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Edvo-Kit #

1116

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Researching Alzheimer's Disease by ELISA

Experiment Objective:

In this experiment, students will explore potential biomarkers for Alzheimer's Disease by analyzing simulated samples from control and Alzheimer's Disease populations.

See page 3 for storage instructions.

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Experiment Components

COMPONENTS

	Storage	Check ✓
A 10X ELISA Wash Buffer	Refrigerator	<input type="checkbox"/>
B ELISA Dilution Buffer	Refrigerator	<input type="checkbox"/>
C Antigen (lyophilized)	Refrigerator	<input type="checkbox"/>
D Primary Antibody (lyophilized)	Refrigerator	<input type="checkbox"/>
E Secondary Antibody (lyophilized)	Refrigerator	<input type="checkbox"/>
F TMB Substrate	Refrigerator	<input type="checkbox"/>
G Stop Solution	Refrigerator	<input type="checkbox"/>

This experiment contains enough reagents for 10 lab groups.

REAGENTS & SUPPLIES

- Small transfer pipets ☐
- Strip tubes (12-well) ☐
- 15 mL plastic tubes ☐
- 1.5 mL snap-top tubes ☐

Requirements

- Automatic micropipettes (5 – 50 μ L) and tips
- Beakers or flasks
- Lab glassware
- Disposable lab gloves
- Safety goggles
- Distilled or deionized water
- Paper towels

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

None of the experiment components are derived from human sources.

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Background Information

In 1906, the German psychiatrist Alois Alzheimer reported the first case of Alzheimer's Disease in a 50 year old woman. Since then, Alzheimer's Disease (AD) has grown to be one of the most recognizable and tragic neurodegenerative diseases. AD is characterized by memory loss and impaired neurological function. It is most commonly diagnosed in populations over 65 years of age, and the risk of developing AD increases with age.

AD is characterized by memory loss and a decline in cognitive ability. Indeed, the first signs and symptoms of AD are only mild cognitive impairments, usually attributed to stress or fatigue. However, these can be quite common in healthy individuals. A diagnosis for AD must be based on sustained short-term memory loss, family history, and behavioral observations over time. Unfortunately, brain imaging techniques including PET and MRI scans are not yet at the point where a diagnosis of AD can be definitely given simply by observing a brain scan. The only definitive diagnosis of AD is post-mortem, by examining brain tissue and looking for molecular markers of AD.

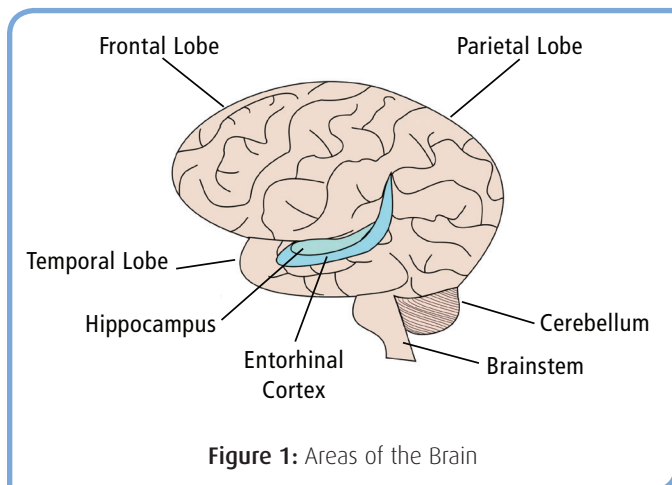
Pathophysiology of AD

The brain is divided into lobes and areas depending on location and function. The 5 major lobes of the brain are the brainstem, parietal lobe, cerebellum, frontal lobe, and temporal lobe (Figure 1). The brainstem is responsible for all of the major functions that keep a person alive, such as heartbeat, blood pressure, and breathing regulation. The parietal lobe is responsible for processing sensory information. The cerebellum regulates motor function and movement. The frontal lobe is responsible for executive decision making and higher-order processing, and the temporal lobe is responsible for memory. Within the temporal lobe lies an area known as the hippocampus. The hippocampus is where all new memories are formed.

