Using Immunology as a Model to Teach Cell Communication

Including: A Practical ELISA For A Typical High School Biology Classroom

"Determination of a Standard Curve and Unknown Concentration of Monoclonal Antibody Using ELISA"

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Abstract:

This educational unit "Using Immunology as a Model to Teach Cell Communication" is developed for advanced high school biology classes (i.e. AP Biology or a second year course) to explore the inherent connections within the fields of Cell Communication and Immunology. This unit explores the processes involved in cell signaling, while simultaneously introducing the students to various levels of vertebrate immune response. Prior to completing the culminating laboratory activity, the students will gain knowledge of the two interrelated fields through a variety of lectures, written assignments, and a computer lab simulation. The final laboratory experiment, "*Determination of a Standard Curve and Unknown Concentration of Monoclonal Antibody Using ELISA*", was developed for a "typical" high school setting and budget; and was designed to complete a macro-scale ELISA (Enzyme-linked Immunosorbent Assay) using typical high school lab equipment. Upon conclusion of this curriculum, students will have developed a deeper understanding of not only Immunology and Cell Communication pathways, but also the concepts underlying an ELISA as a common lab assay used in the fields of immunology and medicine.

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Overview:

This curricular unit is designed to present students in a "typical" high school setting with the following aspects of Cell Biology and Immunology:

- An overview of cell-to-cell recognition using various cell surface molecules
- An overview of cell signaling
- Signal reception and the initiation of transduction
- Signal transduction pathways
- Cellular responses to signals
- Nonspecific defenses against infection based on cell signaling
- Specific immune responses based on cell signaling
- An overview of ELISA (Enzyme-Linked Immunosorbent Assay) assays and their importance
- Quantitative ELISA assay to determine standard and unknown concentrations of monoclonal antibodies (mAb)

The nonspecific and specific human immune responses function greatly on the concepts of cell-to-cell recognition and cell communication pathways. For this reason, the following unit was designed to connect cell-signaling details with the details of the human immune response, including the first, second, and third lines of defense. This unit is developed for Advanced Biology (i.e. a second year course or AP Biology) and should be taught during the instructor's Cell Biology portion of the curriculum.

Science Background:

Teachers should review all areas of cell communication and immunology prior to beginning this unit. Topics for review in the area of Cell Communication include: cell-to-cell recognition, cell signaling, signal reception, transduction, and cellular response as a result of the signal transduction pathway. In regards to the field of Immunology, teachers should review the following topics: first, second, and third lines of defense, specifics of the inflammatory response, phagocytosis, cell-mediated immunity, humoral immunity, and all associated components (i.e. helper T cells, B cells, plasma cells, etc…). Teachers should also review these two disciplines in order to make the inherent connections. A few examples of these "connections" include: (1) the ability of the immune system to recognize "self" and "nonself" based on cell surface molecules to identify foreign cells or molecules, (2) the role of cytokines (i.e. Interleukin 1 and 2) as ligands to trigger signal pathways to elicit a specific immune response, and (3) the concept of antigen recognition by immunoglobulins (also known as antibodies) and the subsequent immune response.

Student Outcomes and Learning Objectives:

Students will be able to:

1. Describe the ligand-receptor interaction and state how such interactions initiate a signal-transduction pathway
2. Compare and contrast G-protein-linked receptors, tyrosine-kinase receptors, and ligand-gated ion channels
3. Describe the three main stages of cell-signaling
4. Describe how signal information is transduced into cellular responses
5. Describe how target cells discriminate among signals and how the same signal can elicit multiple cellular responses

6. List and describe the nonspecific lines of defense in the vertebrate body
7. Distinguish between antigens and antibodies
8. Explain how B cells and T cells recognize specific antigens
9. Describe the mechanism of clonal selection and distinguish between effector and memory cells
10. Distinguish between the primary and secondary immune responses
11. Compare and contrast the structures and functions of cytotoxic T cells and helper T cells
12. Compare the production and functions of class I MHC and class II MHC molecules
13. Distinguish between humoral and cell-mediated immunity
14. Describe the functions of CD4, CD8, cytokines, perforin, interleukin-2 and interleukin-1
15. Diagram and label the structure of an antibody and explain how this structure allows antibodies to recognize and bind to antigens and assist in the destruction and elimination of antigens
16. Describe the production and uses of monoclonal antibodies
17. List some known autoimmune disorders and describe possible mechanisms of autoimmunity
18. Describe the basics of an ELISA assay and practical uses of this type of lab assay

Curriculum Unit Overview:

This curriculum unit includes lectures on cell communication topics and the human immune response. It also includes a computer ELISA lab simulation to be completed in class to introduce the students to the assay prior to the students completing an ELISA in the classroom laboratory, which is also included here. The unit strongly emphasizes the inherent connections between cell communication recognition, signaling, and the human immune system including nonspecific and specific immune responses.

Curriculum Unit Time Requirement

Total Time Requirement = 10 classroom periods (designed for 54 minute class periods)

Overview of 10 Days: (*For details see "Part II: Curriculum Details")

Days 1 and 2:	Cell Communication Lecture and Related Assignment
Days 3, 4, and 5:	Immunology Lecture and Related Assignment
Day 6:	Computer ELISA Lab Simulation from the Internet See Appendix A
Days 7, 8, 9, and 10:	Lab Protocol: "*Determination of a Standard Curve and Unknown Concentrations of Monoclonal Antibody Using An Enzyme-Linked Immunosorbent Assay (ELISA)"* See Appendix B

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Monoclonal Antibody Using ELISA"

Student Prior Knowledge:

Prior to completing the culminating laboratory activity of this unit connecting cell communication with immunology, the students will gain knowledge of the two interrelated fields of Cellular Communication and Immunology through a variety of lectures, written assignments, and a computer lab simulation. The students will understand the following concepts related to the fields of study on which this unit is based.

