

Title: Identification of E.coli at various dilutions using the Enzyme-Linked Immunosorbent Assay

I. Overview

Two very important mechanisms used by vertebrates to protect against toxins, bacteria, viruses, protozoans and cancer are the specific and the nonspecific mechanisms. In the nonspecific mechanism, the body does not distinguish between various infective agents. It uses the external barrier, the skin and the mucous membranes to prevent the entry of the foreign invaders. White blood cells along with antimicrobial agents such as complement and interferons help in defending the body. An inflammatory response is set up to destroy those invaders, which have penetrated the skin. In the specific response mechanism, the body produces antibodies, which target particular invading agents. In making this system work, B and T lymphocytes are responsible for the production of antibodies, a group of proteins called immunoglobulins(Igs). Of the five classes of Igs' the most common type are the IgG. This is a Y-shaped monomer found abundantly in blood and body fluids. It can protect against bacteria, viruses, and toxins and is also able to trigger the complement system (some 20 different proteins) into action.

In this experiment students will be introduced to an immunological assay (the ELISA Test) using E.coli DH5a. Students will be able to see an interaction between E.coli and two different antibodies. They will determine the concentration at which a proper antibody-antigen interaction can demonstrate the presence of the E.coli.

Science Background:

Teachers should review all areas of immunology prior to introduction of this lab. Experiment. Students should have a clear understanding of the molecular basis of antigen –antibody specificity. They should know that the antigens are usually proteins or large polysaccharides. Such things as cell surfaces of transplanted tissues and organs or blood cells act to initiate an immune response. Antibodies only recognize a localized region on the surface of the antigen the epitope and not the entire molecule. Several types of antibodies may be produced to a single antigen. Furthermore the unique Y shaped structure of the antibody should be mentioned in terms of its unique structure (two light and two heavy chains). In addition, It should be emphasized that although all four chains possess a constant C region showing little variation in the primary sequence of the amino acids, it is the tips of each chain or the Y regions which are involved in specificity (the variable region varies from antibody to antibody). Therefore the antigen-bonding site (Y) is responsible for the antibody's ability to recognize the specific antigen epitope and the stem regions C is used to inactivate the antigen.

II. Student Outcomes

After completing the unit on immunology, students will do an experiment using two different antibodies and E.coli. Pre-lab discussion should include a short review of immunology concepts. Students should make predications related to dilution effects on the absorbance of light by the samples in the 96 well plates.

III. Learning Objectives:

- Students will be able to observe some distinct color changes as they start adding the second antibody. The addition of the substrate TMB will interact with the peroxidase located on antibody 2.
- Recording the results attained from the ELISA reader will enable students to attain the exact concentrations of E-coli at various concentrations along the 96 well plate.
- They will be able to analyze concentrations vs. dilutions and finally confirm identification of E-coli in distinct areas of the plate.
- Results based on my lab experiment show clearly that as the dilution of E.coli increases across the plate (left to right starting with row 3-12) it becomes much harder to confirm the existence of E.coli. Best dilution with good identification of E-coli being present was at 1:2, 1:4, and 1:8 dilutions.
- Graphing results attained from the ELISA reader is also an excellent way to confirm E-coli identification.

IV. Student Prior Knowledge and Skills

- Students will be expected to have mastered the topic of immunology.
- They need to know the way in which the body responds to foreign invaders and to recognize the importance of different lines of defense (the skin, the mucous membranes, the sweat glands and white blood cells.)
- Students need to distinguish between a nonspecific and a specific mechanism of response Innate and acquired immunity.
- Most importantly they need to understand the functions of the B and T white blood cells and the way in which these cells respond to an invaders.
- Some basic concepts include the following: B and T cells are located in lymph nodes, spleen and other lymphatic tissue.
- B-cells are responsible for a humoral immune response. The antigen binds to receptors on B cells, which have specific receptors for the antigen epitopes. This response causes a proliferation of B cells into plasma cells. These cells in turn secrete antibodies, which destroy the invaders.
- T-cells are responsible for the cell-mediated response. In this response T receptors will become activated when the pathogens enter cells. T-cell receptors recognize the bound antigens displayed on infected cells.
- Two other cells, which help in the process, are helper T cells and cytotoxic T cells.
- Cytokines are important substances in this process. These substances can start a chain reaction to alert other cells about the presence of a particular antigen. This is accomplished by helper T-cells. The Cytotoxic T-cells destroy other cells. A substance porphyrin is secreted when the T-cells recognize an antigen bound to it. Porphyrin will lyse the invader.

