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

I. Overview

Two very important mechanisms used by

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 unable 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

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

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 Na_2CO_3 mixed with 6.0 g of $NaHCO_3$ 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 Na_2HPO_4 with 1.0 g of KH_2PO_4) 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

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**

- x Reason for studying this unit: In order to understand how the mammalian body reacts to foreign organisms.
- x 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 anitmicrobial 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 call 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.
- x Overview of appropriate science background: Students need to review all concepts covered in the immunology unit prior to completing this lab. Experiment.
- x **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 than 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 than added to each of the 96 well Immulon plates, covered(use CelophaneWrap) and let stand overnight at room temperature.
9. Wells are washed 3X with Phosphate Buffer solution + Tween, padded try 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 than 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 than 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

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 than construct a graph using the average of the concentrations in each of the 9 rows of Immullon plate and than 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) than 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?