Biol 465 Lab #4, Part 2:

Using the ELISA assay to quantify antigen.

You should be familiar with the concept of the ELISA assay and with its use in medicine and research. In the last lab, we used an ELISA to simulate detection of an HIV protein in the serum samples of students. Whereas the exercise only pretended the antigen was an HIV protein, we *did* perform an ELISA exactly as it is done in the laboratory, using a chicken protein as antigen, a monoclonal primary antibody raised in mouse, against the chicken protein, an enzyme (HRP)-linked, goat-anti-mouse secondary antibody, and a substrate (TMB) that is chemically altered by (to produce a blue color) by the enzyme (HRP) on the secondary antibody.

Review the steps in the ELISA and the post lab questions given out last time. ****Draw a cartoon/sketch of what occurred in each of your samples last week. Try to think of a modification that would allow one to <u>detect</u> HIV antibodies (produced in the host) instead of an HIV antigen (produced by the virus). Assume you could bind anything you needed to the wells of the ELISA plates.

Today's lab will be an extension of the ELISA procedure. ELISA is a powerful assay to determine the presence of antigen in a sample, (a positive/negative result), but it can also be used as a *quantitative* assay in experiments in which it is important to know "how much" antigen is present or whether one sample has more or less antigen than another. For example, in an HIV infection, the level of p24 capsid protein is a direct indicator of the number of viral particles in the individual. ELISA is one way of monitoring viremia, or the level of live/infectious virus in blood, by detection of p24 capsid protein. Monitoring approximate numbers of viruses is important in determining the extent of the disease and the effectiveness of treatment. Obviously for ELISA to be quantitative the read-out (degree of color development) must be directly proportional to the amount of antigen present in the sample. Subtle differences in color may not be apparent to the human eye, but ELISA readers (spectrophotometers) can give a numerical output. We will use an ELISA reader today.

The goals of our lab today are:

1. To extend and enhance our understanding of ELISA

*2. To use ELISA to establish a standard curve which relates color development (absorbance read by the ELISA plate reader) to concentration of antigen.

*3. To determine the concentration of antigen from a sample with an unknown concentration.

* Goals 2 and 3 are common practices in all fields of science. Establishing standard curves using any assay allows the measurement of experimental samples.

Procedure

You will use the attached protocol (with some important modifications) for setting up dilutions of antigen for a standard curve. The actual ELISA procedure will be the same as last week. Since this is a quantitative assay we will want to be extremely careful with both the dilution procedure and the ELISA procedure. The plates must be read within 15 minutes of adding the substrate to insure accurate detection of antigen.

Materials

- Student test samples (**Each group take one A sample and one B sample)**
- Concentrated antigen ((100 ng/ml) (**Each group take one tube to be used to make serial dilutions and to use as + control)**
- Phosphate buffered saline (PBS) **Each group take one tube to dilute antigen and to use as – control)**
- Primary antibody (**Each group take one)**
- Enzyme-linked secondary anti-mouse antibody (**Each group take one)**
- Substrate for enzyme (Horseradish peroxidase) (brown tube) (**Each group take one)**

- 12-well ELISA microplate strips **(2 per group)**
- Racks for reagents

- Wash buffer
- Stack of paper towels
- Micropipettor/tips

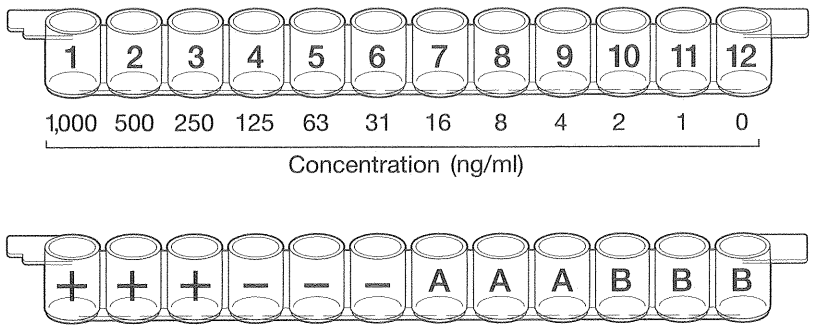
See attached protocol. (You will also need last week's protocol to carry out the ELISA detection of antigen).