Cell Communication:

I. An Overview of Cell Signaling
   a. Communicating cells may be close together or far apart
   b. Examples of cell communication (i.e. yeast reproduction, bacterial endospore formation)
   c. The three stages of cell signaling are reception, transduction, and response

II. Signal Reception and the Initiation of Transduction
   a. A signal molecule binds to a receptor protein, causing the protein to change shape
   b. Most signal receptors are plasma membrane proteins

III. Signal-Transduction Pathways
   a. Pathways relay signals from receptors to cellular responses
   b. Protein phosphorylation, a common mode of regulation in cells, is a major mechanism of signal transduction
   c. Certain small molecules and ions (second messengers) are key components of signaling pathways

IV. Cellular Responses to Signals
   a. In response to a signal, a cell may regulate activities in the cytoplasm or transcription in the nucleus
   b. Elaborate pathways amplify and specify the cell's response to signals

Immunology:

I. Nonspecific Defenses Against Infection
   a. The skin and mucous membranes provide first-line barriers to infection
   b. Phagocytic cells, inflammation, and antimicrobial proteins function early in infection

II. How Specific Immunity Arises
   a. Lymphocytes provide the specificity and diversity of the immune system
   b. Antigens interact with specific lymphocytes, inducing immune responses and immunological memory
   c. Lymphocyte development gives rise to an immune system that distinguishes self from nonself

III. Immune Responses
   a. Helper T cells function in both humoral and cell-mediated immunity
   b. In Cell-Mediated Immunity, cytotoxic T cells counter intracellular pathogens
   c. In Humoral Immunity, B cells make antibodies against extracellular pathogens

IV. Immunity in Health and Disease
   a. Immunity can be achieved naturally or artificially
   b. The immune system's capacity to distinguish self from nonself limits blood transfusion and tissue transplantation
   c. Abnormal immune function can lead to disease
   d. Use of the common ELISA assay helps detect antibody levels in blood and urine tests

Curriculum Lessons:

Day #1:	Cell Communication PowerPoint/Lecture	
Day #2:	Cell Communication PowerPoint/Lecture/ Written Assignment	
Day #3:	Immunology PowerPoint/Lecture	
Day #4:	Immunology PowerPoint/Lecture	
Day #5:	Immunology PowerPoint/Lecture/ Written Assignment	
Day #6:	Howard Hughes Medical Institute Internet Lab Simulation	(see Appendix A)
Day #7, 8, 9, 10:	Laboratory Activity: *"Determination of a Standard Curve and Unknown Concentration of Monoclonal Antibody Using ELISA"*	(see "Part III: Laboratory Activity Details" and Appendix B)

Assessments:

Students will be assessed in this unit in a number of different manners. They will be required to complete written assignments on each topic (cell communication and immunology). In addition, the students will complete the computer lab simulation (included in Appendix A) and related questions that will help introduce the students to the concepts of an Enzyme-Linked Immunosorbent Assay prior to completing the lab activity. The aforementioned Laboratory Activity will also be a required assessment of both a student's lab skills as well as the conceptual written analysis of the lab at the completion of the exercise (See Appendix B). At the conclusion of the unit, students will be assessed with a multiple-choice quiz and the topics will also be included on the semester exam of the course.

Using Immunology as a Model to Teach Cell Communication

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"Determination of a Standard Curve and Unknown Concentration of Monoclonal Antibody Using an ELISA"

Overview of Lab Activity:

This lab entitled "*Determination of a Standard Curve and Unknown Concentration of Monoclonal Antibody Using an ELISA"* was designed with the typical high school biology classroom in mind. Most public high school across the United Stated do not have high priced lab equipment like plate readers with built in spectrophotometers and high speed centrifuges; however, most public high school lab departments have a clinical table top centrifuge and basic spectrophotometer. This lab activity was designed to complete a macro-scale ELISA (Enzyme-linked Immunosorbent Assay) using typical high school lab equipment. In addition, this activity will demonstrate the concepts behind an ELISA as a common lab assay used in the fields of immunology and medicine. The students will perform the assay using various standard antibody concentrations and various unknowns antibody concentrations in order to determine the unknown concentrations using a standard curve and analysis.

Student Expectations and Objectives:

1. Students will gain valuable lab techniques including: micropipetting, centrifugation concepts, spectrophotometry, use of a table top orbital shaker and vortex mixer, precision, and the importance of following lab protocols.

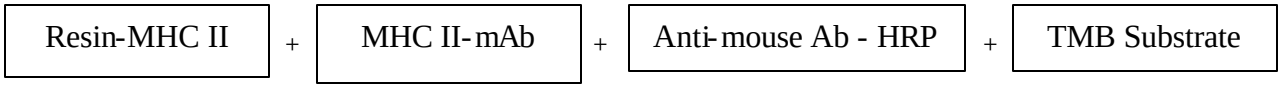
2. Students will also gain valuable knowledge of the key components of Enzyme-Linked Immunosorbent Assays (ELISA) and their usefulness in determining antibody concentrations.

3. Students will learn how to utilize Microsoft Excel to develop a standard scatter plot curve using the standard antibody concentrations, and then use the linear function of Excel to determine unknown antibody concentrations.

Background Information:

Due to the fact that antibodies, secreted by the immune system, are found in the liquid portion of blood, an ELISA (enzyme-linked Immunosorbent assay) is used in many laboratories to determine whether a particular antibody is present in an individual's blood sample. Referring to Figure 1, this lab begins with an antigen (MHC II), which has been conjugated to an affinity resin that can be used in a 15ml conical tube on a macro-scale. The primary antibody (MHCII-mAb) is then introduced to the conjugated resin to allow the antigen and antibody to bind together. Detection then becomes possible when a second antibody (anti-mouse Ab) is added. The secondary antibody has been chemically linked to the enzyme horseradish peroxidase (HRP) to produce a detectable signal (anti-mouse Ab-HRP) as the substrate is introduced. Now that the "resin-antigen-primary antibody-secondary antibody-HRP" complex has been established, the signaling system, i.e. the enzymes' substrate (TMB) is added to induce a color change, which is quantifiable using spectrophotometry. The absorbance readings of the various color solutions reflect the amount of primary antibody present in the various samples. Using the absorbance data and Microsoft Excel, a standard curve can be plotted for the 6 standard antibody concentrations. Finally, the unknown antibody concentrations can be determined using the linear extrapolation of the standard curve.