In 1953, a temporal lobectomy (removal of the temporal lobe) was performed on Patient HM. Patient HM had intractable epilepsy, meaning he had seizures that could no longer be treated with medicine. The seizures were caused by overactive neurons in his temporal lobe, so his doctor decided to remove that area of his brain. While the surgery was successful and his seizures halted, he lost the ability to form new memories. All memories that he had before the surgery were intact, but he could not store any new information. This unfortunate surgery is how we now know that all new memories are formed within the temporal lobe, specifically in the hippocampus.

How exactly memories are formed and stored in the brain is still not well understood. However, what we do know is what other areas of the brain the hippocampus communicates with. The hippocampus sends information to the frontal lobe, which is able to process information and make executive decisions. The area of the brain that connects the hippocampus and the frontal lobe is called the entorhinal cortex. The entorhinal cortex is able to send information back and forth between the hippocampus and the frontal lobe, making it a potentially important area for consolidating memories. It is now well-accepted that AD begins in the entorhinal cortex and progressively spreads throughout the brain.

What is happening within the entorhinal cortex during AD? The major symptom is broad neuronal death, caused by the accumulation of mutated and misfolded proteins. There are two major proteins that play a role in disease progression: A β and tau. A β is a small peptide (protein) that is cleaved from a larger protein known as Amyloid Precursor



sor Protein (APP). When APP is cleaved by a certain protease, it forms A β peptides. A β peptides can all clump together to form plaques, or areas of dense A β accumulation. This can inhibit protein transport, mitochondrial activity, and many other processes that are critical to the cell. Tau, on the other hand, is a microtubule protein. Microtubules are the “railroad tracks” of a cell, and tau binds to microtubules to stabilize them in the axon. Normally, tau is not found outside of the axon. However, during AD tau is mislocalized to both the cell body and the dendrites. This mislocalized tau is also improperly regulated, and forms neurofibrillary tangles. These tangles are also very detrimental to the cell. When detecting AD in postmortem tissue, scientists use a stain for plaques and tangles. If these are present, it means that the patient had AD.

Between A β plaques and tau tangles, an AD neuron becomes overwhelmed and dies off. Once a neuron dies, it can never regenerate. This is different from most other cells in the body and is what makes brain injuries and neurodegenerative diseases so difficult to treat. It also makes them difficult to diagnose, because if a few neurons die others may be able to compensate for them. Therefore, symptoms may not show until many neurons have died and function can no longer be restored. Most clinical trials for drugs to treat AD begin upon diagnosis. However, it has been shown that by the time someone is diagnosed the disease has already irreversibly damaged the brain. Therefore, many scientists and research groups are working on tests which can detect and diagnose AD earlier, before the brain has massive neuronal loss.

Studying Alzheimer's Disease

The brain is bathed in a clear colorless liquid known as cerebrospinal fluid (CSF). CSF surrounds the brain and fills the ventricles (otherwise empty space) and middle of spinal cord. CSF is produced by special cells lining the ventricles and is similar to blood plasma. CSF is high in electrolytes and helps maintain homeostasis in the brain.

When A β accumulates into plaques during Alzheimer's Disease, the plaques occupy the extracellular space in the brain and cause damage to neurons. These plaques begin appearing up to 20 years before symptoms start. However, clinical trials for new drugs can't begin until a patient has already been diagnosed. At this point too many neurons have died to have complete memory restoration. Therefore, scientists are trying to find early warning signs of Alzheimer's Disease so that it can be stopped before too many neurons die off. One question is whether or not we can measure A β levels in the CSF, and if so, what happens in a disease state? Does the level of A β stay the same, increase, or decrease in patients with Alzheimer's disease, and can that become an early marker of detection?

In this experiment, you will be testing simulated CSF samples from patients with Alzheimer's Disease and healthy age-matched controls. It is important to use age-matched controls in case the level of A β in the CSF changes with age. To test the samples, you will be using the Enzyme-Linked Immunosorbent Assay (ELISA).

