PRACTICAL 5: ELISA: AN IMPORTANT CLINICAL ANALYTICAL TECHNIQUE

5.1 Introduction

The **aims** of this practical are:

- to introduce antibody structure
- to illustrate how antibodies can be used to provide techniques of high sensitivity, specificity and adaptability for the detection of very low levels of clinically important biomolecules
- to demonstrate the use of one such technique for the detection of **cotinine**, a metabolite of nicotine and a useful marker for the management of smoking-related respiratory, cardiovascular and periodontal disease.

5.2 Theory and background

By now you should appreciate the use of chromogenic reactions for the detection and quantitative determination of molecules of clinical importance. The simple procedures you have seen that involve the direct or indirect determination of some property of the material of interest (e.g. enzyme activity, or size) are limited in terms of **sensitivity** (OK for mM or perhaps μM), **specificity** (ISE membranes may allow the passage of more than one ion, or a chromogenic substrate may be acted upon by more than one enzyme) and **generality of use** (the material of interest must have some measurable property – not always the case).

It would be ideal if you could have a technique that was extremely sensitive (nM or less), was totally specific for the compound you wished to measure, and could be adapted to measure virtually anything *i.e.* a method that detected merely the **presence** of something and not a property. This is where **antibodies** are extremely useful.

Antibodies

A complete description of antibody synthesis, structure and function is beyond the scope of this introduction. You should refer to any standard text book for further information, for example Chapter 14 of "Biochemistry" by L. Stryer (4^{th} edition, Freeman).

Antibodies are proteins synthesised and secreted by B lymphocytes in response to the presence of an **antigen** in the blood. The antigen is usually a "foreign" protein, *e.g.* a bacterial or viral surface protein. Antibodies belong to the **immunoglobulin** class of proteins and there are

various subtypes (*e.g.* IgG, IgM). They have a **Y**-shaped structure (see Figures below). The important thing about them from the practical point of view is that they are designed to fit the antigen like a key fits a lock in the same way that enzymes and their substrates have a high specificity for each other. However, the specificity and affinity of an antibody for an antigen is often much higher than that of an enzyme for its substrate. Thus, the perfect antibody "anti-**X**" will recognize and bind very tightly to compound "**X**" but will not touch compound **Y**, even though **X** and **Y** might be quite similar.

Since antibodies are proteins, if you inject an IgG from one species (e.g. rabbit) into another species (e.g. goat, therefore "non-self"), you will get an antibody to the antibody (goat anti-rabbit IgG). The practical importance of this will become evident below. You will now see that we ought to be able to use antibodies to detect specific antigens with high sensitivity and specificity.

The immune system normally only makes antibodies to large molecules such as proteins. However you can fool it into making an antibody to a small molecule by first attaching the small molecule to a "**non-immunogenic**" protein, i.e. a protein that does not stimulate the production of an antibody against itself (they do exist). This is also useful (see below).

Radioimmunoassay (RIA)

One application of antibodies (Abs) that is particularly suited to the measurement of compounds of low molecular weight is the **radioimmunoassay**. This method requires you to have a radioactive standard of the compound you wish to measure *e.g.* [^{3}H]oestradiol or [^{125}I]thyroxine. At its simplest, the control assay contains *e.g.* [^{3}H]oestradiol plus an anti-oestradiol Ab which bind tightly to each other. When a coagulant is added to precipitate the protein (Abs are proteins) all the radioactivity is also precipitated. The test assay contains [^{3}H]oestradiol, anti-oestradiol Ab and the sample with the unknown amount of oestradiol. The oestradiol in the sample displaces some of the radioactive oestradiol from the Ab (they have the same affinity for the Ab) so when the coagulant is added some radioactivity remains unprecipitated. This can be separated by centrifugation and measured. The amount of radioactive oestradiol released is equal to the amount of non-radioactive oestradiol in the sample.