V. Time Requirements

One week of classroom discussion on the topic of immunology should follow a two-day/three hour lab. Each day.

VI. Materials and equipment

To be used for 1 class of 30 students. Students should work in pairs

- 96-well plates (Immulon 4HBx)
- Sterile petri dishes with LB agar – you need 15 plates
- Antibody 1(Rabbit IgG anti-E.coli) should be kept in the freezer at –20 C until needed. Dilute the antibody 1 (1:1000 dilution) by adding 20 uL of Ab 1 to 20 ml of PBS+ Tween
- Antibody 2(Goat IgG anti-rabbit) should also be kept at –20 until needed. 2Ab (1:8000 dilution) can be made by adding 2.5 uL of the antibody to 20 ml of PBS + Tween.
- 3,3,5,5 tetramethylbenzidine may be used directly from the bottle
- 15Inoculating loops
- 15bunsen burners
- 15 Immulon 4HBx plates
- 15pipettors (2-20 uL; 10-100uL; 100-1000uL)- with disposable tips.
- E.coli bacteria DH5 alpha type. Kept in the refrigerator until ready to plate.
- **Other necessary equipment include:** Incubator set at 37 C; Centrifuge (capable of spinning at 5000RCF at 5 C)- Time 10 min.; Heat bath set at 60 C; Spectrophotometer (set at 660nm); ELISA reader (set at 405 nm)

Laboratory procedure:

1. E.coli (DH5 a) is cultured on LB sterile agar plates. The plates are incubated overnight at 37 C.
2. Growth is collected using a sterile inoculating loop and transferred to a solution of Carbonate/Bicarbonate at pH 9.6 (about 50 ml)
3. The tubes are transferred to a hot water bath at 60 C and kept for approx. ½ hour until all bacteria is killed.
4. The tubes are centrifuged for 10 min.at 5C at RPM 5000 RCF (5520) and washed 3 times in Carb/ Bicarb. The bacteria will form into a small white palette and adhere to side of the tube.
5. The palette is than suspended in Carb/Bicarb solution and read by a spectrophotometer at 660nm at conc. Of 0.3
6. 50 uL of each organism is added each of the 96 well plates (Immulon 4HBx) covered and incubated overnight at room temperature. (An assay Design Sheet should be used by each student to record what is placed in each well)
7. The wells are washed with 1x Phosphate Saline Buffer (PBS) solution + Tween, patted dry and incubated uncovered at 37C to dry. This procedure is done when a “white powdery” appearance is noted. The procedure usually takes about ½ hour.
8. 225 uL of Bovine Serum Albumin (2%)BSA made up in 1x PBS is added to each well and incubated for 1.5 hours. (This step allows for blocking unoccupied sites on each well surface to prevent antibody from binding directly to the immulon well).
9. Wells are washed 3 times with 1x PBS+ Tween, patted dry and 100uL of antibody 1(1:1000 dilution which is 20uL of the antibody in 20 ml of PBS + Tween) (IgG

- Rabbit anti E-coli) is added to each well starting with the second column and diluted across to row 12. The first column will be used to set up different controls. (Please see assay sheet). Incubate overnight.
10. Wash 3 times with Phosphate Saline Buffer, add antibody 2 (1:8000 dilution which is 2.5 uL of antibody 2 in 20 uL of 1x PBS + Tween)) IgG Goat anti rabbit Horse Raddish-Peroxidase conjugate to each well starting with the second row. Dilute repeatedly rows 3—12 . Wait 30 minutes.
 11. Wash 3 times with the Phosphate Saline Buffer and add 100 uL liquid substrate 3,3,5,5-Tetramethylbenzidine (TMB) to each well. Incubate at room temperature for 10 minutes.
 12. Stop the reaction by adding 50 uL of 2 M sulfuric acid.
 13. Read the 96 well plate at 405 nm using the ELISA reader.

VII. Advance Preparation

- a. Carbonate/Bicarbonate solution at pH 9.6 (3.2 g of Na₂CO₃ mixed with 6.0 g of NaHCO₃ in 1L of Dist. Water
- b. Phosphate Saline Buffer(PBS) . (Mix 40 g of NaCl with 1 g of KCl with 5.7 g of Na₂HPO₄ with 1.0 g of KH₂PO₄) dissolve in 500 ml of Dist. Water. This is 10x. You need to dilute to 1x from 10 x.
- c. 2% Bovine Serum Albumin BSA in PBS. (Dissolve 2g of Bovine Serum Albumin in 90 ml of Dist. water and 10 ml of 10x PBS)
- d. PBS + 0.01% Tween (100ml of 10x in 900 ml of Dist. Water (1L) add 0.5 ml of Tween-20 used in washing the plates
- e. 2M H₂SO₄(dissolve conc. H₂SO₄ 14 ml in 125 ml of distilled water)

All solutions must be filtered with the exception of antibody 1 solution and antibody 2 solution.