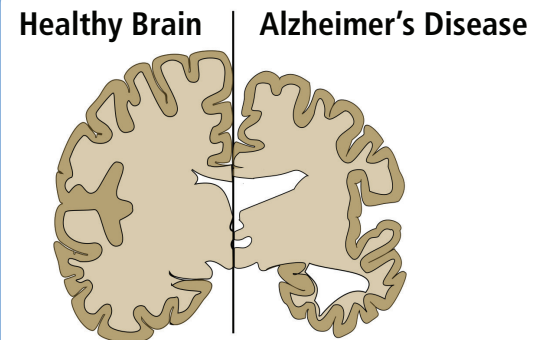
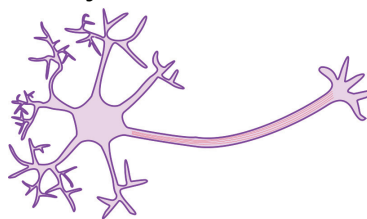
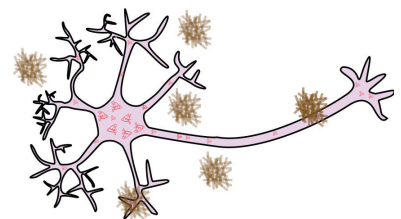


Figure 2: Healthy vs. Diseased Brain

Healthy Neuron



Alzheimer's Disease



Normal Tau
(axonal)

Tau tangles
A-beta plaques

Figure 3: Healthy vs. Diseased Neurons

Description of the Immunological Screening

The Enzyme-linked immunosorbent assay (ELISA) test was originally developed for antibody measurement from blood. These immunoassays have been adapted to successfully detect samples that contain proteins in CSF. This ELISA simulation experiment has been designed to detect the amount of A β circulating in simulated patients' CSF. ELISAs are performed in microtiter plates which are generally made of polystyrene or polyvinyl chloride. The plates are somewhat transparent and contain many small wells in which liquid samples are deposited. First, samples(s) are added to the wells where proteins are adsorbed by hydrophobic association to the walls of the well. The excess, unbound protein is then washed away. After washing away unadsorbed material, the unoccupied sites on the wells are blocked with proteins, typically bovine serum albumin to ensure the plate is saturated with proteins.

The ELISA assay requires antibodies to visualize the protein of interest. Antibodies (also called immunoglobulins, or Igs) are specialized proteins that allow the immune system to distinguish between "self" and "non-self" proteins or polysaccharides. Antibodies

used in scientific research are produced as an immune response when animals (i.e. rabbits, mice and guinea pigs) are injected with an antigen. The immune response will produce antibodies that are specific to the antigen, which are then purified from the serum. The traditional ELISA requires two antibodies. One antibody, called the primary, recognizes the protein of interest. For example, an ELISA that detects A β protein would use an antibody that specifically binds to A β . The secondary antibody recognizes the primary antibody – if a rabbit produced our primary antibody, we would use a secondary antibody that recognizes rabbit antibodies. The secondary antibody is covalently linked to an enzyme called Horseradish Peroxidase (HRP) that lets us detect the presence of the antibody-antigen complex (Figure 4). HRP has a high catalytic activity – its substrate turnover rate exceeds 100 per second – allowing us to quickly detect even the smallest amount of antigen.

In this lab, you will use an ELISA to test simulated CSF samples taken from patients with Alzheimer's Disease and age matched controls. This rapid test can be completed in less than one hour and can detect the presence of A β protein in the CSF. You will create a standard curve to compare the patient and control samples to.

