EDVOTEK & PLTW Experiment #468

Mystery Infection

(MI 1.1.5)

Experiment Objective:

In this experiment, students will master the experimental concepts and methodology involved with a quantitative enzyme-linked immunosorbent assay (ELISA). This ELISA experiment is designed to simulate the quantification of bacterial meningitis antigens in the cerebral spinal fluid (CSF) of patients.

See page 3 for storage instructions.

Version 468.190508

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Table of Contents

Experiment Components

	COMPONENTS	Storage	Check (√)
A	10x ELISA Wash Buffer (PBST)	Refrigerator	❑
B	ELISA Dilution Buffer	Refrigerator	❑
C	Whey Antigen (Lyophilized)	Refrigerator	❑
D	Anti-Whey Primary Antibody (Lyophilized)	Refrigerator	❑
E	Secondary Antibody (Lyophilized)	Refrigerator	❑
F	TMB Substrate	Refrigerator	❑
G	Stop Solution	Refrigerator	❑

REAGENTS & SUPPLIES

• Small transfer pipets	Room Temperature	❑
• Strip tubes (12 well)	Room Temperature	❑
• 15 mL Plastic tube	Room Temperature	❑
• 1.5 mL Snap-top tubes	Room Temperature	❑

Experiment #468 is designed for 10 groups.

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.

Requirements

- Paper towels
- Distilled or deionized water
- Beakers or flasks
- Lab glassware
- Disposable lab gloves
- Safety goggles
- Adjustable volume micropipettes (50 µL volume) and tips

Introduction

Given Sue's diagnosis, all of the patients from the past two days need to be called back in for immediate testing. School officials are concerned about a possible outbreak of bacterial meningitis on campus. In order to diagnose bacterial meningitis, it is necessary to obtain a sample of cerebral spinal fluid using a spinal tap. Since this procedure is extremely invasive and painful, only those patients doctors feel are at greatest risk for the disease will be tested. Use the spinal fluid samples to identify those who are infected with meningitis and to trace how this disease may have spread amongst the students on campus. Devise a plan to halt the spread of the disease before it is too late!

In Human Body Systems, you investigated the workings of the immune system and learned how antibodies, specific proteins produced in response to invading antigens, circulate to keep us healthy. Antibodies seek out and attach themselves to invaders, flagging them for destruction by the immune system. These antigens are molecules foreign to the body and can include bacteria, viruses and fungi. Since antibodies are extremely specific to the antigens they attack, these proteins can be used in the laboratory to help identify disease agents. One test, the Enzyme-Linked Immunosorbent Assay (ELISA), combines targeted tagging with antibodies and an enzyme reaction that produces a visible color change to test for the presence of disease antigens or antibodies produced in response to that antigen. The ELISA assay can even detect disease agents in body fluids before the body has a chance to mount an immune response and produce antibodies. An ELISA can provide **qualitative results**, indicating whether a patient is positive or negative for the presence of the antigen or antibody, or an ELISA can provide **quantitative results**, determining how much of the detected substance is present.

In this lab, you will use ELISA to test simulated cerebral spinal fluid (CSF) samples taken from patients at Sue's school for the presence of bacterial meningitis. This rapid test can be completed in less than one hour and can detect antigens of the *Neisseria meningitidis* bacteria. Your job is to determine which college students are infected with this deadly bacterium and to propose a strategy for halting further spread. For those who are infected, use quantitative data to propose a chain of infection. Use data from the ELISA test and from the patient histories to trace how this disease may have spread from person to person.

Background Information

Antibodies (also called immunoglobulins, or Igs) are specialized proteins that allow the immune system to distinguish between "self" and "non-self" proteins or polysaccharides. These Y-shaped molecules comprise four linked polypeptide chains: two identical "heavy chains" and two identical "light chains" (Figure 1). The antigen binding sites are located at the ends of the short arms of the Y. The amino acid sequence region is variable, allowing for each antibody to recognize a unique **epitope** (a particular location within an antigen).

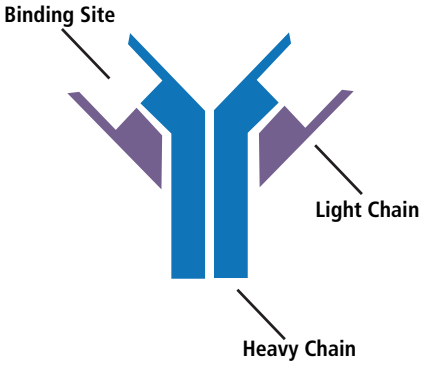


Figure 1: Structure of an antibody

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. This solution will contain a mixture of antibodies because different immune cells will create antibodies that recognize different epitopes of the antigen. This heterogeneous mixture of antibodies is called a **polyclonal antibody**. If we isolate and culture individual immune cells from these animals, we can create **monoclonal antibodies**. These antibodies are directed against a single epitope, and thus are very specific. Because of their specificity, monoclonal antibodies can be used to detect the presence of specific biomolecules (i.e. peptides, proteins, antigens and hormones) in a complex sample. To quantitatively detect the presence of molecules within a sample, scientists use the Enzyme Linked Immunosorbent Assay (ELISA). These samples can be single proteins or complex mixtures like cellular lysates. The ELISA is commonly used for medical diagnostics, as it can be used to identify antigens in blood, urine, spinal fluid, and other biological samples. An ELISA can be designed to provide qualitative or quantitative results. In a qualitative ELISA, the results will indicate if a sample is positive or negative for the antigen. This type of assay is simple to perform and is useful for situations where the exact concentration of molecules is not necessary, such as pregnancy or drug tests. Alternatively, quantitative ELISAs use a standard curve to determine the precise concentration of a substance in the sample.