Figure 1:



Conjugated Resin **Primary Antibody** **Secondary Antibody-Enzyme** **Substrate**

Time Requirements (overview):

Four class periods are needed for the lab protocol and analysis. (This is based on a 54-minute class period.)

Day #1: Preparation of control resin and MHCII-Resin with the primary antibody (i.e. MHCII monoclonal antibody)

Day #2: Introduction of the Secondary Antibody with enzyme attached (i.e. anti-mouse Ab-HRP)

Day #3: Introduction of enzyme substrate (i.e. TMB) and spectrophotometry

Day #4: Analysis: Class Data, Microsoft Excel scatter plot, linear determination of unknown antibody concentrations, and written analysis questions

Equipment List:

1. Clinical Table Top Centrifuge
2. Micropipettes and Tips
3. Spectrophotometer (required 450nm absorbance readings)
4. Orbital Shaker (optional but useful)
5. Vortex Mixer (optional)

Materials per Student Lab Group:

1. 4 – 1.5ml Microcentrifuge Tubes
2. 4 - 15ml Conical Tubes
3. Transfer Pipettes
4. Parafilm Wax (8- ½ inch strips)
5. Permanent Marker
6. Test Tube Rack

Stock Solution/Class of 12 Stations:

(Based on class of 24 students divided into 12 groups consisting of 2 students each)

1. 6 standard primary antibody concentrations (see Teacher Protocol for details)
2. 4 unknown primary antibody concentrations (see Teacher Protocol for details)
3. 1XPBS-Tween solution (see solution preparation for details)
4. Secondary Antibody: Antimouse Ab-HRP (see Teacher Protocol for details)
5. TMB Substrate
6. 1M HCL
7. 1XPBS-5%skim milk solution (see solution preparation for details)

Ordering Information:

Product	Company	Approximate Cost
Resin	GE Healthcare	$166.00 for 25ml
TMB Substrate	Sigma Scientific	$47.10 for 100ml
15ml conical tubes	USA Scientific	$124.00 for 500
1.5ml microcentrifuge tubes	USA Scientific	$30.00 for 500
Tips	USA Scientific	$38.50 for 1000
Transfer Pipettes	USA Scientific	$38.00 for 500

* The MHC II, primary antibody, and secondary antibody-HRP can all be purchased commercially or produced in a university lab setting if available to you. Please email questions to Ann_Brokaw@admin.rockyriver.k12.oh.us regarding these 3 items.

Teacher Preparation Time:

(For details of preparation see **Teacher Protocol**)

Resin Conjugation:

Total time required is approximately 3 hours not including sitting overnight in refrigerator. Please note, this conjugation can be done in advance and kept from year to year (no more than 3 total years conjugated at once.)

Standard and Unknown Antibody Preparation:

Total time for dilutions is approximately 1.5 hours

Secondary Antibody (Antimouse Ab-HRP) Dilution:

Total time for dilution is approximately 30 minutes

1XPBS-Tween:

Total time for dilution is approximately 20 minutes if 1X PBS is already available.
Total time including preparation of 1X PBS is 1.5 hours.

1XPBS-5%Skim Milk:

Total time for dilution is approximately 30 minutes assuming 1XPBS is available. (Can use same 1XPBS as in 1XPBS-Tween preparation above)

Total Preparation Time:

Including Resin Conjugation ~ 6 hours over 2 days

Not including Resin Conjugation ~ 3 hours

Safety Issues:

Lab Gloves are recommended on days #1, 2, and 3 to maintain relatively sterile conditions.

Laboratory Goggles are required for days #1, 2, and 3.

All solutions can be safely poured and rinsed down standard sinks for disposal.

Disposal of all plastic ware (i.e. microcentrifuge tubes, conical tubes) can be safely placed in standard trash receptacles.

Student Protocol:

(See the following pages for a copy of the student protocol. A copy of the student protocol, including analysis questions, is also provided in Appendix B for teacher originals for copying.)

Determination of a Standard Curve and Unknown Concentrations of Monoclonal Antibody Using ELISA

"A Practical ELISA For A Typical High School Biology Classroom"

Student Objectives:

1. Students will understand the concept and procedure of an enzyme-linked immunosorbent assay (ELISA).
2. Students will develop advanced lab skills.
3. Students will understand how to develop a standard curve using Microsoft Excel.
4. Students will understand how to determine unknowns from the standard curve using the Microsoft Excel software.
5. Student will develop an understanding of practical uses of ELISA assays in today's society.

Student Procedure Day #1:

1. Obtain 4 prepared microcentrifuge tubes (2 containing control resin, 2 containing cconjugated MHCII-Resin) from the teacher.

2. Hold the microcentrifuge tubes up to the light and confirm that each tube contains roughly the same amount of resin compared to the other tubes. (The resin has settled to the bottom.)

3. Using a sharpie, label the 4 microcentrifuge tubes:
    a. Label one control tube and one MHCII-Resin tube "Standard"
    b. Label the other control tube and MHCII-Resin tube "Unknown"
    c. Label ALL tubes with your initials and class period

4. Obtain the stock tube of your assigned "Standard" and "Unknown" from the teacher. Do NOT write on these tubes.

5. To each of the "Standard" tubes, add 135ul of the standard MHCII-mAb concentration assigned to your lab group (i.e. SF). Cap the tubes.

6. Change tips on the micropipette!

7. To each of the "Unknown" tubes, add 135ul of the unknown MHCII-mAb sample assigned to your lab group (i.e. UC). Cap the tubes.