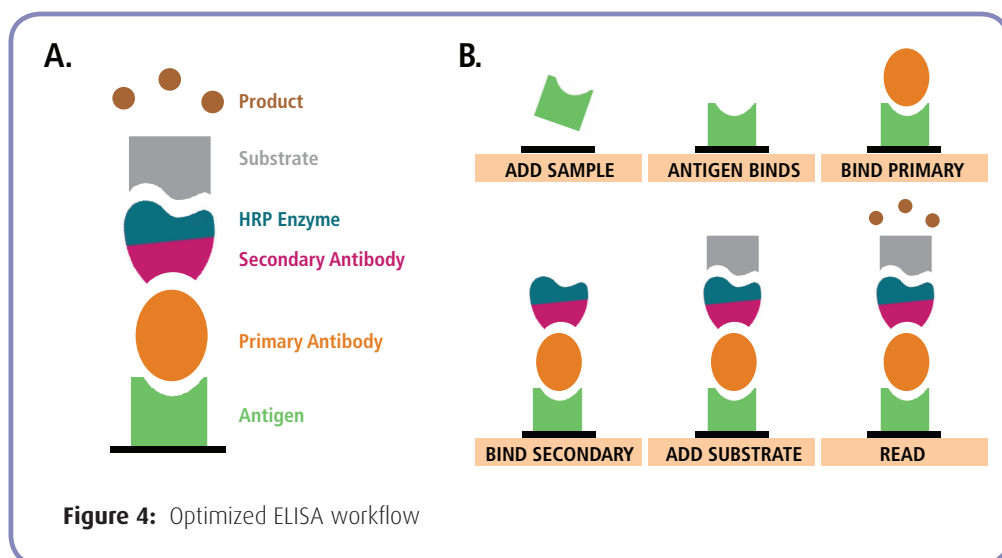


Figure 4: Optimized ELISA workflow

**Do you think that patients with Alzheimer's disease have less A β in their CSF or more?
What could be the potential reasons for each?
Before starting, form a hypothesis to investigate this question.**

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will explore potential biomarkers for Alzheimer's Disease by analyzing simulated samples from control and Alzheimer's Disease populations.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.



LABORATORY NOTEBOOKS

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

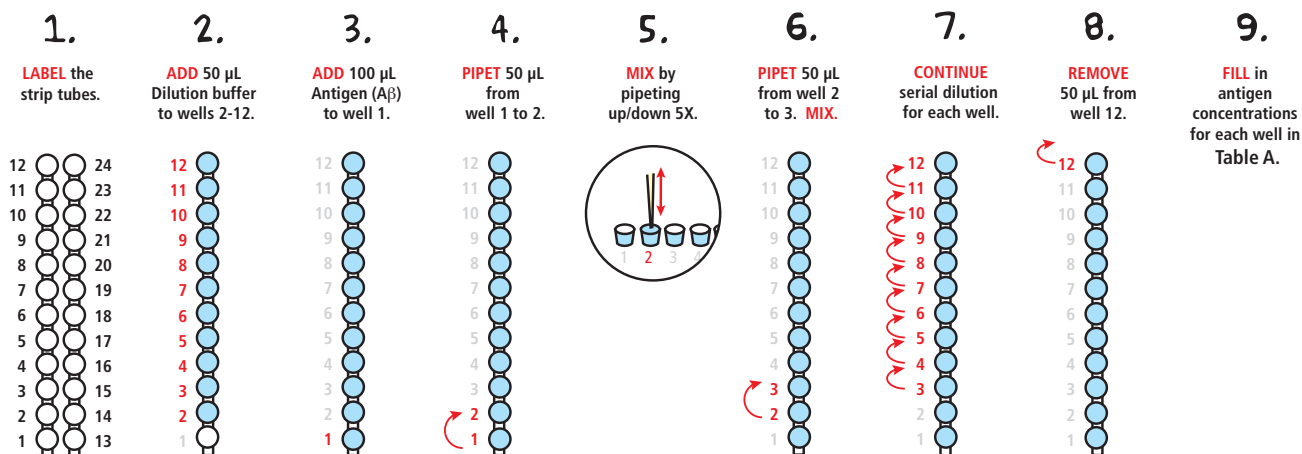
During the Experiment:

- Record (draw) your observations, or photograph the results.

After the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Module I: Performing the ELISA



PREPARATION OF STANDARD CURVE:

- OBTAIN** two 12-well strip tubes. Using a fine-tipped marker, **LABEL** one strip with numbers 1-12 and the other with numbers 13-24. The strip with wells 1-12 will be your "Standard Curve Strip". The strip with wells 13-24 will be your "Patient/Control strip". Set the strip with wells 13-24 off to one side for now.
- Use a micropipette to **ADD** 50 µL Dilution Buffer to wells 2-12.
- ADD** 100 µL of A β (Antigen) to well #1. The A β is provided at a concentration of 100 µg/mL.
- PIPET** 50 µL from well #1 into well #2.
- Fully **MIX** the sample by gently pipetting up and down 5 times.
- Using the same pipette tip, **TRANSFER** 50 µL from well #2 into well #3 and **MIX** as in step 5.
- Continue this procedure to serially **DILUTE** the remaining samples through well #12.
- REMOVE** and **DISCARD** 50 µL of the diluted antigen from well #12.
- Set the strip off to the side where it will not be disturbed. **FILL** in the dilutions and antigen concentrations for each well in Table A, below, or on your student worksheet.