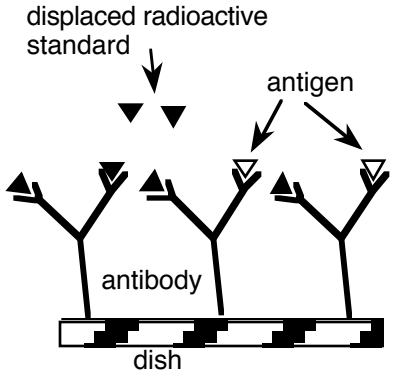


Figure 1. RIA

In a variation of the method **(Figure 1)**, the Ab is "**immobilised**" (attached) to the base of a small well in a plastic dish, or "**multiwell plate**" (certain plastics bind proteins very tightly so this is easy). In the control assay, when radioactive oestradiol is added to the well it all sticks to the Ab and so is "sucked" out of solution. Any non-radioactive oestradiol in the test sample reduces the amount of radioactive oestradiol removed from solution and again this can be measured and compared to the control.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA is ideally suited to assaying protein factors e.g. insulin or luteinising hormone (LH) but can be adapted to measure small molecules as well. There are several variations of the method. For example, the protein factor (test sample, *e.g*. containing LH) is first immobilised on the bottom of a multiwell plate well **(Figure 2)** and then any remaining binding capacity the plastic well has for protein is swamped ("**blocked**") by some non- interfering protein *e.g*. albumin. Next the "**first antibody**", *e.g.* anti-LH, is added. The amount of this that sticks in the well depends only on the amount of LH already there (the well having been blocked). A "**second antibody**" is then added which recognises and binds to the first antibody (typically the first antibody will be of the IgG class raised in a rabbit or a mouse and the second antibody will be raised by injecting another animal *e.g*. a goat with rabbit or mouse IgG to produce *e.g*. goat anti-rabbit IgG). This second antibody will already have been covalently attached ("**conjugated**") to an enzyme such as **alkaline phosphatase** or **peroxidase** which can catalyse a **chromogenic** reaction. When the colour reagents are added the amount of colour that develops depends on the amount of enzyme, which ultimately depends on the amount of LH originally immobilised from the unknown sample — **high** LH, **high** colour development.

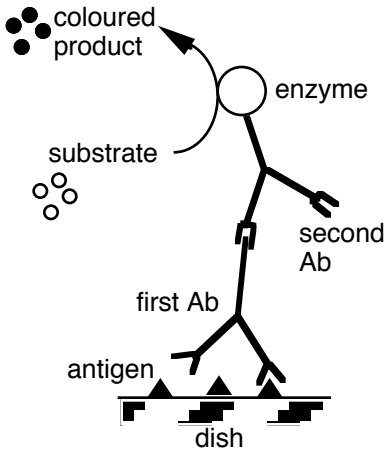


Figure 2. ELISA

A variant of this which you will use today, (**competitive ELISA**), combines the strategies of RIA and ELISA and can be used to measure small, non-protein molecules which by themselves do not bind to plastic dishes. The small molecule standard, *e.g.* **cotinine**, is first covalently attached to an inactive protein carrier that allows it to bind to the plastic. The first antibody (e.g. **rabbit anti-cotinine**) is added in the presence of the sample containing the unknown amount of cotinine. The unknown (free in solution) competes for binding to the first antibody and so reduces the amount of first antibody that binds to the well and, therefore, the extent of the final colour reaction – the **more** cotinine in the sample, the **less** the colour that eventually develops.

Cotinine

Cigarette smoking is a major contributory factor in cancer and in lung, cardiovascular and periodontal disease. In order to correlate disease progression with tobacco use, it is necessary to have a reliable quantitative marker. **Nicotine** is the major alkaloid in tobacco but has a half –life in plasma of only 30 min, so cannot readily be used as a biochemical marker of tobacco use. Nicotine is rapidly converted in the body to the much more stable metabolite **cotinine**, which

has a half-life of between 10 and 30 h, so its level remains relatively constant in active smokers over long periods of time. It is therefore used to monitor smoking behaviour and smoking status in both active and passive smokers. A typical serum cotinine level in smokers is around 450 µg/l, while in non-smokers it can be up to 20 µg/l due to passive smoking. These are low levels so a sensitive assay is required.

- *In this practical, you will use a competitive ELISA to measure the cotinine level in the serum of smokers, non-smokers (unexposed), and passive smokers who live with smokers*

Cotinine is first immobilised in the wells of a multiwell plate by covalently linking it to a synthetic protein (polylysine) that binds very tightly to the plastic. After blocking, a rabbit anti-cotinine Ab is added to the wells along with a diluted serum sample and a series of cotinine standards of known concentration. The amount of this "first Ab" that binds is inversely proportional to the amount of cotinine in the serum. The amount of first Ab is determined by the addition of peroxidase-conjugated goat anti-rabbit IgG followed by a colorimetric assay for peroxidase enzyme activity after washing out all unbound material from the wells. Hence a **low** level of peroxidase activity in the well means a **high** level of cotinine in the added serum.

