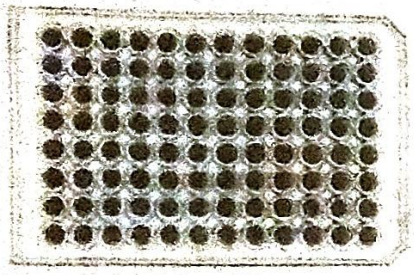
BIO 121 - Secondary Immune Response Lab

This lab exercise will examine the immune response to a harmless test antigen in two volunteers. The volunteers will be given an injection of a harmless substance and the production of immunoglobulin G (IgG) antibodies to the antigen will be measured. Patient 1 will receive a single dose of the antigen on Day 0 of the test. Patient 2 will receive 2 doses of the antigen, one dose on Day 0 and the second dose on day 55. The purpose of this exercise is to examine any differences in the immune response between someone receiving one dose only and someone receiving two doses and the difference in responses between the first and second dose. Patient 1 is a required control for this study.

The amount of IgG produced by each of the patients will be measured using an Enzyme-Linked ImmunoSorbent Assay (ELISA) test. This type of test will be used to quantify the amount of antibodies produced by each patient. It uses the simple principle of an antibody-antigen reaction. Since antibodies are small and invisible with common lab instruments, this procedure will use a color reaction to visualize and quantify the amount of antibodies. In today's lab we will utilize the Sandwich ELISA test to gather data. The sandwich ELISA uses several different antibodies to measurement the antigen of interest.

How a Sandwich ELISA Test is Performed

The experiment uses two important tools, a multiwell plate to perform the experiment and a device (plate reader - spectrophotometer) that can read the color intensity, using the correct UV wavelength, inside each well.



The ELISA protocol is described with the different chemical components of this experiment. The "sandwich" is made of antibodies on the outside and antigen in the middle. You start with a horse-antibody (Y) to the test antigen - that antibody will be chemically attached to the plastic plate (Figure 1). Then the test antigen (◎) is applied to the wells and allowed to attach to the horse-antibody (Y) (Figure 2). It is important to remember that excess material always needs to be removed to prevent inaccurate readings. This is called the rinsing step and occurs after adding each component. The next step would be to collect blood samples at regular daily intervals from the patients. These samples are purified for antibodies (Y) – antiserum – and added to the wells, producing the sandwich (Figure 3).

The color reaction is achieved by using an enzyme-linked antibody (Ⓔ-Ⓐ) that has 2 special features: it attaches to the patient antibody and has an enzyme that converts a colorless molecule (■ - substrate) into a colored product (✱) (Figure 4). The amount of enzyme-linked antibody attaching to the well is directly proportional to the amount of patient antibody in the well (Figure 5). The enzyme-linked antibody will convert the substrate into a colored product with varying intensities depending on the amount of patient antibody present (see reaction plate – Diagram 2). The color intensity will be measured using the plate reader spectrophotometer. The color value will be converted into an actual antibody amount using the standard curve extrapolation method (Table 1).

You will use the standard curve provided to determine antibody values and plot those values on a graph paper in Excel or on this form. Using your graphed results complete the lab by answering the questions.

ELISA Protocol

1. Allow the horse-antibody to test antigen to attach to well (Figure 1).
2. Bind the test antigen to the horse-antibody in the well (Figure 2).
3. Obtain a sample of patient blood, purify for antibodies, add to the well (Figure 3).
4. Add the enzyme-linked antibody to the well (Figure 4).
5. Add the colorless substrate to the well and allow the reaction to proceed (Figure 5).
6. The reaction will produce a color that may not be visible, although stop the enzyme reaction.
7. Using the Plate reader, gather the optical density (OD) values for each well.

List of chemicals

Y – Horse-antibody to test antigen

Y – Human antibodies produced in patients exposed to test antigen

- Test antigen

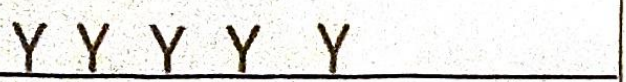
Ⓔ-Y - Enzyme-linked antibody to human antiserum

- Colorless substrate for enzyme

- Colored product of enzyme

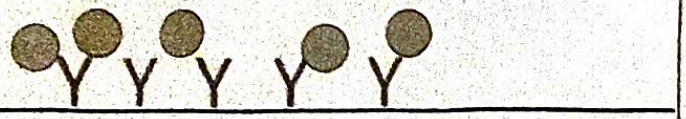
In this type of ELISA an antibody to our test antigen (horse-antibody to test antigen) is allowed to bind to the bottom of the wells of a 96 well plate.