Table A: Dilutions and Concentrations

Well #	1	2	3	4	5	6
Dilution	---					
Concentration	100 µg/mL					
Well #	7	8	9	10	11	12
Dilution						
Concentration						

Module I: Performing the ELISA, continued

10.
RETRIEVE
Control & Patient
Samples.

RECORD
the Control patient and
AD patient numbers
your group will be
testing in
TABLE B.

11.
ADD 50 μ L
Positive Control
to wells 13-15.

Replace Tip

12.
ADD 50 μ L
Negative Control
to wells 16-18.

Replace Tip

13.
ADD 50 μ L
Control Patient
to wells 19-21.

Replace Tip

14.
ADD 50 μ L
AD Patient Sample
to wells 22-24.

Replace Tip

15.
INCUBATE
for 5 min.

16.
INVERT
onto paper
towels and **TAP**.

17.
ADD
wash buffer
to each well.

18.
INVERT
onto paper
towels and **TAP**.

19. **REPEAT** wash steps 17 & 18.

LOADING THE CONTROL AND PATIENT SAMPLES:

10. **RETRIEVE** the samples provided by your instructor (positive control, negative control, control patient sample and AD patient sample). **RECORD** the Control patient and AD patient numbers your group will be testing in Table B on page 11.
11. Using a micropipette, **ADD 50 μ L** of Positive Control sample to wells 13-15. **REPLACE** the pipette tip.
12. **ADD 50 μ L** of Negative Control sample to wells 16-18. **REPLACE** the pipette tip.
13. **ADD 50 μ L** of Control Patient sample to wells 19-21. **REPLACE** the pipette tip.
14. **ADD 50 μ L** of AD Patient sample to wells 22-24.
15. **RETRIEVE** the Standard Curve strip from Step 9. **INCUBATE** both strips for 5 minutes at room temperature.

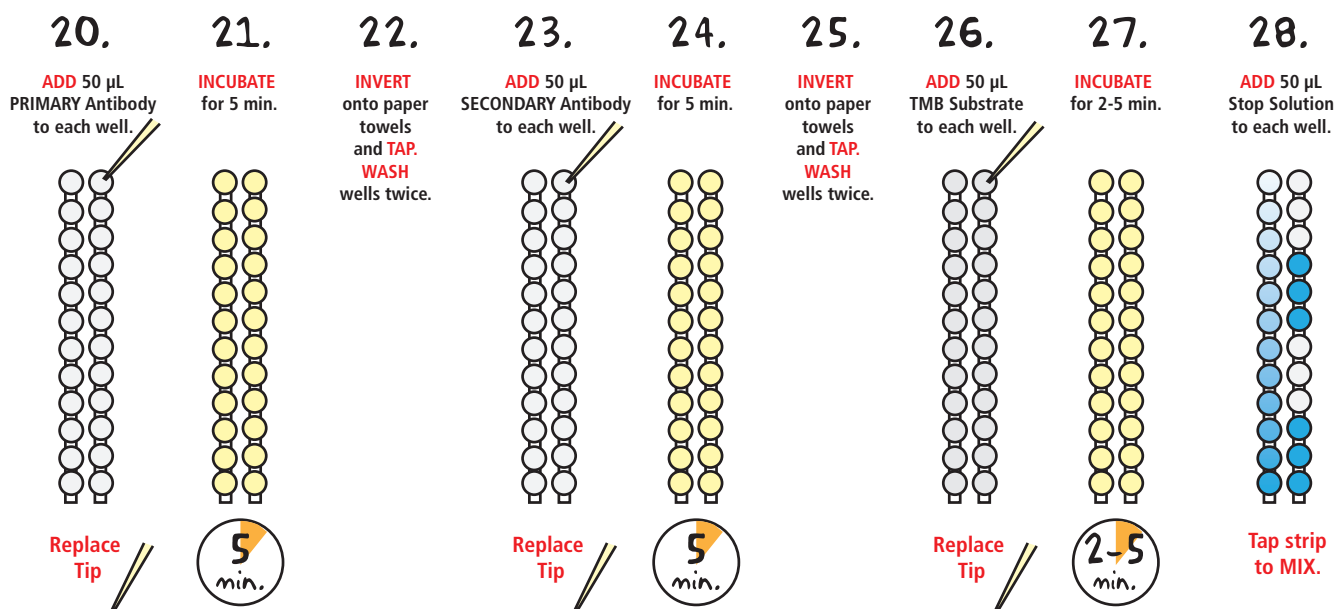
During this incubation, the proteins in the sample are binding to the plastic walls of the wells.