Each group will prepare a series of dilutions and assay an unknown sample A and an unknown sample B in triplicate using two 12-well microplate strips. The strips will be read in an ELISA plate reader (a modified spectrophotometer) in Dr. Super's lab, Moore 216. The blue color will be quantified by absorbance at 655 nm. Students will use absorbance values to roughly compare diluted sample concentrations and to roughly estimate the concentration of unknown samples, A and B. Then they will create a standard curve using the serially diluted samples and extrapolate from the curve a concentration for A and B.

No write up! Just review the concepts.

Student Lab Procedure

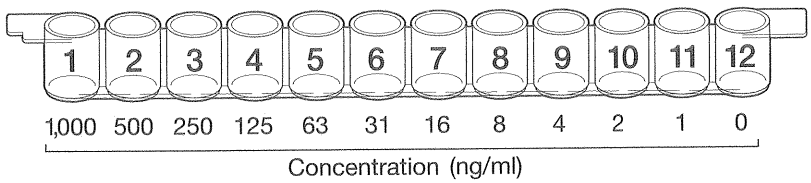
1. Label the outside wall of each well on one 12-well strip with the numbers 1–12. Label the first three wells of a second 12-well strip with a "+" for the positive controls, the next three wells with a "–" for the negative controls, the next three wells with the initials of one of your sample tubes, and the last three wells with the initials of the second sample tube.



2. Use a pipet to add 50 µl of PBS from the yellow tube labeled "PBS" to wells labeled #2 through #12.
3. Add 100 µl from the yellow tube labeled "1,000 ng/ml AG" to the well labeled #1.
4. Perform serial dilution from well #1 through well #11 in the following manner:
   a. Pipet 50 µl out of well #1 and add it to well # 2. Pipet up and down gently three times to mix the sample in well #2.
   b. Using the same pipet tip, transfer 50 µl from well # 2 to well # 3 and mix the sample in well # 3.
   c. Using the same pipet tip, transfer 50 µl from well # 3 to well # 4 and mix the sample in well # 4.
   d. Repeat this transfer and mixing step, moving to the next well each time. STOP when you reach well # 11; discard the 50 µl of solution from well #11 into a waste container.
5. In the second microplate strip, use a fresh pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
6. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
7. Use a fresh pipet tip to transfer 50 µl of each of your team's samples into the appropriately initialed three wells.
8. Wait 5 minutes to allow the proteins in the samples to bind to the plastic wells, then proceed exactly as in protocol II, starting at step 5 on page 56.

Analysis of Results

The concentration of 1x antigen is 1 microgram per milliliter (µg/ml) or 1,000 nanograms per ml (ng/ml). Note: If adapting this protocol for a quantitative ELISA antibody test, the concentration of primary antibody in 1x serum is also 1 µg/ml. Thus, the concentrations of protein in the wells of the dilution series are:



If using a microplate reader, insert the microplate strips firmly back into the strip holder in the correct orientation, secure the plate into the microplate reader, close the lid, and read using a 655 nm filter.

Microplate readers measure the amount of light at a specific wavelength (in this case, 655 nm) that is absorbed by the liquid in the wells of the microplate. The absorption of light by the liquid is directly related to the intensity of the colored product in the wells, which in turn is determined by the amount of enzyme activity in the wells. The amount of enzyme activity is governed by the amount of antigen that originally bound to the wells.

Create a standard curve by plotting the known concentrations of each well on the y-axis and the corresponding absorbance values from the microplate reader on the x-axis (See example on next page). (Note: The unit of measurement for absorbance is absorbance units, or AU.) Since the resulting curve will be logarithmic, you will need to linearize it by plotting the data on semilog graph paper. A sheet is provided on the next page.

Calculate the concentrations of the test samples by drawing vertical lines from their absorbance values on the x-axis to the standard curve. Read horizontally from the points where the vertical lines intersect with the standard curve to the concentration values on the y-axis.

Please note that the substrate – enzyme reaction loses correlation between antigen concentration and absorbance value when unoxidised substrate becomes scarce either due to too concentrated antigen (see 1000 ng/ml datapoint on example graph on next page) or after too long a reaction time. Thus microplates should be read within 15 min of adding substrate.

Note: The enzymatic reaction can be stopped after 5 min by adding 0.18 M sulfuric acid to the wells. The blue solution will turn yellow. The yellow solution is read using a microplate reader with a 450 nm filter. Stopping the run after 5 min produces a more linear reading of diluted samples.

If no microplate reader is available, visually compare the intensity of the blue color in test wells to the intensities of the dilution series of known concentrations. Identify which wells of known concentration most closely match the test wells and, from this, approximate the concentration of antigen in the test samples.

Semilog graph paper