The traditional ELISA requires two antibodies. One antibody, called the **primary**, recognizes the antigen of interest. For example, an ELISA that detects the HIV virus would use an antibody that recognizes one of the virion's coat proteins. 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 2A). HRP has a high catalytic activity – its substrate turnover rates exceed 10^6 per second – allowing us to quickly detect even the smallest amount of antigen.

To perform an ELISA, the samples are added to the wells and the antigens are allowed to adsorb to the surface through hydrophobic associations (Figure 2B). ELISAs are performed in transparent plastic microtiter plates, which allow scientists to easily visualize the results. These plates contain many small wells into which the samples are deposited and analyzed.

Background Information, continued

In this experiment, positive samples will contain simulated meningitis antigens that can stick to the wells. After briefly washing the wells to remove unbound sample, the primary antibody is added to each well. If the antigen is present, the primary antibody will bind to it and remain attached after washing. Next, a secondary antibody is added and will only adhere where primary antibody has already bound. Finally, the substrate TMB is added to each well - if the secondary antibody is present the HRP enzyme will catalyze a reaction that produces a colored product from the TMB. The data collected will allow students to diagnose patients as positive or negative for bacterial meningitis, and to quantify the levels of bacterial antigen in the positive patients.

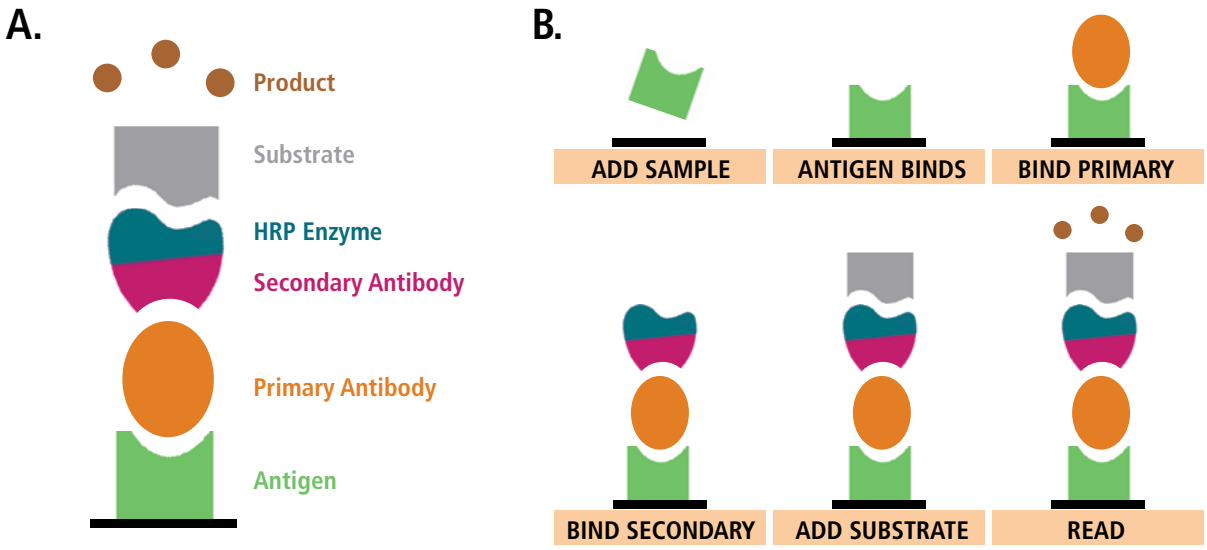


Figure 2: Optimized ELISA workflow

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will master the experimental concepts and methodology involved with a quantitative enzyme-linked immunosorbent assay (ELISA). This ELISA experiment is designed to simulate the quantification of bacterial meningitis antigens in the cerebral spinal fluid (CSF) of patients

OVERVIEW

The ELISA will utilize 12-well plastic strips – the first strip will be used to create a standard curve, while the second strip will allow you to quantify the amount of antigen in control and patent samples.

The standard curve will be created using a 100 µg/mL solution of antigen and dilution buffer. At every dilution you will cut the concentration of antigen in half, resulting in a wide range of protein concentrations across the dilution series. This standard curve will then be compared to control and patient samples to determine who is expressing the bacterial meningitis antigen.