8. Change tips on the micropipette!

9. Obtain a stock solution of 1xPBS-5%Skim Milk from the teacher

10. <u>You MUST change tips between each tube in this step</u>. To each of the 4 tubes, add 265ul of 1XPBS-5%Skim Milk solution, so that the total volume in each tube is now 500ul. Cap the tubes. Hold tubes up to confirm that the tubes have roughly the same amount of solution.

11. Invert the tubes gently mixing the solution. Also, flick the tip of each tube gently with your finger to re-suspend the resin into the solution.

12. Seal the 4 tubes with the small pieces of parafilm wax provided.

13. Place the 4 tubes on the orbital shaker for one hour. (The teacher will place tubes overnight in refrigerator after one hour of shaking.)

14. Obtain 4-15ml conical tubes with lids.

15. Label them accordingly:
    a. "Control Standard", the assigned code (i.e. SB), and your initials
    b. "Standard", the assigned code, and your initials
    c. "Control Unknown", assigned code, and initials
    d. "Unknown", assigned code, and initials

16. Place them in assigned tube rack until next day.

17. Properly clean up your lab station.

Student Procedure Day #2:

1. Pipette 10ml of 1XPBS-T (1X PBS-Tween) to each of the 4 conical tubes labeled at the conclusion of day #1.

2. You MUST change tips will each transfer. Transfer the 500ul of resin-MHCII-mAb in each of the 4 microcentrifuge tubes to the respective 15ml conical tube. Try to use the same tip with each tube to use a small amount of the 1X PBS-T to "rinse" out the microcentrifuge tube to minimize the amount of resin lost in the transfer. (The 4 microcentrifuge tubes get properly disposed.)

3. Cap and invert the conical tubes several times to resuspend the resin. (This is the wash step.)

4. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

5. You MUST use a new pipette for each tube. Using small transfer pipettes, carefully remove the supernatant from each tube. Dispose of each pipette after ONE use.

6. Add 10ml of 1XPBS-T to each conical tube.

7. Repeat steps #3-5 above (this is the second wash procedure).

8. Vortex the stock solution of anti-mouse Ab-HRP solution for a few seconds.

9. Add 1ml anti-mouse Ab-HRP (in 1XPBS) to each of the 4 conical tubes.

10. Cap tubes and gently invert tubes to resuspend resin in the anti-mouse Ab-HRP solution.

11. Place the 4 conical tubes on the orbital shaker for one hour. (The teacher will place tubes overnight in refrigerator after one hour of shaking.)

12. Properly clean up your lab station.

Student Procedure Day #3:

1. After removing tubes from the orbital shaker, add 10ml of 1XPBS-T to each conical tube.

2. Cap and invert the conical tubes several times to resuspend the resin. (This is the wash step.)

3. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

4. You MUST use a new pipette for each tube. Using small transfer pipettes, carefully remove the supernatant from each tube. Dispose of each pipette after ONE use.

5. Repeat steps #1-4 above (this is the second wash procedure).

6. <u>You MUST change tips between each tube.</u> Add 300ul TMB substrate to each of the 4 conical tubes. (The TMB substrate should be at room temperature.)

7. Cap tubes and gently shake by hand to mix.

8. Place the tubes on the orbital shaker for 20 minutes. (You should see a color change over this 20 minute time period.)

9. <u>You MUST change tips between each tube.</u> Add 300ul 1M HCl to each of the 4 conical tubes to stop the reaction between the HRP enzyme and the TMB substrate.

10. Cap the tube and gently shake by hand to mix.

11. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

12. <u>You MUST change tips between each tube.</u> Remove liquid supernatant to spectrophotometer cuvettes. ***Keep track of which cuvette has which standard or unknown solution, perhaps line them up in the test tube rack respectively, the cuvettes CANNOT be labeled. ***

13. Add 2.4ml of distilled water to each of the 4 cuvettes to dilute the samples for the spectrophotometer.

14. Calibrate the spectrophotometer with 3ml of distilled water in a separate cuvette.

15. Read and record (Table 1.1) the absorbance of each cuvette at 450nm. ***Be sure to keep track of which cuvette is which. ***

16. Properly clean up your lab station.

<u>Student Procedure Day #4:</u>

1. According to your teacher's instructions, collect class absorbance data for all the standard concentrations and the unknown concentrations. (Table 1.1)

2. Calculate class average absorbance for each standard and each unknown concentration.

3. Using Microsoft Excel, the absorbance values, and the scatter plot function; develop a standard curve for the 6 standard antibody concentrations. (Print the scatter plot.)

4. Using Microsoft Excel, linear extrapolation, and the scatter plot from #2 above, determine the concentration of the 4 unknown antibody concentrations. (Record in Table 1.2)

5. Answer the analysis questions.

Table 1.1: Absorbance data (A^{450}) from ELISA assay for standards MHCII-mAb concentrations and unknown MHCII-mAb concentrations.

A. Raw Data: All Lab Groups Absorbance Reading and Calculated Absorbance Differences:

Period	Group Number	Standard	MHCII-Resin Absorbance	Control-Resin Absorbance	Absorbance Difference	Unknown	MHCII-Resin Absorbance	Control-Resin Absorbance	Absorbance Difference
2nd	1	SA				UA			
	2	SB				UB			
	3	SC				UC			
	4	SD				UD			
	5	SE				UA			
	6	SF				UB			
	7	SA				UC			
	8	SB				UD			
4th	9	SC				UA			
	10	SD				UB			
	11	SE				UC			
	12	SF				UD			
	13	SA				UA			
	14	SB				UB			
	15	SC				UC			
	16	SD				UD			
7th	17	SE				UA			
	18	SF				UB			
	19	SA				UC			
	20	SB				UD			
	21	SC				UA			
	22	SD				UB			
	23	SE				UC			
	24	SF				UD			.