REMOVAL OF SAMPLE AND WASHING THE WELLS:

16. **INVERT** the strips over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strips 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.
17. Using a transfer pipet, **ADD** Wash Buffer to fill each well (200 μ L), being careful not to overfill.
***NOTE:** To minimize cross-contamination it is important that you avoid spilling buffer into neighboring wells.*
18. **REPEAT** step 16 to **REMOVE** the wash buffer.
19. Using the same transfer pipet, **REPEAT** the wash a second time. **INVERT** the strips onto fresh paper towels and **TAP**.

The wash buffer contains a blocking compound to saturate the plastic, which allows us to skip a formal blocking step.

Module 1: Performing the ELISA, continued



ADDITION OF PRIMARY AND SECONDARY ANTIBODIES:

20. Using a new micropipette tip, **ADD** 50 µL of Primary Antibody (1°AB) to each well.

21. **INCUBATE** for 5 minutes at room temperature.

During this incubation, the primary antibody (anti-A β) is binding to any A β immobilized in the well.

22. **INVERT** onto paper towels and **TAP. WASH** the wells twice as in steps 16-19.

23. Using a new micropipette tip, **ADD** 50 µL of the Secondary Antibody (2°AB) to each well.

24. **INCUBATE** for 5 minutes at room temperature.

During this incubation, the secondary antibody is binding to the primary antibody.

25. **INVERT** onto paper towels and **TAP. WASH** the wells twice as in steps 16-19.

ADDITION OF SUBSTRATE:

26. Using a new micropipette tip, **ADD** 50 µL of Substrate to each well.

27. **INCUBATE** the plate for 2-5 minutes at room temperature, or until color no longer changes in the wells with the highest antigen concentrations.

NOTE: It is important that the reaction is not allowed to proceed for more than 10 minutes as the enzymatic reaction can saturate at the highest concentrations of substrate.

During this incubation, the HRP bound to the secondary antibody is catalyzing a reaction with the substrate.

28. Using a new micropipette tip, **ADD** 50 µL of Stop Solution to each well. Gently tap tubes to **MIX**.

29. **PROCEED** immediately to Module II: Analysis of Quantitative ELISA.

Module II: Analysis of Quantitative ELISA Results

1. **OBSERVE** the color of the reactions in your positive and negative samples to confirm that the ELISA has succeeded. *NOTE: Placing the strip tubes on a white sheet of paper or light box can enhance the contrast between wells.*
2. Using the standard strip as a reference, **ESTIMATE** the concentration of your control patient and AD patient samples. **RECORD** the results in Table B.
3. **SHARE** your findings with other groups and record the concentrations for each patient in Table C.

Table B: Identification of Patient Samples						
Well #	13	14	15	16	17	18
Sample	Positive Control			Negative Control		
Concentration						
Well #	19	20	21	22	23	24
Sample	Control Patient # _____			AD Patient Sample # _____		
Concentration						

Table C: Concentration of Patient Samples					
Group #	AD Patient	Concentration	Group #	Control #	Concentration
	Patient #1			Control #1	
	Patient #1			Control #1	
	Patient #2			Control #2	
	Patient #2			Control #2	
	Patient #3			Control #3	
	Patient #3			Control #3	
	Patient #4			Control #4	
	Patient #4			Control #4	
	Patient #5			Control #5	
	Patient #5			Control #5	

Study Questions

EXPERIMENTAL ANALYSIS QUESTIONS:

1. Do the control patients or the AD patients have more A β in their CSF?
2. Come up with a hypothesis to explain your findings from question 1.
3. If you were researching Alzheimer's Disease biomarkers, what would be your next experiment?

STUDY QUESTIONS:

1. What is Alzheimer's Disease?
2. What are the major risk factors for Alzheimer's Disease?
3. Why is it difficult to find drugs to treat Alzheimer's Disease?
4. Why do we need to compare to age-matched controls?
5. How could biotechnology help us cure Alzheimer's Disease?

