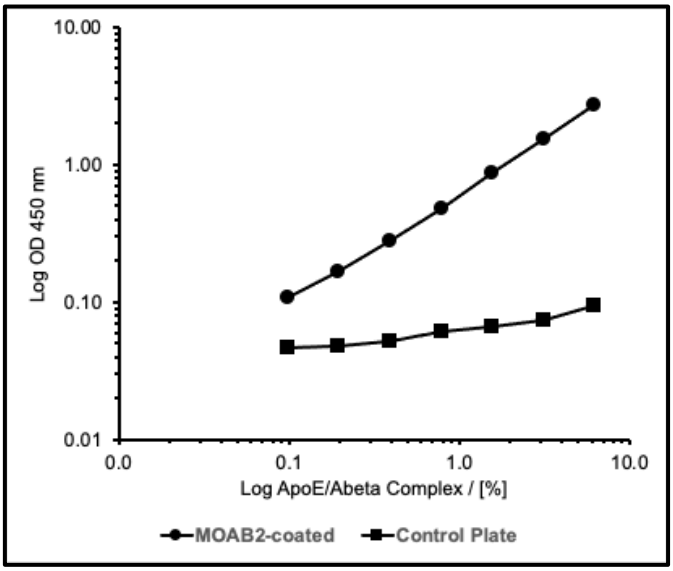
1. **Determine unknown concentration of ApoE/Aβ in your sample.** The ApoE/Aβ complex concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of ApoE/Aβ complex in the sample.
2. If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual ApoE/Aβ complex concentration in the sample

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each ApoE/Aβ complex 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, ApoE/Aβ complex standards were run following the assay protocol. The TMB reaction was stopped after 9 minutes.

Typical optical densities for diluted standards are summarized in the following table:

ApoE/Aβ complex / (%) MOAB-2 plate	Mean OD 450 nm
6.25	2.67
3.13	1.54
1.56	0.86
0.78	0.48
0.39	0.28
0.20	0.16
0.10	0.11
Blank	0.06

Specificity

The Biosensis ApoE/Aβ complex ELISA detects ApoE/Aβ complexes as validated and described by Tai *et al.* (2014). It will not detect monomeric Aβ or oligomeric Aβ (as also demonstrated in Tai *et al*., 2013).

For more data and applications of the ApoE/Aβ complex ELISA kit, please refer to Tai *et al.* (2013) and Tai *et al.* (2014).

13. Informational References

Kim, J., Basak, JM & Holtzman, DM (2009), **The role of apolipoprotein E in Alzheimer's disease**. *Neuron* **63**:287–303.

Munson GW *et al.* (2000), **SDS-stable complex formation between native apolipoprotein E3 and beta-amyloid peptides**. *Biochemistry* **39** (51):16119-24.

Spinney, L. (2014), **Alzheimer's disease: The forgetting gene.** *Nature* **510**:26–28.

14. Specific References

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.

Tai ML *et al.* (2014), **Soluble ApoE/Aβ complex: mechanism and therapeutic target for APOE4-induced AD risk**. *Molecular Neurodegeneration*. 2014; 9:2.

15. Other Related Products

MOAB-2 is a monoclonal antibody (cat# M-1586-100) for specific detection of Aβ peptides in applications such as IHC, WB and IF. MOAB-2 antibody does not cross-react with APP.

For quantification of oligomeric Aβ peptides, a MOAB-2 based ELISA is exclusively available from Biosensis (cat# BEK-2215-1P/2P).

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Note that calculations are based on MOAB-2 antibody-coated plate.

Standard curve, blank and controls:

- Standard (6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.2%, 0.1%): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test samples) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test samples on the "test only plate" for a total available number of 174 tests per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 test samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty test samples at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 samples, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty test samples. In summary: 60 samples, 4 tests per sample, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 test sample experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty test samples, tested in duplicate (two draws per sample, 1 dilution, 4 wells per test, total of 240 wells). The unused 8-well strips can be used for other assays later.

Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:50 & 1:100 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:50 = 240, PLUS 120 x 2 @1:100 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty test samples tested in duplicate at two dilutions (two draws per test, 2 dilutions, 8 tests/wells per test, total of 480 tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 test samples with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty test samples, 2 draws per sample experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

For the two dilutions per sample, sixty test samples, 2 draws per sample experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or 3 x 2-plate kits would need to be ordered to ensure enough wells for all sixty test samples.

Appendix B: Troubleshooting Guide

This ApoE/Aβ complex 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
High 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