During the experiment you will use an adjustable volume pipette to add the different components to the plastic strips. It is important to use good, accurate pipetting techniques to ensure the correct amount is added to each well. In addition, replacing the tip between components and using care to prevent overflowing the wells during the washes will help to prevent cross-contamination.

Before starting the Experiment:

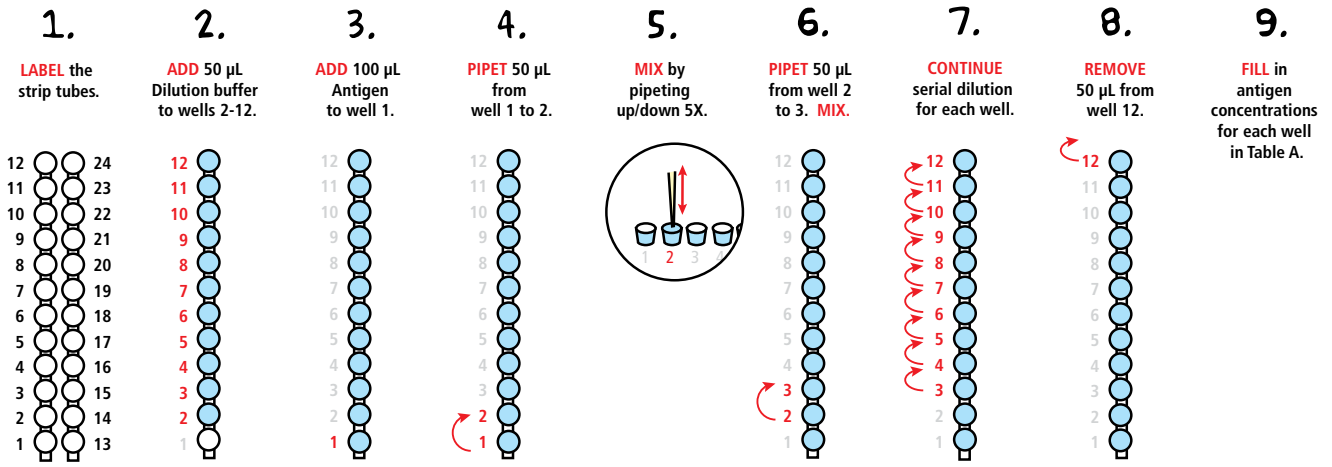
- Carefully read the introduction and student protocol.
- Examine the Student Worksheet (page 16) and ensure that you have all of the required components and understand how they will be used.

Laboratory Safety:

- Gloves and goggles should be worn at all times.
- Do not mouth pipet reagents - use adjustable volume pipettes or pipet pumps to measure and transfer liquids.

Wear gloves and safety goggles

Module 1: Performing a Quantitative ELISA



Preparation of Standards:

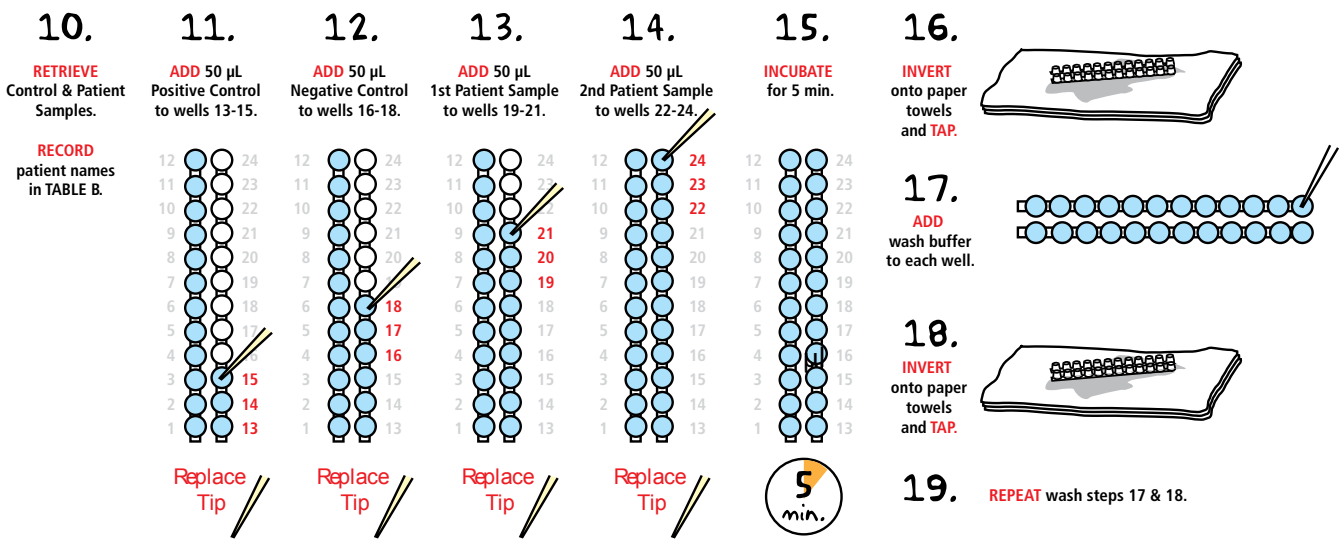
1. **OBTAIN** two 12-well strip tubes. Using a fine-tipped marker, **LABEL** one strip with numbers 1-12 and the other with numbers 13-24. Set the strip with wells 13-24 off to one side for now.
2. Use a micropipette to **ADD** 50 µL Dilution Buffer (Dil. Buf. - pink label) to wells 2-12.
3. **ADD** 100 µL of Antigen (yellow label) to well #1. The antigen is provided at a concentration of 100 µg/mL. ***NOTE: You can pipette 50 µL into the well twice to reach 100 µL.***
4. **PIPETTE** 50 µL from well #1 into well #2.
5. Fully **MIX** the sample by gently pipetting up and down 5 times.
6. Using the same pipette tip, **TRANSFER** 50 µL from well #2 into well #3. **MIX** as in step 5.
7. Continue to serially **DILUTE** the remaining samples through well #12.
8. **REMOVE** and **DISCARD** 50 µL of the diluted antigen from well #12.
9. 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 (page 16).