B. Compiled Data: All Lab Groups' Absorbance Differences and Averages:

Absorbance Differences:								
	Result #1	**Result #2**	**Result #3**	**Result #4**				**Average**
SA								
SB								
SC								
SD								
SE								
SF								
	Result #1	**Result #2**	**Result #3**	**Result #4**	**Result #5**	**Result #6**		**Average**
UA								
UB								
UC								
UD								

C. Graphical Analysis:

1. Using Excel develop a scatter plot for the class average absorbance of the 6 standard antibody concentrations. (See example)

2. Add a regression line into the scatter plot (i.e. a trend line).

3. Save your graph and data on the same page. Print this page.

4. Using the graphing calculators, find the equation of the line, and using the absorbance values (Y variable) determine the 4 unknown antibody concentrations (X variable). Fill the values into the following table:

	Unknown A	**Unknown B**	**Unknown C**	**Unknown D**
Primary Antibody Concentration				

D. Analysis Questions:

Directions – answer the following questions in complete sentences in the space provided.

1. Why is the control resin necessary?

2. Identify the following reagent by name:
    a. Primary Antibody =
    b. Secondary Antibody =
    c. Enzyme =
    d. Substrate=

3. Why is each standard and each unknown tests multiple times in all classes?

4. What is the purpose of washing the resin before adding each reagent?

5. What is the purpose of adding the 1M HCl at the end of Day #3? Explain

6. Explain what is meant by a false positive result. Name one error that would result in a false positive result.

7. Explain what is meant by a false negative result. Name one error that would result in a false negative result.

8. Using your ELISA knowledge, the textbook, and the Internet if needed, explain how this particular type of assay is issued to diagnose HIV, the virus associated with AIDS.

Teacher Protocol, Instructions, and Answer Keys:

Determination of a Standard Curve and Unknown Concentrations of Monoclonal Antibody Using An ELISA Assay

"A Practical ELISA Assay For A Typical High School Biology Classroom"

Overview:

This lab is meant to utilize typical high school lab department equipment and supplies to illustrate, on a macro-scale, an ELISA technique so that students are exposed to a common immunological lab experiment. Teacher preparation time is extensive, but materials can be used from year to year if kept in appropriate conditions. In addition, the cost of the resin, the primary antibody, secondary antibody, and substrate can seem somewhat large at first glance, but the materials, if stored properly, can be used for 5 to 10 years. This practical ELISA can be a great experience for your students and can certainly help students develop critical lab skills for future lab work (i.e. micropipetting, centrifugation, spectrophotometry, etc…).

Resin Conjugation:

- Need 200µl of MHCII-Resin and 200µl of control resin per lab group
- Need 2400µl (2.4ml) per class of 24 students in 12 lab groups

MHCII-Resin Conjugation and Control Resin Preparation:

1. Resin (NHS-activated Sepharose 4 Fast Flow) – *using 5 ml of resin and isopropyl alcohol in which it is stored.*
    a. Spin the 5ml in 15 ml Falcon tube down at 1000rpm, 5 minutes
    b. Pipette off alcohol supernatant (waste)
    c. Wash resin with 10-15 times volume of resin with cold 1mM HCl, invert tube to resuspend (*might need to was twice with smaller volumes, resuspend, spin, and remove supernatant each time*)
    d. Spin down each wash, 1000rpm, 5 minutes
    e. Remove wash supernatant (waste)
    f. Neutralize pH with 5ml 1M $NaHCO_3$ invert tube to resuspend
    g. Spin, 1000rpm, 5 minutes
    h. Remove supernatant (waste)

2. Mix MHCII into Resin (pipette in) **OR** If making "control resin," add a volume of ethanolamine to resin equal to the MHCII volume.

3. Add 400µl 2M NaCl

4. Add 400µl 1M $NaHCO_3$

5. Resuspend by inverting tube

6. Shake on orbital shaker in fridge (4C) overnight (2^{nd} option is shake at room temp for 2-4 hours)

7. After shaking overnight, block un-reacted groups
    a. Spin down coupled resin, 1000 rpm, 5 min.
    b. Remove supernatant (waste)
    c. Resuspend in 10ml 0.5M Ethanolamine
    d. Shake for at least 1.5 hours in room temperature

8. Wash Resin-MHCII Coupled Medium
    a. Spin down blocked medium and remove supernatant (waste)
    b. Alternate washes between 2 different buffers (high and low pH)
        i. 0.1M Tris-HCl pH 8-9
        ii. 0.1M Acetate, 0.5M NaCl pH 4-5
    c. Wash 3 volumes buffer:1 volume resin medium
    d. Complete alternate wash cycle repeated 5 times
    e. Store in 1XPBS (store in 1:1 volume resin to 1XPBS)

MHCII-mAb (Primary Antibody) Standards and Unknowns

- MHCII-mAb is the primary antibody for the ELISA
- Prepare the standard and unknown dilutions prior to completing the lab. Store the dilutions in the freezer until day of use.

Standard Dilutions:

1. SA 0.01µg/µl
2. SB 0.005 µg/µl
3. SC 0.001 µg/µl
4. SD 0.0005 µg/µl
5. SE 0.0001 µg/µl
6. SF 0.00005 µg/µl

Unknown Dilutions:

1. UA 0.0073 µg/µl
2. UB 0.0021 µg/µl
3. UC 0.00061 µg/µl
4. UD 0.000089 µg/µl

Anti-mouse Ab-HRP (Secondary Antibody-Enzyme) Dilutions

Use a 1:2,500 Dilution of Stock Antibody-Enzyme Complex from a supply company

TMB Substrate

Use TMB substrate directly from the manufacturer's bottle. No dilution necessary.