Figure 1



Allow a standard known amount of test antigen to bind to the antibody attached to the well.

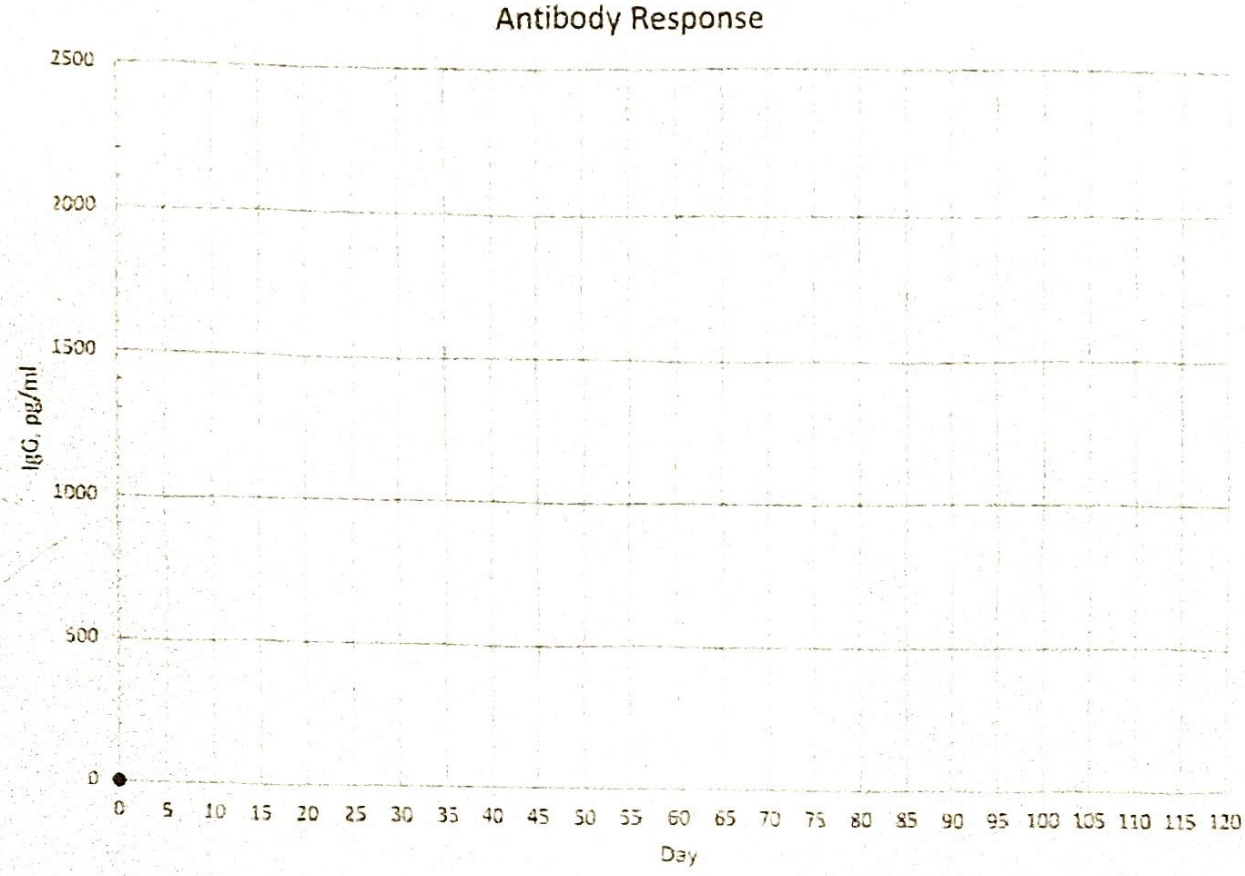
Figure 2



The antiserum, obtained from Patient 1 in 7-day intervals is added to the corresponding wells and allowed to bind to the test antigen. Remember that only IgG antibodies can bind to the test antigen. It is the concentration of this antibody molecule that we want to know.

Figure 3

^(= 1.5/1000



Post-lab questions

1. Why is the response to the second antigen exposure for Patient 2 so much larger than the first antigen exposure?

2. What type of cell is expected to be produced in response to the initial exposure to the antigen?

3. The ELISA test used in this exercise measures IgG levels. What other major type of antibody molecule is produced in response to an antigen?

4. The response of Patient 2 to the second exposure of the antigen is the basis for what medical procedure?

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Determine the concentration of IgG for both patients

You will need to use the Standard Curve (shown above) to convert the OD450 readings (in the chart) for each data point into an amount of IgG. You will them graph the pg/ml of IgG for each day for each patient.

