

1) What is IgM used in response to? You said pathogenic invasion in class but you were not completely sure.

Answer: IgM's cause lysis of bacteria. For additional info, see Table 5.1 in Mikkelsen.

2) When cleaved with pepsin, what happens to the CH2 and CH3 regions of an antibody? Slide 9 only shows the F(ab)₂ region.

Answer: They remain as separate fragments. They are not shown in the figure because they cannot be used in immunoassays. Only the F(ab)₂ retain the paratope.

3) For competitive immunoassays, why is the unbound fluorescently labeled antigen measured instead of the antibody-bound fluorescently labeled antigen? It seems that by measuring the unbound antigen that the data is collected inversely.

Answer: The unbound labeled antigen fraction is washed off. What is measured is the bound fluorescently labeled assay. The higher the amount of native (sample) antigen, the lower the amount of bound fluorescently labeled assay. Thus, lower fluorescence means higher amounts of antigen in the sample.

4) What is the best control for a non-competitive immunoassay? Primary antibody + antigen or primary antigen + secondary antigen?

Answer: I assume that we are talking about a sandwich assay with a labeled secondary antibody. Usually more than one control must be used. I will not give a direct answer, but encourage to consider the following in selecting the various needed controls.

- i. There may be autofluorescence in the plate, even when the antigen and the secondary antibody are absent.
- ii. The secondary antibody may non-specifically bind to the plate even when the antigen is absent, giving rise to a fluorescence response. The same effect is noticed if the washes are not effective.
- iii. The immunoassay may not work because the antibodies have been denatured or mishandled.
- iv. No fluorescence detection may be seen because the fluorophores have photobleached.

5) When would polyclonal antibodies be used over monoclonal antibodies?

Answer: Polyclonal are better for the experiment is not designed to investigate a specific epitope. For example, I would use a polyclonal for a Western blot given that proper controls are used. I would use a monoclonal for an affinity CE experiment.

4/3 Lecture-

6) Could you clarify the difference between the weighted x&y reciprocal values used in slide 8?

Answer: See Electrophoresis, 1997, 18, 82-91. Weighted regressions are always used when the error associated with data point in an experimental data set has a different relative. The error is usually inversely proportional to the weight. That is, the higher the error the less weight the corresponding data point should have.

4/8 Lecture-

7) Partial Filling Technique confuses me and I am not sure if its because I am looking to closely at it. What the goal with this method and what does the spectrum shown on slide 3 tell me?

Answer: See the reference in that slide. The link is in the blog or grab it directly from the library website. Like any other affinity CE techniques, the goal is to measure the interaction between the analyte and a ligand. The technique is useful when the sample must be kept in a plug with different composition to that in which the interaction will be measured (black plug). This is very useful if you have a storage solution for the sample and a biological solution for the plug. There is an electropherogram in this slide. This electropherogram can be used to measure the shift in mobility associated with the complex formation.

4/10 Lecture-

8) What is the difference between a TAG and a FLAG? When would you use a TAG and when would you use a FLAG (slide 6)?

Answer: A FLAG peptide (KYKDDDDKK) is commonly used to mark proteins. Expression vectors are used to add this peptide to proteins. Upon expression these proteins are identified by means of an anti-FLAG antibody. This approach was used by Ho in 2002.

The TAG is the generic name and could include, glutathione S-transferase, His6, calmodulin-binding peptide, and common **epitope** tags (Haemagglutinin, myc, FLAG). This approach was used by Gavin, 2002. See Mass Spectrometry Reviews, 2004, 23, 350-367.

Since the FLAG requires the use of antibody, I would use that approach when I would like to have high selectivity.

9) On slide 11, it appears as though all of the pools (active and inactive) are being pooled to form a library. Doesn't this combine all of the fractions, even those which are not active? What is the point of this?