Instructor's Guide

Notes to the Instructor

Current research shows that one high specificity marker for Alzheimer's Disease is a combination of decreased A β 42 (there are many different oligomers of A β , and A β 42 is the most toxic) and an increased level of tau in the CSF. Therefore, you will create 5 AD patient samples with low A β levels and 5 control patient samples with high A β levels to distribute to your class. Your class should be divided into 10 groups so that each patient sample is tested twice.

It is necessary to prepare the samples, antibody, and buffers prior to performing the experiment. All components should be kept at 4° C in the dark until needed.

Preparation for:	What to do:	When:	Time Required:
Module I: Performing the ELISA	Prepare 1X wash buffer.	Any time before lab.	5 min.
	Prepare antigen.	Up to one week before lab.	5 min.
	Prepare primary antibody.	Up to one week before lab.	5 min.
	Prepare secondary antibody.	Day before lab.	5 min.
	Prepare ABTS.	Up to one week before lab.	5 min.
	Aliquot reagents.	Up to one week before lab.	30 min.

Red = Prepare immediately before module.
 Yellow = Prepare shortly before module.
 Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations

Preparation of 1X ELISA Wash Buffer

1. Add all of the 10X ELISA wash buffer (Component A) to 525 mL of distilled water and mix well. Label as "1X wash buffer."
2. Dispense 55 mL into small beakers for each group and label "Wash Buffer".

Preparation of Dilution Buffer

- Dispense 1 mL of the ELISA Dilution Buffer (Component B) into 10 microcentrifuge tubes and label as "Dilution Buffer".

Pre-Lab Preparations, continued

Preparation of Antigen

1. Transfer 7 mL of ELISA Dilution Buffer (Component B) to a 15 mL conical tube and label as "A β ".
2. Carefully remove the stopper from the vial of Antigen (Component C). Transfer 0.5 mL from the conical tube "A β " to the vial of Antigen (Component C). Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of reconstituted Antigen (Component C) to the conical tube "A β ". Mix well.
4. Label 10 microcentrifuge tubes "A β " and dispense 0.15 mL per tube.

Preparation of Negative and Positive Control Samples

1. Label 10 microcentrifuge tubes as "Positive Control" and dispense 175 μ L "A β " (prepared above) per tube.
2. Label 10 microcentrifuge tubes as "Negative Control" and dispense 175 μ L of ELISA Dilution Buffer (Component B) per tube.

Preparation of Patient Samples

Each student group will be responsible for testing one control patient sample and one AD patient sample.

Control Patient Samples:

- Prepare the simulated control patient samples by mixing 2 mL "A β " (prepared above) with 500 μ L ELISA Dilution Buffer (B) in a 15 mL conical tube. Label 10 microcentrifuge tubes "Control" with numbers 1-5 (with each number duplicated once). Dispense 175 μ L per tube.

AD (Alzheimer's Disease) Patient Samples:

- Prepare the simulated AD patient samples by mixing 3.7 mL ELISA Dilution Buffer (B) and 100 μ L A β (prepared above) in a 15 mL conical tube. Label 10 microcentrifuge tubes "Patient" with numbers 1-5 (with each number duplicated once).

Pre-Lab Preparations, continued

Preparation of the Primary Antibody

1. Transfer 14 mL of ELISA Dilution Buffer (Component B) to a 15 mL conical tube. Label the conical tube "1°AB".
2. Carefully remove the stopper from the vial of Primary Antibody (Component D). Transfer 0.5 mL from the conical tube "1°AB" to the vial. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of reconstituted Primary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "1°AB" and dispense 1.3 mL per tube.

Dilution of the Secondary Antibody

1. Transfer 14 mL of ELISA Dilution Buffer (Component B) to a 15 mL conical tube. Label the conical tube "2°AB".
2. Carefully remove the stopper from the vial of Secondary Antibody (Component E). Transfer 0.5 mL from the conical tube "2°AB" to the vial. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of reconstituted Primary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "2°AB" and dispense 1.3 mL per tube.

Preparation of TMB Substrate

- Dispense 1.3 mL of TMB Substrate (Component F) into 10 microcentrifuge tubes. Label the tubes "Substrate".

Preparation of Stop Solution

- Dispense 1.3 mL Stop Solution (Component G) into 10 microcentrifuge tubes. Label the tubes "Stop".