Wear gloves and safety goggles

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 1: Performing a Quantitative ELISA, continued



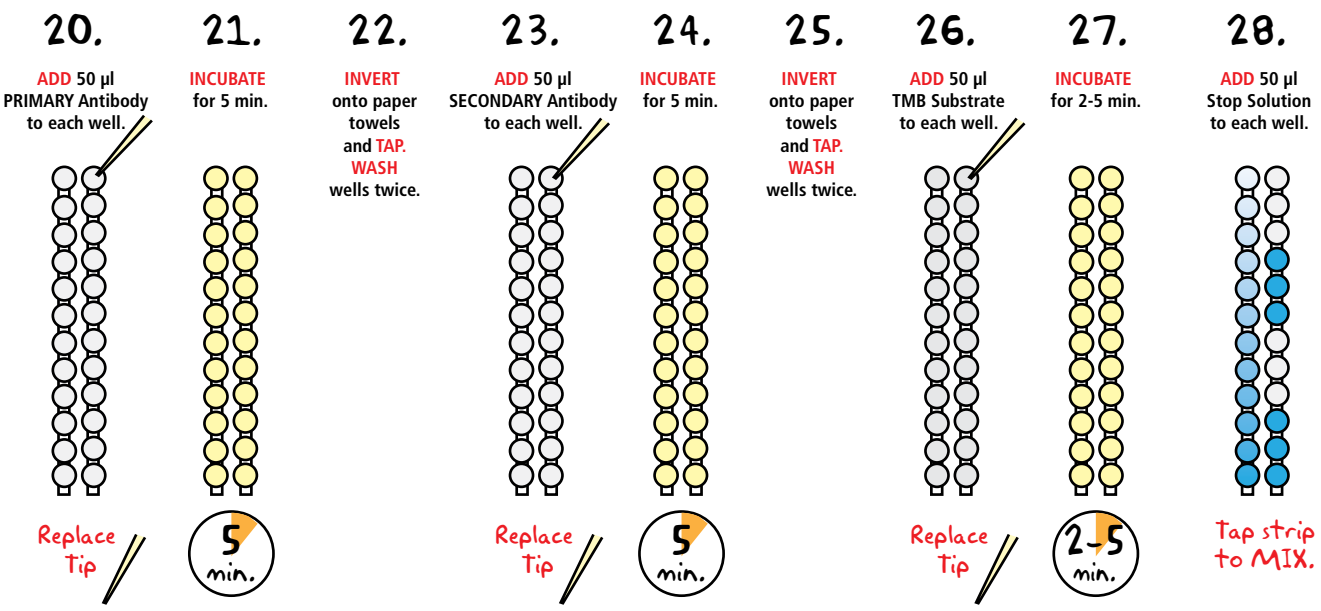
Loading the Control and Patient Samples:

10. **RETRIEVE** the Control and Patient Samples (white labels) provided by your instructor. **RECORD** the names or letter code of the patients in the indicated fields on the student worksheet (page 16).
11. Using a micropipette, **ADD** 50 µL of Positive Control (+ CTRL) sample to wells 13-15. **REPLACE** the pipette tip.
12. **ADD** 50 µL of Negative Control (- CTRL) sample to wells 16-18. **REPLACE** the pipette tip.
13. **ADD** 50 µL of your first Patient Sample to wells 19-21. **REPLACE** the pipette tip.
14. **ADD** 50 µL of your second Patient Sample to wells 22-24.
15. **RETRIEVE** the Standard Curve strip from Step 9. **INCUBATE** both strips for 5 minutes at room temperature.

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, 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**.