Solutions Preparations

1. 10X PBS
    a. Add 10.9g Na_2HPO_4
    b. Add 3.2g NaH_2PO_4
    c. Add 90g NaCl
    d. Add water until the level reaches 1000ml
    e. pH the solution to 7.2

2. 1X PBS
    a. Add 100ml of 10X PBS
    b. Add 900ml of Distilled Water

3. 1X PBS-Tween (1X PBS-T)
    a. For total volume 500ml
    b. Add 49.95ml 10X PBS
    c. Add 449.55ml Distilled Water
    d. Add 0.5ml Tween

4. **1M HCl**
    a. $M_1V_1 = M_2V_2$
    b. Example: Making 50ml of 1M HCl: (12M HCl)(X) = (1M HCl)(50ml), X=4.2ml

5. **1XPBS-5%Skim Milk**
    a. Add 50mg of powdered skim milk
    b. Add 1ml of distilled water
    c. Vortex mixture

Class Lab Group Organization:

This organization is based on a class of 24 students divided into 12 lab groups consisting of 2 students each. Each lab group will be assigned four microcentrifuge tubes. Two of the tubes will contain 200ul of control resin (100µl each), and two of the tubes will contain 200ul of conjugated resin (MHCII-Resin) (100µl each). The lab groups will also be assigned one standard antibody sample and one unknown antibody sample. The assignments are organized into Table 1.2.

Table 1.2

Lab Group	Tube #1 100µlControl Resin + Standard Sample	Tube #2 100µl MHCII-Resin + Standard Sample	Tube #3 100µl Control Resin + Unknown Sample	Tube #4 100µl MHCII-Resin + Unknown Sample
1	SA	SA	UA	UA
2	SB	SB	UB	UB
3	SC	SC	UC	UC
4	SD	SD	UD	UD
5	SE	SE	UA	UA
6	SF	SF	UB	UB
7	SA	SA	UC	UC
8	SB	SB	UD	UD
9	SC	SC	UA	UA
10	SD	SD	UB	UB
11	SE	SE	UC	UC
12	SF	SF	UD	UD

General Preparation Suggestions:

1. Prior to beginning the lab in class, aliquot 100ul of Control Resin to 48 microcentrifuge tubes each, and aliquot 100ul of conjugated MHCII-Resin to 48 additional microcentrifuge tubes. Label all tubes according to the type of resin it contains. (Again, numbers are based on a class of 24 students divided into 12 lab groups consisting of 2 students each.)

2. Prior to starting the lab in class, prepare all standard and unknown antibody dilutions, and aliquot the six standards and 4 unknowns to microcentrifuge tubes so that lab groups have the required volume in a tube separated from the rest of the class. Keep all antibody dilutions frozen until day of use.

3. Prepare stock solutions of the following lab supplies for ease of use for the lab groups:
    a. 1XPBS-5%skim milk
    b. 1XPBS-Tween solution
    c. 1M HCl
    d. Anti-mouse Ab-HRP

4. If possible, place the following lab equipment in a central locations easily accessible by all lab groups:
    a. Table top orbital shaker
    b. Clinical centrifuge(s)
    c. Vortex mixer
    d. Micropipettes (if not enough for every group)

5. Be sure to allow the TMB substrate to warm up to room temperature prior to use.

6. Be sure to allow the spectrophotometer to warm up for at least 15 minutes prior to use.

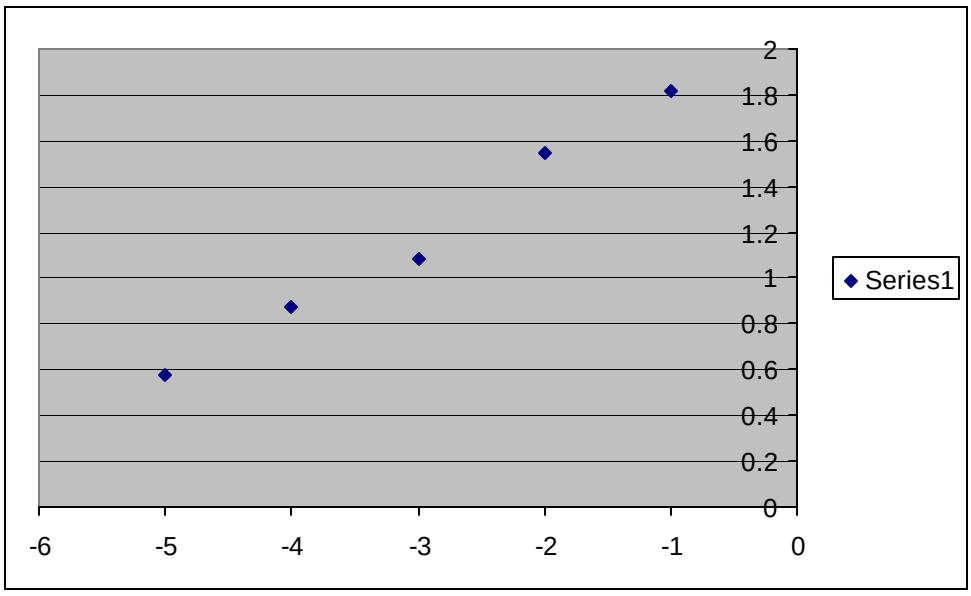
Standard Curve Preparation Suggestions:

1. When using Microsoft Excel, convert the standard antibody solutions to log10 in order to produce a scatter plot. See example below:

mAb	log10 [mAb]	Absorbance*
0.1	-1	1.82
0.01	-2	1.55
0.001	-3	1.08
0.0001	-4	0.87
0.00001	-5	0.58

* this is sample data for illustration purposes only

2. When using Microsoft Excel to develop the scatter plot, use the "Insert Chart" option and select "XY Scatter" as the type of chart to use. See example below based on above sample data:



3. To determine the unknown antibody concentrations, determine the line of regression for the scatter plot (either using Microsoft Excel or a graphing calculator) and obtain the equation of the line ($y = mx + b$). The absorbance readings for each of the unknown antibody samples represents the y in the equation, then solve for x. The x value is the log10 [mAb]; therefore, calculate the inverse log to obtain the actual antibody concentration of the unknown samples.