Each Lab Group Should Receive:

- 2 12-well strip tubes
- 1 Beaker of Wash Buffer (55 mL)
- 1 Tube Dilution Buffer (1 mL)
- 1 Tube A β (0.15 mL)
- 1 Tube Positive Control (175 μ L)
- 1 Tube Negative Control (175 μ L)
- 1 Tube Control Patient Sample (175 μ L)
- 1 Tube AD Patient Sample (175 μ L)
- 1 Tube Primary Antibody (1.3 mL)
- 1 Tube Secondary antibody (1.3 mL)
- 1 Tube Substrate (1.3 mL)
- 1 Tube Stop Solution (1.3 mL)
- 1 Transfer pipet
- 1 Empty beaker for waste

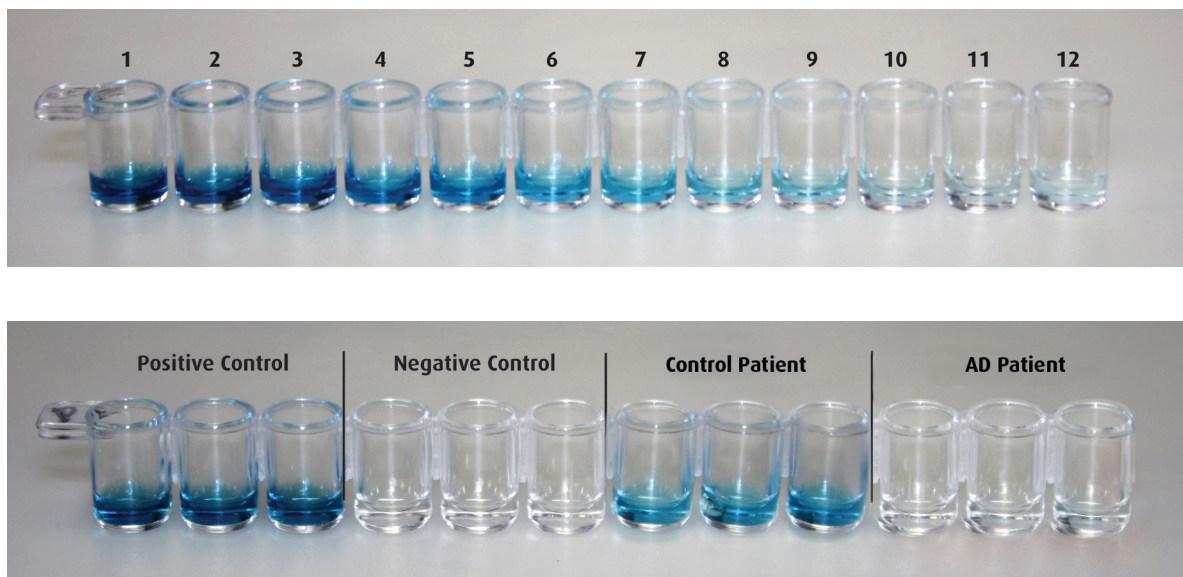
Avoiding Common Pitfalls

1. Students should be advised to be very careful when transferring solutions into and out of the 12-well strips.
2. Use only clean or appropriately labeled pipets.
3. Do not attempt to empty the 12-well strips by shaking it out. This will not work - it will result in contaminating adjacent wells.
4. Wash the wells gently and slowly, without force.

Experiment Results and Analysis

The presence or absence of A β , and its levels in each patient population, should be confirmed in the positive and negative control samples. A positive reaction, as indicated by a blue color in the well, indicates infection with the bacteria. Conversely, samples from negative patients will show no color change.

Patient samples should be compared to the standard curve to determine the concentration of antigen in each reaction. Typical results from an example experiment can be seen below:



We encourage your students to look at biomarker research for Alzheimer's Disease as an extension activity. We suggest starting at PubMed, the NIH's database for scientific journals: www.ncbi.nlm.nih.gov/pubmed and searching for Alzheimer's Disease biomarkers.

If your class is interested in doing other experiments related to neurodegenerative diseases, we recommend Cat# 1115 Detecting Risk Factor's for Alzheimer's Disease Using Western Blot, and Cat# 1125 Diagnosing Huntington's Disease Using PCR.

**Please refer to the kit
insert for the Answers to
Study Questions**