Module 1: Performing a Quantitative ELISA, continued



Addition of Primary and Secondary Antibodies:

20. Using a new micropipette tip, **ADD** 50 µL of Primary Antibody (1°AB - green label) to each well.
21. **INCUBATE** for 5 minutes at room temperature.
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 - orange label) to each well.
24. **INCUBATE** for 5 minutes at room temperature.
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 TMB substrate (blue label) 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.
28. Using a new micropipette tip, **ADD** 50 µL of Stop Solution (brown label) to each well. Gently tap tubes to **MIX**.
29. **PROCEED** immediately to Module II: Analysis of Quantitative ELISA.

Module 2: 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. **DETERMINE** an initial positive or negative diagnosis for the patient samples and **RECORD** it below or in your lab notebook.
   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, **ESTIMATE** the concentration of your patient samples and record the results in Table B.
3. **SHARE** your findings with other groups and record the concentration for each patient in Table C.

TABLE B: Identification of Patient Samples

Well #	13	14	15	16	17	18
Sample Name	Positive Control			Negative Control		
Concentration						
Well #	19	20	21	22	23	24
Sample Name						
Concentration						
Diagnosis						

Table C: Concentration of Patient Samples

Patient	Concentration
Sue	
Jill	
Anthony	
Wanda	
Maggie	
Maria	
Arnie	
Marco	
Alvin	

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATIONS:

Some of the components can be prepared ahead of time, aliquoted, and stored in the refrigerator (4° C) until needed. Adhesive labels have been provided for use on the lids of the microcentrifuge tubes to assist with labeling. See the table below for information on advanced preparation of reagents. A Student worksheet has been provided (page 16) to help students collect the necessary reagents and perform the experiment.

Component:	What to do:	When:	Label Color:
10X ELISA Wash Buffer (A)	Dilute to 1X solution and aliquot	Anytime before the experiment. Cover and store in the refrigerator.	------
ELISA Dilution Buffer (B)	Aliquot for standards and samples (table 2)	Anytime before the experiment. Store tubes in the refrigerator.	Dilution = Pink (10 tubes) Samples = White (32 tubes)
Whey Antigen (C)	Rehydrate and aliquot for Antigen and positive control	Up to one week before performing the experiment.	Antigen = Yellow (10 tubes) Positive = White (10 tubes)
Anti-Whey Primary Antibody (D)	Rehydrate and aliquot	Up to one week before performing the experiment.	Green (10 tubes)
Secondary Antibody (E)	Rehydrate and aliquot	On the same day as performing the experiment.	Orange (10 tubes)
TMB Substrate (F)	Aliquot	Up to one week before performing the experiment.	Blue (10 tubes)
Stop Solution (G)	Aliquot	Up to one week before performing the experiment.	Brown (10 tubes)

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

MODULE I: PERFORMING A QUANTITATIVE ELISA

Preparation of Wash Buffer

1. Add contents of the 10x ELISA Wash Buffer (PBST) (A) to 525 mL of distilled water. Mix well.
2. Dispense 55 mL of the diluted Wash Buffer into a small beaker for each group. Label the beakers "Wash buffer".

Preparation of Dilution Buffer:

- Dispense 1 mL of the ELISA Dilution Buffer (B) into 10 microcentrifuge tubes and label as "Dilution Buffer" using the pink "Dil. Buf." labels. Reserve the remaining buffer for preparation of the antigen samples.

Preparation of Control and Patient Samples:

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label the tube as "Antigen".
2. Transfer 0.5 mL of the ELISA Dilution Buffer from step 1 into the vial of Whey Antigen (C). Pipette up and down or vortex to mix.
3. Transfer the entire contents of the reconstituted Whey Antigen back into the 15 mL conical tube.
4. Dispense 120 µL of the Whey Antigen into 10 microcentrifuge tubes. Label the tubes as "Antigen" using the yellow labels.
5. Dispense 175 µL of Whey Antigen into an additional 10 microcentrifuge tubes. Label the tubes as "Positive Control" using the white "+ CTRL" labels.
6. Dispense 175 µL of the Dilution Buffer (B) into 10 microcentrifuge tubes. Label the tubes as "Negative Control" using the white "- CTRL" labels.

After rehydration, the whey antigen stock will be a 100 µg/mL solution, and must be diluted to create the patient samples. Label one microcentrifuge tube with each patient name and prepare the indicated samples using Dilution Buffer (B) and Whey Antigen. The patients' identities can be revealed at the start of the lab by using the provided white labels or obscured by using a simple "A" through "I" marking directly on the microcentrifuge tube.

7. To prepare the positive simulated patient samples (Sue, Jill, Maria, and Marco), perform the indicated dilutions in **Table 2** using unlabeled microcentrifuge tubes. Each tube will contain enough sample for up to 3 groups. Briefly vortex or flick the tubes to mix.
8. Using the provided white labels, label 3 microcentrifuge tubes for each of the positive patients. Aliquot 175 µL of each sample into the appropriate tubes.
9. To prepare the negative patient samples, label 2 microcentrifuge tubes for each of the negative patients and aliquot 175 µL of Dilution Buffer (B) into the appropriate tubes.

Each group should receive one positive and one negative patient, randomly distributed to ensure each patient is tested at least twice.