Answer: The library approach pools all the yeast(+) colonies in one vial to form a library. The bait protein is in yeast (-). Most of the colonies will contain proteins that are not true 'preys' for a given bait in the colonies. For example, even when all the yeast (-)

cells harboring bait 1 will mate with any yeast (+) cells in the library only a handful of yeast (+) colonies will have the correct preys and later result in expression of the LacZ and UrA3 gene. The approach is advantageous because there is no waste in having to test separately each bait against each prey. The main consideration is that as soon as the mating of yeast (+) and yeast (-) is done, the newly formed yeast must be separated, extremely diluted, and hope that each new cells will be placed in a separate well. Only those express the genes of interest (color resulting from the enzymatic marker) are selected for identification of the genes that led to the expression of the prey protein [the bait proteins are known].

Added April 25, 2008

1) The antibody fragment Fc has immunogenic properties but when the disulfide bridge/hinge region is cleaved, this immunogenic property is lost (comparing the resulting CH2 and CH3 fragments of the antibody from papain and pepsin cleavage). So how does the cleavage of this bridge cause the loss of immunogenic activity?

Answer: 'Immunogenic' is a concept that is more adequate to describe antibody response while they are still circulating in the body. 'Affinity' should be used to define interactions between antibodies and isolated targets (e.g. molecules, cell surfaces, etc.) that contain the epitope. Only isolated antibodies are treated with papain or pepsin. Since Fc does not have any binding affinity toward an epitope, it does not matter if the F_C fragment is cleaved from the original antibody. What is important is that the Fab or (Fab)₂ fragment maintain an unaltered paratope (V_L and V_H domains). This is usually the case.

2) For running controls for the non-competitive antibody sandwich assay, you mentioned in your previous question-answer email that photobleaching of the fluorescent probes would be possible. How would you run a control for this? How would photobleaching occur?

Answer: When a fluorophore is in the excited state, it may experience vibrational relaxation and then fluoresce, intersystem crossing and possibly phosphoresce, thermal relaxation and never emit a photon, or be destroyed due to ionization, reaction, or molecular reorganization. The last category of processes causes the loss of the fluorophore. This causes the decrease of fluorescence intensity with time, as the fluorophores are cycling through excitation and emission cycles; this is photobleaching.

In order to determine conditions that prevent photobleaching, people determine the illumination time and power of the excitation source that does not cause a change in fluorescence intensity and use these conditions in their experiment. Typically, illumination times are in the ms range with irradiation power $\sim 1 \text{ mW/m}^2\text{s}$.

3) The glucose sensor works on the basis of hydrogen peroxide detection, correct?

Answer: The coupling reactions are: glucose gets oxidized, glucose oxidase gets reduced; oxygen gets reduced when glucose oxidase gets oxidized. The final product that will be detected, usually electrochemically, is hydrogen peroxide.

4) Do we need to study other 4 group's process for Kd determination, LOD, etc. In terms of the exam, what should I be taking from Exercise 20-21?

Answer: There is no need to look at other people's work, unless you feel that this will make you understand better the various approaches to calculate Kd values. Keep in mind that some of the answers that were posted may not be necessarily correct, unless the group decided to update them after getting back their exercise.

Please look at the summary that compares the results from the various groups. It is important to understand why there is variation in the outcome of these results.

Lastly, the main goal of posting and summarizing these results is to make sure that everybody knows how to obtain a Kd using a Scatchard plot or a non-linear fitting. The range (lowest (called LOD in this case) and highest values) of Kd that can be determined for another system with similar experimental conditions should be based on the actual measurements so the fundamental principle should be the same for each group.

5) I was going through "partial filling technique", and I was wondering what does delta M mean. I know that M means the mobility ratio of protein and marker, but what does delta M mean?

Answer: Refer to slide 3, lecture 21, April 8, there are two peaks in the electropherogram. The first peak is a neutral marker to correct for variations in EOF. The second peak is the relevant one. Delta M refers to the difference between a given M value and the M value when $[L]=0$. This is very similar to the first example in this unit in which we did talk about using ACE to determine the K between Brij 35 and several porphyrins. The main difference in the expressions is that in this "partial filling technique example" they correct for EOF variations and changes in viscosity do not seem to be an issue. For further explanations, the reference **Electrophoresis 2001, 22, 1419** was not sufficient; it was necessary to go to the original paper.