Use the graph below to plot the amount of IgG for each patient. Put both data sets (Patient 1 and Patient 2) on the same graph. Please use a different color line or different dashed lines for each Patient.

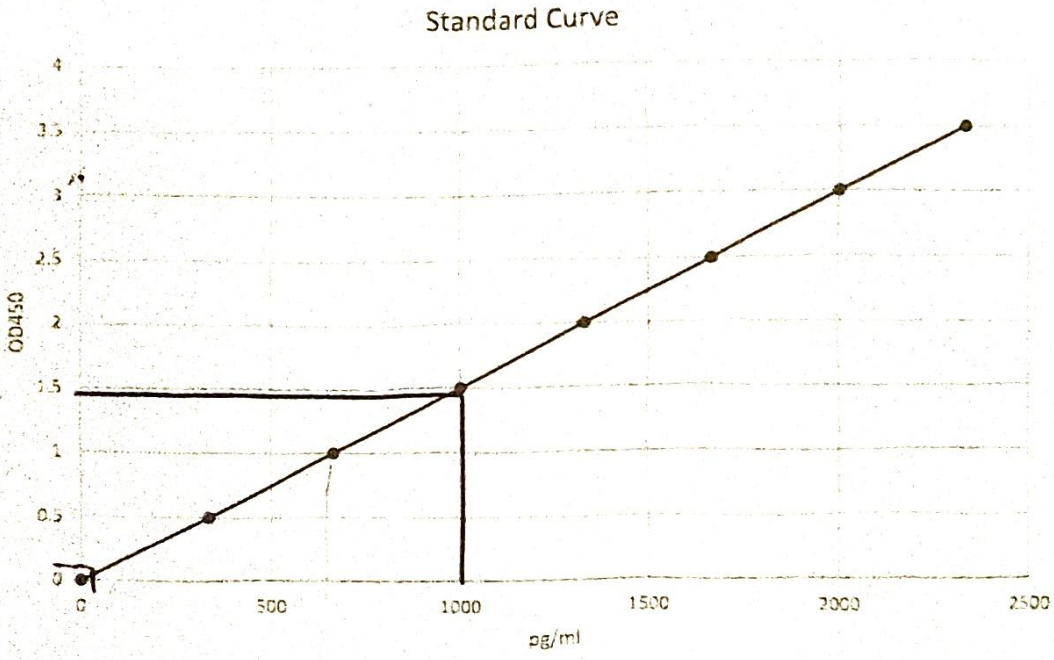
Table 1. OD readings

Day	OD 450 Patient 1	OD 450 Patient 2
0	0.002	0.0018
7	0.01	0.019
14	0.1	0.1
21	0.25	0.2
28	0.2	0.25
35	0.1	0.1
42	0.06	0.06
49	0.01	0.01
56	0.01	0.5
63	0.01	1
70	0.01	3.2
77	0.01	3.1
84	0.01	2.3
91	0.01	2.2
98	0.01	2.1
105	0.01	2
112	0.01	1.8
119	0.01	1.8

Table 2. Extrapolated antibody content

Day	IgG, pg/ml Patient 1	IgG, pg/ml Patient 2
0	0	0
7		
14		
21		
28		
35		
42		
49		
56		
63		
70		
77		
84		
91		
98		
105		
112		
119		

Table 1. Standard curve for antibody quantity



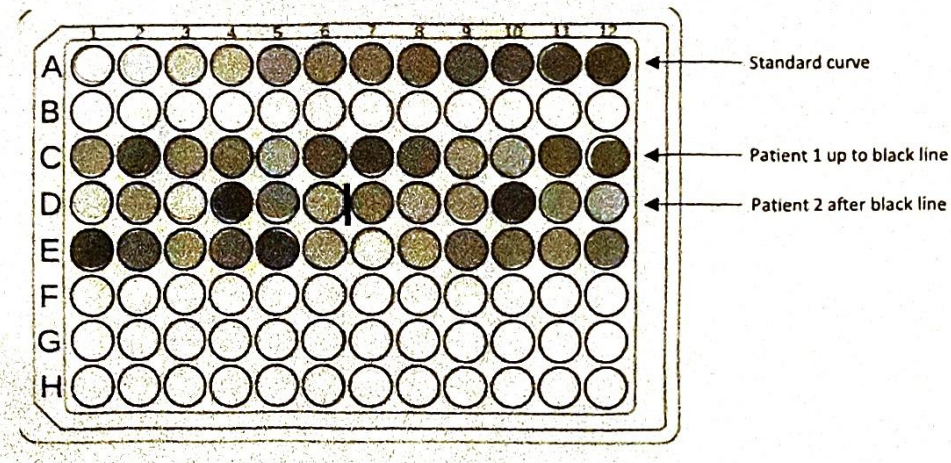
Standard curve extrapolation

A standard curve is created by preparing a set of wells on your plate with a known and increasing amount of antibody. The enzyme reactions will then produce a colored reaction in each well with more intensity as antibody quantity increases. Using the standard curve, it is easy to determine the amount of IgG that any well reading represents. A reading of 1.5 (on the Y axis) represents an amount of IgG of 1000 pg/ml.