Patient and control samples can be made up to a week before performing the experiment. Store on ice or refrigerate until needed. ***DO NOT FREEZE.***

	Patient Name	Vol. of Dilution Buffer (B)	Vol. of Antigen (C)	Final Concentration	Number of Aliquots (175 µL/tube)
Positive	Sue - A	300 µL	300 µL	50 µg/mL	3
	Jill - B	525 µL	75 µL	12.5 µg/mL	3
	Maria - C	580 µL	20 µL	3.33 µg/mL	3
	Marco - D	590 µL	10 µL	1.66 µg/mL	3
Negative	Maggie - E	400 µL	---	---	2
	Anthony - F	400 µL	---	---	2
	Arnie - G	400 µL	---	---	2
	Wanda - H	400 µL	---	---	2
	Alvin - I	400 µL	---	---	2

Table 2: Dilutions of Patient Samples

Preparation of the Primary Antibody

1. Transfer 14 mL of ELISA Dilution Buffer (B) into a 15 mL conical tube. Label the tube as "Primary Antibody".
2. Transfer 0.5 mL of the ELISA Dilution Buffer from step 1 into the vial of Anti-Whey Primary Antibody (D). Pipette up and down or vortex to mix.
3. Transfer the entire contents of the reconstituted Primary Antibody back into the 15 mL conical tube and invert.
4. Dispense 1.3 mL of the Primary Antibody into 10 microcentrifuge tubes. Label the tubes as "Primary Antibody" using the green "1°AB" labels. Store on ice or refrigerate until needed. ***DO NOT FREEZE.***

Preparation of the Secondary Antibody

Prepare on the same day as needed for the experiment.

1. Transfer 14 mL of ELISA Dilution Buffer (B) into a 15 mL conical tube. Label the tube as "Secondary Antibody".
2. Transfer 0.5 mL of the ELISA Dilution Buffer from step 1 into the vial of Secondary Antibody (E). Pipette up and down of vortex to mix.
3. Transfer the entire contents of the reconstituted Secondary Antibody back into the 15 mL conical tube.
4. Dispense 1.3 mL of the Secondary Antibody into 10 microcentrifuge tubes. Label the tubes as "Secondary Antibody" using the orange "2°AB" labels. Store on ice or refrigerate until needed. Do not freeze.

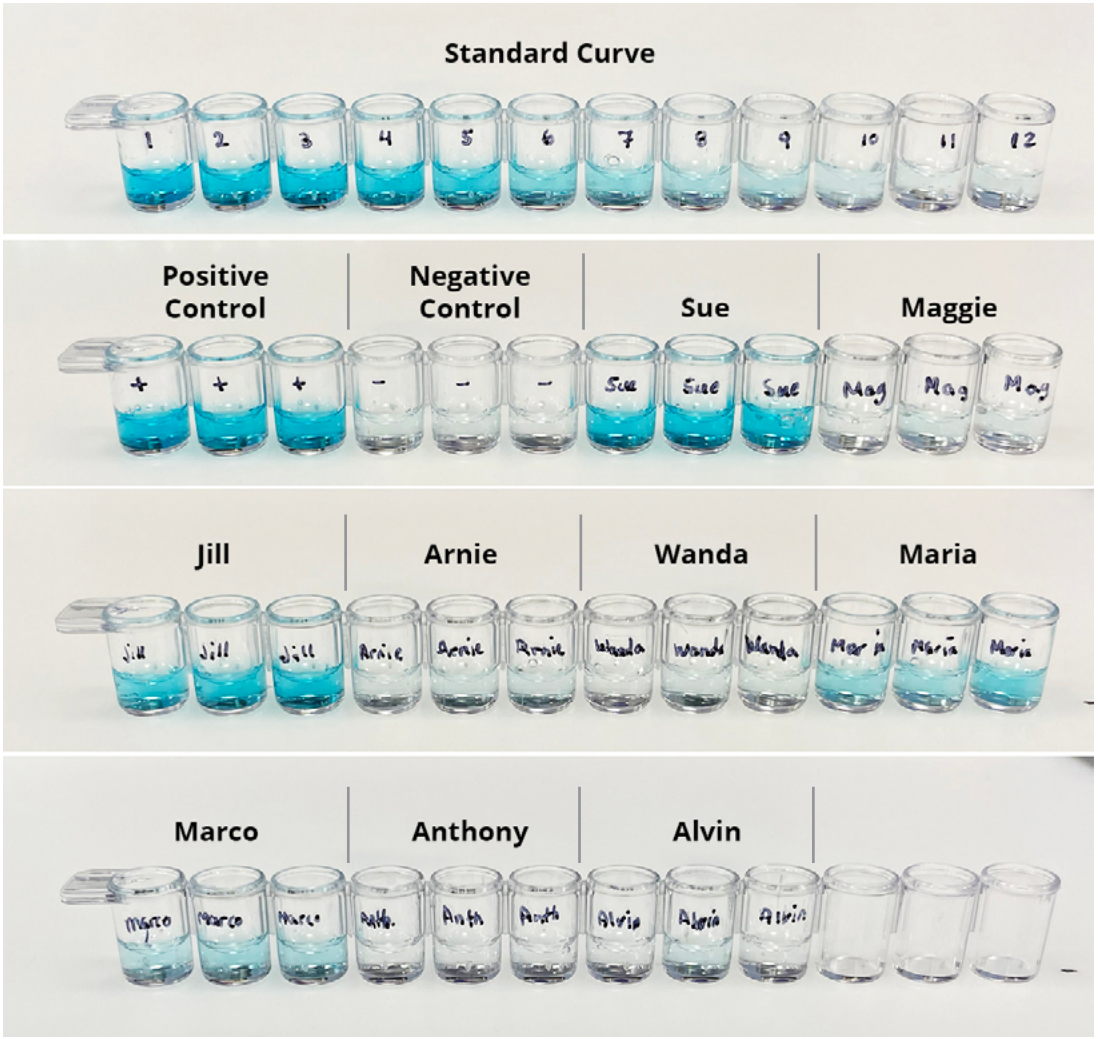
Preparation of TMB Substrate and Stop Solution