Appendix A:
Howard Hughes Medical Institute
Biointeractive Lab Simulation
ELISA Computer Activity

Howard Hughes Medical Institute: Biointeractive Lab
Immunology Lab

Introduction:

1. Go to *www.hhmi.org/biointeractive/vlabs/*
2. Scroll down to bottom and click on *Immunology Lab*
3. Maximize the screen if you wish

Diagnosis:

1. Where are antibodies found?

2. How can they be used in the laboratory?

3. What does ELISA stand for?

4. What are ELISA assays used for in labs?

5. What are the 3 important limitations of an ELISA? Explain each.
   a.

   b.

   c.

Background:

1. What determines if a patient has an infectious or autoimmune disease?

2. What does a positive result indicate?

3. The watery fluid of the blood is called _____.

4. What is allowed to react with the target antigen?

5. Detection is possible when _____.

6. Once isolated the secondary antibody can be _____
   _____

7. What is the signaling system?

8. What happens when the appropriate chemical (substrate) is added?

9. How is the test quantified?

10. Therefore, the amount of color reflects _____
_____.

Lab Notebook:
Proceed through the entire lab simulation protocol. Be sure and read captions below the pictures (left side) and the information in the lab notebook (right side).

Be sure to "start over" to begin the lab. You CANNOT skip any steps.

Answer the following questions as you proceed.

1. Define *Systemic Lupus Erythematosus (SLE)*.

2. From Figure 1 (click on it), what are the 4 Steps of an ELISA protocol?
   a.
   b.
   c.
   d.

3. What does a centrifuge do?

4. What are you preparing in Step 2?

5. (Step 3) What has the ELISA plate been pre-treated with? And why?

6. (Step 4) What is the positive control?

7. What is a primary antibody? (define)

8. (Step 4) What is the negative control?

9. (Step 4) Why is it necessary to have a positive and a negative control?

10. (Step 5) Why incubate the plate?

11. (Step 6) Why wash the plate?

12. (Step 7) What is a secondary antibody? (Define)

13. (Step 7) What is the attached enzyme in this assay?

14. (Step 7) What is the specific substrate for HRP? What color does it produce?

15. (Step 10, in "why") How can the yellow color be quantitatively measured? At what wavelength?

16. **Results** (**Indicate on this page and on the computer which boxes turned colors**.)

	A	**B**	**C**	**+ (pos)**	**- (neg)**
1:2					
1:10					
1:100					

17. Did you complete the ELISA correctly? (Yes/No) _____

    If **yes**, proceed to #18.

    If **no**, proceed to #19.

18. What do the results indicate about:
    Patient A _____
    Patient B _____
    Patient C _____

19. Explain what you did wrong and what you will need to do next time. Did your incorrect procedure provide you any results? Explain what went wrong.

Appendix B:
Student Lab Protocol

Determination of a Standard Curve and Unknown Concentrations of Monoclonal Antibody Using ELISA

"A Practical ELISA Assay For A Typical High School Biology Classroom"

Determination of a Standard Curve and Unknown Concentrations of Monoclonal Antibody Using ELISA

"A Practical ELISA Assay For A Typical High School Biology Classroom"

Student Objectives:

1. Students will understand the concept and procedure of an enzyme-linked immunosorbent assay (ELISA).
2. Students will develop advanced lab skills.
3. Students will understand how to develop a standard curve using Microsoft Excel.
4. Students will understand how to determine unknowns from the standard curve using the Microsoft Excel software.
5. Student will develop an understanding of practical uses of ELISA assays in today's society.

Student Procedure Day #1:

1. Obtain 4 prepared microcentrifuge tubes (2 containing control resin, 2 containing MHCII-Resin) from the teacher.

2. Hold the microcentrifuge tubes up to the light and confirm that each tube contains roughly the same amount of resin compared to the other tubes. (The resin has settled to the bottom.)

3. Using a sharpie, label the 4 microcentrifuge tubes:
   a. Label one control tube and one MHCII-Resin tube "Standard"
   b. Label the other control tube and MHCII-Resin tube "Unknown"
   c. Label ALL tubes with your initials and class period

4. Obtain the stock tube of your assigned "Standard" and "Unknown" from the teacher. Do NOT write on these tubes.

5. To each of the "Standard" tubes, add 135ul of the standard MHCII-mAb concentration assigned to your lab group (i.e. SF). Cap the tubes.

6. Change tips on the micropipette!

7. To each of the "Unknown" tubes, add 135ul of the unknown MHCII-mAb sample assigned to your lab group (i.e. UC). Cap the tubes.

8. Change tips on the micropipette!

9. Obtain a stock solution of 1xPBS-5%Skim Milk from the teacher

10. <u>You MUST change tips between each tube in this step</u>. To each of the 4 tubes, add 265ul of 1XPBS-5%Skim Milk solution, so that the total volume in each tube is now 500ul. Cap the tubes. Hold tubes up to confirm that the tubes have roughly the same amount of solution.

11. Invert the tubes gently mixing the solution. Also, flick the tip of each tube gently with your finger to re-suspend the resin into the solution.

12. Seal the 4 tubes with the small pieces of parafilm wax provided.

13. Place the 4 tubes on the orbital shaker for one hour. (The teacher will place tubes overnight in refrigerator after one hour of shaking.)

14. Obtain 4-15ml conical tubes with lids.

15. Label them accordingly:
    a. "Control Standard", the assigned code (i.e. SB), and your initials
    b. "Standard", the assigned code, and your initials
    c. "Control Unknown", assigned code, and initials
    d. "Unknown", assigned code, and initials

16. Place them in assigned tube rack until next day.

17. Properly clean up your lab station.

Student Procedure Day #2:

1. Pipette 10ml of 1XPBS-T (1X PBS-Tween) to each of the 4 conical tubes labeled at the conclusion of day #1.

2. You MUST change tips will each transfer. Transfer the 500ul of resin-MHCII-mAb in each of the 4 microcentrifuge tubes to the respective 15ml conical tube. Try to use the same tip with each tube to use a small amount of the 1X PBS-T to "rinse" out the microcentrifuge tube to minimize the amount of resin lost in the transfer. (The 4 microcentrifuge tubes get properly disposed.)