Preparation time 3 hours. This includes making up all solutions needed and setting up the lab for the students.

Estimated cost for purchasing equipment and chemicals: **\$ 3000.00**

Look into the possibility of working with a nearby research lab. for the use of the high power centrifuge and the ELISA reader. All other equipment and chemicals may be purchased at the above cost.

Safety concerns: Proper handling and disposal of chemicals needed.

(Company from which chemicals are purchased will share with you the method of disposal)

Gloves, apron and goggles are recommended for all students.

A freezer / refrigerator is needed to store all chemicals.

Distilled water is needed for preparation of all reagents.

Vacuum filtration is needed if available.

Technical skills: Students need to know how to micropipette properly. Need to read directions to make sure that the time intervals between various steps are kept accurately.

They need to make sure that antibodies are always in the refrigerator prior to use. Most importantly they need to dilute across the 96 well plate vary carefully.

IX. What is expected from Students. Students need to be given the lab. Experiment one day before the actual lab. Experiment. They need to prelab the introduction, safety precautions and the procedure.

After completing the lab (3 hour labs for two days) a post lab. Discussion should follow. Students will need to write up the lab results and generate a graph using the assay sheet. Conclusions based on the results should also be included and discussed in class.

X. Data results: The experiment should be done to include at least 3 trials. Results from each trial should generate concentrations from the ELISA reader. The assay sheet should be used to generate a graph. See attached data from all my trials. Some possible areas of error could be in dilution technique, wait time between addition of antibodies; not washing 3X with BSA and blotting properly; forgetting to apply blocking agent; not interpreting the results properly.

XI. Classroom Discussion. What is an antigen? What is an antibody? General structure for each. How do they interact? What do these results show? Interpreting the graph. Creating scenarios where this particular test for E. coli may be useful. How is dilution important in this test? What does dilution do to interpreting the results properly?

XII. Assessment: A general test on immunology(multiple choice questions/short essays) including some lab questions concerning the ELISA test and how it may be used in a practical sense to understand the specificity aspect of the interaction of an antigen with an antibody.

Additional Notes for Curriculum Developers

Identification of E.coli at various dilutions using the ELISA test. Title

Table of contents: Introduction; Safety regulations (Use the data sheet from the Company from which the chemicals are purchased); Materials and Equipment used; Procedure; Observations; (include assay sheet for results/graph paper;) Conclusions.

Number of pages 5

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STUDENT SECTION

I. Rationale

- Reason for studying this unit: In order to understand how the mammalian body reacts to foreign organisms.
- Introduction: In vertebrates there are two very important mechanisms that are used to protect against toxins, bacteria, viruses, protozoan and cancer. In the nonspecific mechanism, the body uses external barriers, the skin and the mucous membranes to prevent entry of the foreign invaders. White blood cells along with antimicrobial agents such as complement and interferon help in defending the body. An inflammatory response is set up to destroy those invaders, which have penetrated the skin. In the specific mechanism, the body produces antibodies. These antibodies are a group of proteins called immunoglobulins(Igs). There are five such classes. Of these the IgG 's are the most common. The antibodies will target specific invading agents. In making the system work, B and T lymphocytes are responsible for the production of antibodies and other specific substances, which can destroy the invading organisms.
- Overview of appropriate science background: Students need to review all concepts covered in the immunology unit prior to completing this lab. Experiment.
- **Procedure:**
 1. Procure sterile petri dishes containing LB agar
 2. Using the Bunsen burner sterilize the inoculating loop and streak E.coli (DH5alpha) across the plate. (The E.coli culture must be at -80 prior to use)
 3. Incubate overnight at 37 C
 4. Examine the bacterial colonies and then transfer the growth to 50 ml of Carbonate/Bicarbonate solution . Mix well
 5. Tubes are transferred to a hot water bath for ½ hour until the bacteria is heat killed.
 6. The tube is centrifuged for 10 min. at 5C (RPM 5000RCF) . A small palette will appear on the side of the centrifuge tube. Decant solution leaving the palette cover with more Carb/Bicarb.and centrifuge again. Repeat one more time.
 7. The palette is suspended in Carb/Bicarb and read by the spectrophotometer at 660nm at Optical Density of 0.3.
 8. 50 ul of E.coli is then added to each of the 96 well Immulon plates, covered(use CellophaneWrap) and let stand overnight at room temperature.
 9. Wells are washed 3X with Phosphate Buffer solution + Tween, padded dry and incubated uncovered at 37C until dry. A white powdery appearance will be seen when ready.
 10. 225 ul of BSA (Bovine Serum Albumin in 2% Phosphate Buffer solution) is then transferred to each of the wells to block the antibody from binding directly to the immulon plate surface. Waiting time is 1.5 hours.
 11. The wells are washed 3 times using the PBS+ Tween combination and then they are padded dry by shaking the contents of the wells out. Use several pieces of paper towel. 100 ul of antibody 1 (IgG rabbit anti E.coli) 1:1000 dilution This means adding 20 ul antibody in 20 ml of PBS + Tween) is added to each row