1. Dispense 1.3 mL of TMB (F) into 10 microcentrifuge tubes. Label the tubes as "TMB" using the blue labels.
2. Dispense 1.3 mL of Stop Solution (G) into 10 microcentrifuge tubes. Label the tubes as "Stop" using the brown labels.
   NOTE: Stop Solution can precipitate when stored at 4° C. If precipitate is seen, gently warm the solution to resuspend before aliquoting.

Experiment Results and Analysis

The presence or absence of the *Neisseria meningitidis* antigen 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:



Appendix A: Student Worksheet

Each Student Group should receive:		Label	Check (✓)
Amt.	*Component*		
1	Tube containing 120 µL Antigen	Yellow	❑
4	Tubes containing 175 µL of the experimental samples: positive control, negative control, patient 1, patient 2	White	❑
1	Tube containing 1.3 mL of the Primary Antibody	Green	❑
1	Tube containing 1.3 mL of the Secondary Antibody	Orange	❑
1	Tube containing 1.3 mL of the TMB Substrate	Blue	❑
1	Tube containing 1.3 mL of the Stop Solution	Brown	❑
1	Tube containing 1 mL Dilution Buffer	Pink	❑
1	Beaker containing approximately 45 mL Wash Buffer	---	❑
1	Adjustable volume micropipette and tips	---	❑
1	Small transfer pipet	---	❑
2	12-well strip tubes	---	❑
1	Stack of paper towels for washes and waste	---	❑

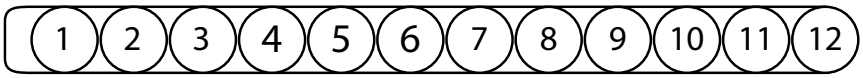
TABLE A: Dilutions and Concentrations

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

PATIENT IDENTIFICATION

Patient 1: _____ **Patient 2:** _____

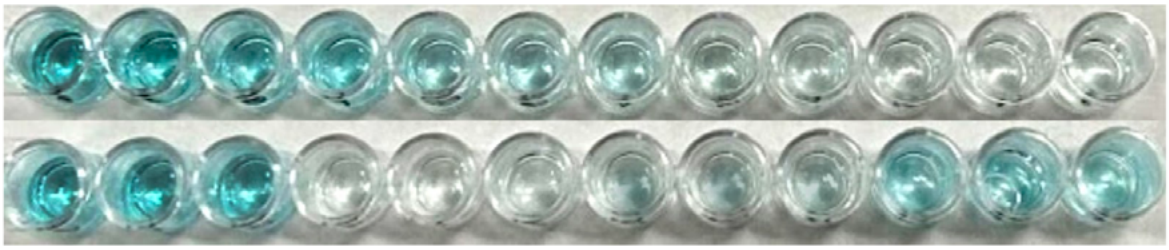
Strip Tube Templates:
Strip tubes may be placed on the templates below to help keep track of the identity of each well.



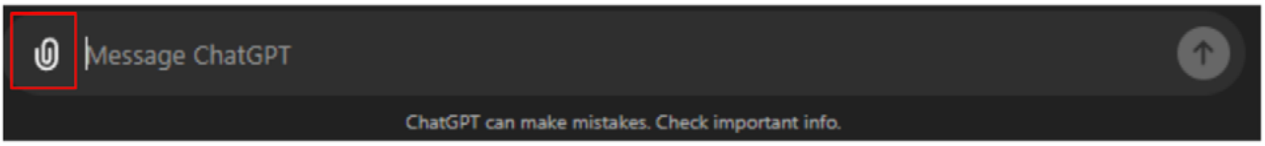
Appendix B:
Using ChatGPT to Analyze Your ELISA

ChatGPT can be used to analyze the results of your quantitative ELISA. The process uses an image of your ELISA strips and examines the intensity of color in each sample. Follow the steps below to use AI to examine your results!