The Elisa data from the study is shown below. A blood sample from each of the patients was taken on the indicated day and the concentration of IgG directed against the test antigen was determined by a sandwich ELISA.

A typical 96 well plate used for this type of assay is shown. The standard curve is shown in wells A1 through A12. Well A1 has the lowest concentration and well A12 the highest concentration of the antigen. The other wells are showing what a typical ELISA test looks like.

Diagram 2. Results of Sandwich ELISA for Patient 1 and Patient 2 exposed to antigen



The enzyme-linked antibody is added to the well and allowed to bind to patient IgG antibodies.

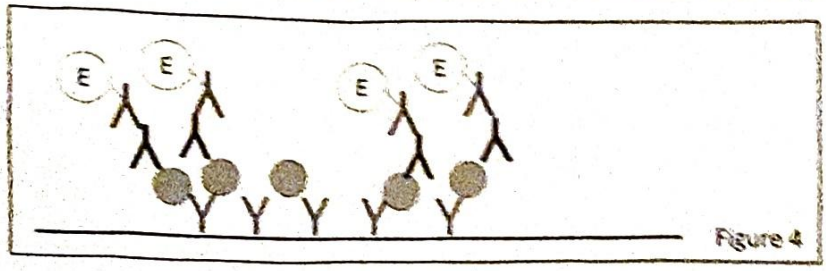
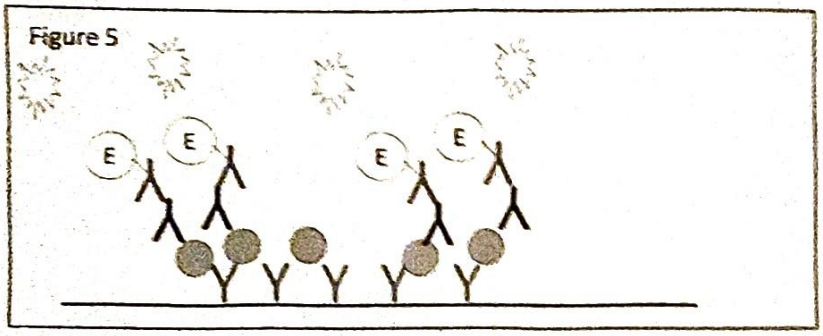
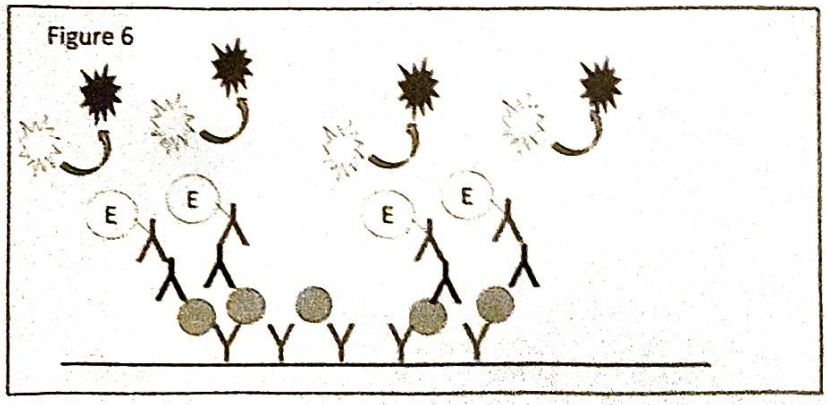


Figure 4

A colorless substrate for our enzyme is added to the plate and the enzymatic reaction is allowed to occur. The reaction is stopped after several minutes.



The amount of the colored product is determined by measuring the OD540 using a plate reading spectrophotometer.



DATA INTERPRETATION

The amount of colored product is determined by measuring the amount of light that can pass through the solution in the well. This reading is the Optical Density. In our case the wavelength of light used is 540 nm, so we will be using the OD450. The darker the color in the well (which is read as the OD450) the higher the amount of the IgG to the test antigen produced by the patients. The relationship between the OD450 and the amount of IgG is determined by a standard curve. A known amount of test antigen is added to a well and the assay is run. Normally several dilutions of the test antigen are used to generate the standard curve. The standard curve for our study is shown below.