3. Cap and invert the conical tubes several times to resuspend the resin. (This is the wash step.)

4. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

5. You MUST use a new pipette for each tube. Using small transfer pipettes, carefully remove the supernatant from each tube. Dispose of each pipette after ONE use.

6. Add 10ml of 1XPBS-T to each conical tube.

7. Repeat steps #3-5 above (this is the second wash procedure).

8. Vortex the stock solution of anti-mouse Ab-HRP solution for a few seconds.

9. Add 1ml anti-mouse Ab-HRP (in 1XPBS) to each of the 4 conical tubes.

10. Cap tubes and gently invert tubes to resuspend resin in the anti-mouse Ab-HRP solution.

11. Place the 4 conical tubes on the orbital shaker for one hour. (The teacher will place tubes overnight in refrigerator after one hour of shaking.)

12. Properly clean up your lab station.

Student Procedure Day #3:

1. After removing tubes from the orbital shaker, add 10ml of 1XPBS-T to each conical tube.

2. Cap and invert the conical tubes several times to resuspend the resin. (This is the wash step.)

3. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

4. You MUST use a new pipette for each tube. Using small transfer pipettes, carefully remove the supernatant from each tube. Dispose of each pipette after ONE use.

5. Repeat steps #1-4 above (this is the second wash procedure).

6. You MUST change tips between each tube. Add 300ul TMB substrate to each of the 4 conical tubes. (The TMB substrate should be at room temperature.)

7. Cap tubes and gently shake by hand to mix.

8. Place the tubes on the orbital shaker for 20 minutes. (You should see a color change over this 20 minute time period.)

9. You MUST change tips between each tube. Add 300ul 1M HCl to each of the 4 conical tubes to stop the reaction between the HRP enzyme and the TMB substrate.

10. Cap the tube and gently shake by hand to mix.

11. Place the 4 tubes in the centrifuge, close lid, and spin tubes for 5 minutes at 1000 rpm.

12. You MUST change tips between each tube. Remove liquid supernatant to spectrophotometer cuvettes. ***Keep track of which cuvette has which standard or unknown solution, perhaps line them up in the test tube rack respectively, the cuvettes CANNOT be labeled. ***

13. Add 2.4ml of distilled water to each of the 4 cuvettes to dilute the samples for the spectrophotometer.

14. Calibrate the spectrophotometer with 3ml of distilled water in a separate cuvette.

15. Read and record (Table 1.1) the absorbance of each cuvette at 450nm. ***Be sure to keep track of which cuvette is which. ***

16. Properly clean up your lab station.

Student Procedure Day #4:

1. According to your teacher's instructions, collect class absorbance data for all the standard concentrations and the unknown concentrations. (Table 1.1)

2. Calculate class average absorbance for each standard and each unknown concentration.

3. Using Microsoft Excel, the absorbance values, and the scatter plot function; develop a standard curve for the 6 standard antibody concentrations. (Print the scatter plot.)

4. Using Microsoft Excel, linear extrapolation, and the scatter plot from #2 above, determine the concentration of the 4 unknown antibody concentrations. (Record in Table 1.2)

5. Answer the analysis questions.

A. Raw Data: All Lab Groups Absorbance Reading and Calculated Absorbance Differences:

Period	Group Number	Standard	MHCII-Resin	Control-Resin	Absorbance	Unknown	MHCII-Resin	Control-Resin	Absorbance
			Absorbance	Absorbance	Difference		Absorbance	Absorbance	Difference
2nd	1	SA				UA			
	2	SB				UB			
	3	SC				UC			
	4	SD				UD			
	5	SE				UA			
	6	SF				UB			
	7	SA				UC			
	8	SB				UD			
4th	9	SC				UA			
	10	SD				UB			
	11	SE				UC			
	12	SF				UD			
	13	SA				UA			
	14	SB				UB			
	15	SC				UC			
	16	SD				UD			
7th	17	SE				UA			
	18	SF				UB			
	19	SA				UC			
	20	SB				UD			
	21	SC				UA			
	22	SD				UB			
	23	SE				UC			
	24	SF				UD			.

B. Compiled Data: All Lab Groups' Absorbance Differences and Averages:

Absorbance Differences:								
	Result #1	**Result #2**	**Result #3**	**Result #4**				**Average**
SA								
SB								
SC								
SD								
SE								
SF								
	Result #1	**Result #2**	**Result #3**	**Result #4**	**Result #5**	**Result #6**		**Average**
UA								
UB								
UC								
UD								

D. Graphical Analysis:

1. Using Excel develop a scatter plot for the class average absorbance of the 6 standard antibody concentrations. (See example)

2. Add a regression line into the scatter plot (i.e. a trend line).

3. Save your graph and data on the same page. Print this page.

4. Using the graphing calculators, find the equation of the line, and using the absorbance values (Y variable) determine the 4 unknown antibody concentrations (X variable). Fill the values into the following table:

	Unknown A	**Unknown B**	**Unknown C**	**Unknown D**
Primary Antibody Concentration				

D. Analysis Questions:

Directions – answer the following questions in complete sentences in the space provided.

1. Why is the control resin necessary?

2. Identify the following reagent by name:
   i. Primary Antibody =
   ii. Secondary Antibody =
   iii. Enzyme =
   iv. Substrate=

3. Why is each standard and each unknown tests multiple times in all classes?

4. What is the purpose of washing the resin before adding each reagent?

5. What is the purpose of adding the 1M HCl at the end of Day #3? Explain

6. Explain what is meant by a false positive result. Name one error that would result in a false positive result.

7. Explain what is meant by a false negative result. Name one error that would result in a false negative result.

8. Using your ELISA knowledge, the textbook, and the Internet if needed, explain how this particular type of assay is issued to diagnose HIV, the virus associated with AIDS.