1. Properly **CROP** your ELISA results. Your image should contain only the wells on your microtiter plate and nothing else. This is important because ChatGPT will create a grid over the image and if it's not properly cropped the grid will be misaligned. A properly cropped image can be found at **https://www.edvotek.com/site/pdf/ELISA_example.pdf**



2. Once you have your image cropped, **OPEN** chatGPT. **UPLOAD** the image you just cropped by clicking on the attachments button (the paperclip) in the bottom left corner next to the chat box.



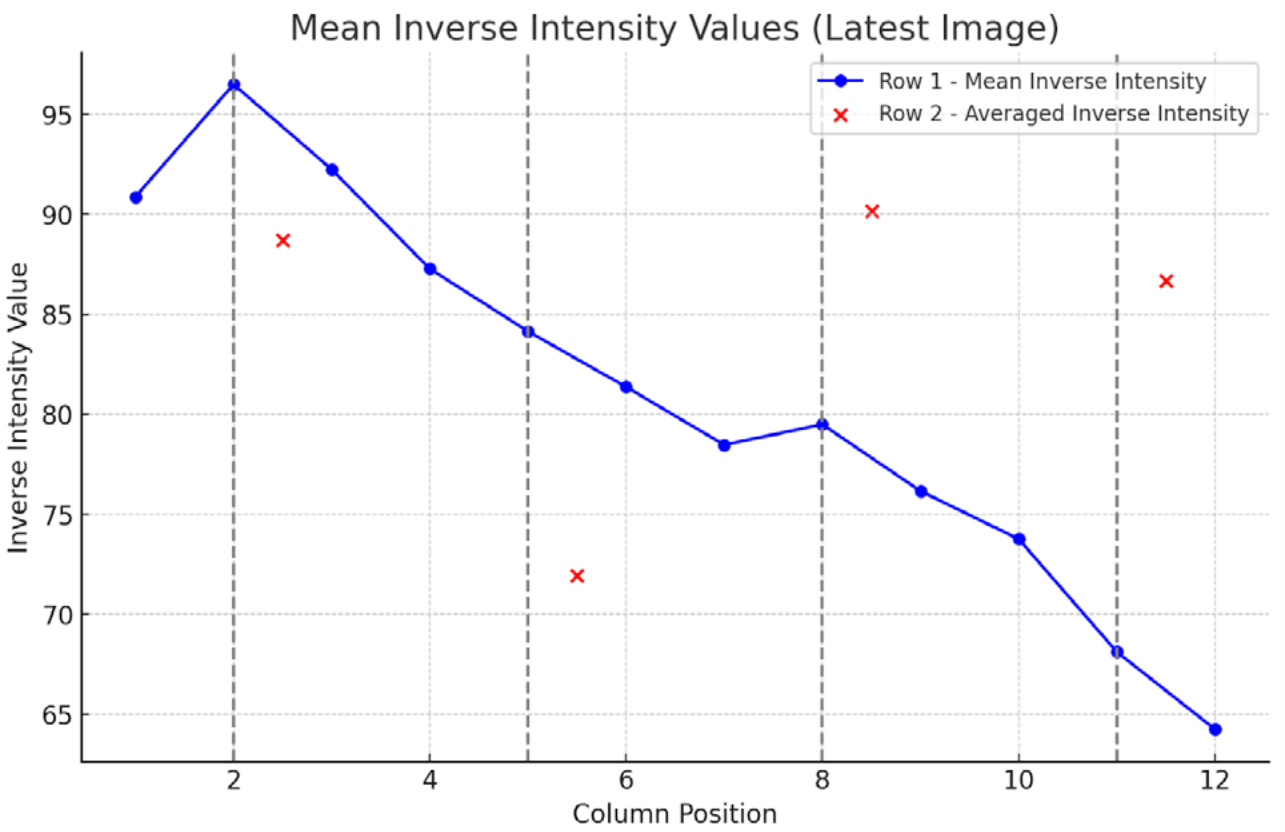
Then, **COPY AND PASTE** the following prompt into the chatbox:

"Create a 12x2 grid over this image. Then, only for the second row, find the average mean inverse pixel intensity value for columns 1, 2, and 3, and then find the average mean inverse pixel intensity value for columns 4, 5, and 6, and then find the average mean inverse pixel intensity value for columns 7, 8, and 9, and then find the average mean inverse pixel intensity value for columns 10, 11, and 12. Then create a line graph of the mean inverse intensity values of the first row and a dot graph of the averages of the mean inverse intensity values of the second row. Put both the dots and line on one graph with the x axis showing the row position and the y-value showing the inverse intensity value for each point. Put vertical lines on the same graph at the following x values: 2, 5, 8, 11. Finally, provide a chart with all data values."

Continued

Appendix B:
Using ChatGPT to Analyze Your ELISA, continued

Be patient, this can sometimes take some time. The results should look something like this:



The graph will show the top row of the ELISA, representing your standard curve, as a blue line. The triplicate samples will be graphed as red points that represent the average value. Finally, the program should provide a list of the data for you to use to determine the value of the unknown samples.