- with the exception of row 1 (used as control) and diluted across to row 12. Let stand overnight wrapped in plastic wrap.
12. Wash 3X with PBS + Tween as before, pad it dry and add antibody 2 (IgG goat anti-rabbit Horse Radish peroxidase conjugate) 1:8000 dilution which is 2.5 ul of antibody 2 in 20 ul of PBS + Tween. Add to each well starting with the second row. Dilute across row 2-12. Wait 30 minutes.
 13. Wash 3 times as before. Add 100 ul of liquid substrate 3,3',5,5'-Tetramethylbenzidine (TMB) to each well. Let stand at room temperature for 10 minutes.
 14. Stop the reaction by adding 50 ul of 2M sulfuric acid to each well
 15. Read the 96 well plate using the ELISA reader at 405 nm.

II. Materials needed for a class of 30 students

- 15 96 well plate (Immulon)
- E. coli strain- DH5 alpha
- 15 LB agar plates
- 15 Inoculating loops
- 15 Bunsen burner
- 1 Incubator at 37 C
- 1 Water bath at 60 C
- 1 L. PBS solution 10X dilute to 1X
- 2 L. 1X + Tween (100ml of 10XPBS in 900ml of Distilled water add 0.5 ml of Tween-20)
- 1 L BSA solution in 2% PBS (1 X)
- 1 L Carbonate/Bicarbonate solution at pH 9.6
- 15 Multichannel pipettes (if available) or single pipettes 15 (10ul – 100ul)
- 15 Reagent reservoirs
- 15 Wash bottles filled with PBS+ Tween
- 100ml TMB (3,3',5,5'- Tetramethylbenzidine substrate (kept in the refrig. until needed))
- 1ml IgG Rabbit anti E.coli antibody 1
- 1ml IgG Goat anti –rabbit antibody 2 all antibodies should be in the refrigerator until needed at –20 C
- Centrifuge 5000RCF at 5C
- Spectrophotometer (660 nm at 0.3 OD)
- ELISA reader at 405 nm

Safety: Goggle and apron needed. Gloves need not be worn until the addition of sulfuric acid. Discarding chemicals properly (Teacher must see Data Sheet from Company selling the chemicals)

III. Procedure:

See (I.) (Rational) Procedure is outlined step by step.

See attached graphs based on my 4 trials if needed

IV. Data Collection:

If students have access to the ELISA reader they will get a printout of results based on the dilutions used Antibody 1 (1:1000) antibody 2 (1:8000) vs. Dilutions of E.coli. They can then construct a graph using the average of the concentrations in each of the 9 rows of Immulon plate and then plot conc. Of E.coli vs. dilutions. See attached graph. If the ELISA reader is not available (I made contact with a research hospital nearby- University of Massachusetts Medical Hospital) then they can use the spectrophotometer and get individual readings at 405 nm. The contents of each well must be put in a separate cuvette for reading. This is Time consuming!

VIII. Discussion/ Analysis

Based on your data obtained from the ELISA reader find an average value for each vertical column with the exception of the first column. Plot the averages on a graph. Use the X-axis to show the dilutions. Use the Y-axis to show E.coli concentrations.

Based on your observations why is this test a good way to show the relationship between an antigen/antibody interactions?

How can this test be used in a clinical laboratory setting?

What are some of the difficulties that a laboratory technician may encounter in assessing the results of this lab?