5.3 Experiment: Determination of cotinine in serum samples

Reagents: **Serum samples A, B and C from smokers, passive smokers and non-smokers**
PBS (phosphate buffered saline)
Stock cotinine solution (500 µg/litre in PBS)
PBS-Tween (PBS containing 0.05% Tween-20)
First antibody (rabbit anti-cotinine in PBS + 1% dried milk protein)
Second antibody (peroxidase-conjugated goat anti-rabbit IgG) in PBS + 1% dried milk protein)
Substrate reagent (0.04% *o*-phenylenediamine, 0.02% hydrogen peroxide in 80 mM citrate buffer pH 5.0)
12.5% H_2SO_4

Procedure: (WORK IN PAIRS)

Make sure you have read and understood the theory behind competitive ELISA before proceeding with this experiment.

ELISA tests are commonly carried out in 96-well plates which can be processed automatically. Sample plates will be available in the lab for inspection. Each pair of students is provided with two 8-well strips which will be processed manually. Wells 2 to 8 in these strips have already

been treated overnight with cotinine attached to a synthetic protein (polylysine). Well 1 (a negative control) was treated with buffer. The excess cotinine was then removed and all 8 wells blocked with PBS (phosphate buffered saline) containing 1% dried milk protein. The strips are provided to you with the blocking solution still in place. (Dried milk protein is used to saturate all the remaining protein binding sites in the wells simply because it is cheap!).

Note: **Each student in each pair should be responsible for one of the two strips. One strip will be used to construct a standard "calibration" curve, the other will be used for the test serum samples.**

1. Collect two 8-well strips. These are provided with blocking solution in them. Remove the blocking solution from each well of both strips by suction (apparatus will be demonstrated).

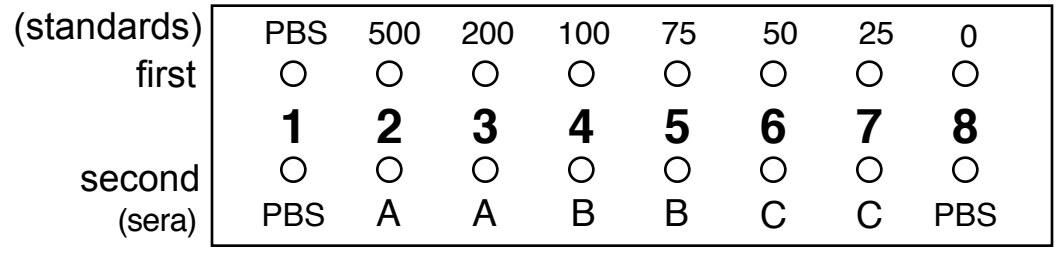
2. Pipette 100 µl of PBS-Tween into each well of both strips. Remove again by suction. Repeat this procedure twice more. (The wells are now "washed" free of all unbound protein). Put the strips to one side while you prepare a series of standard solutions in test tubes.

3. Using the 500 µg/litre stock cotinine solution, prepare a series of standard solutions **in small plastic test tubes** to give the following 7 concentrations by diluting the stock with PBS. Each standard dilution should have a final volume of 0.5 ml. (Only one set per pair required)

   Standards: 500, 200, 100, 75, 50, 25 and 0 µg/litre cotinine

4. Take the **first** strip and carefully pipette 10 µl of PBS into well 1 and 10 µl of the 7 standards into wells 2 to 8 (one standard per well, the 500 µg/l in well 2, the 200 µg/l in well 3 etc. — see figure below).

5. Take the **second** strip, and carefully pipette 10 µl of PBS into well 1, 10 µl of serum A into wells 2 and 3, 10 µl serum B into wells 4 and 5, 10 µl serum C into wells 6 and 7 and 10 µl PBS into well 8. **Together**, your two well strips should be as in the figure below, with sera A, B and C being measured in duplicate:



6. Now add 40 µl **first antibody** solution to all 8 wells of **both** strips and incubate at room temperature for 20 min. Tap the strips **gently** every 2-3 min to help mix the contents.

7. After 20 min, remove the first antibody by suction and wash each well twice with PBS-Tween using the procedure described in Step 3.

8. Add 50 µl **second antibody** solution to each well and incubate for 10 min at room temperature with occasional gentle mixing by tapping.

9. After 10 min, remove the second antibody by suction and wash each well twice with PBS-Tween using the procedure described in Step 2.

10. Add 75 µl substrate reagent to each well **of the first strip only** and tap to mix.

11. Watch the colour develop for **exactly 1 min** (continue tapping) then quickly but carefully add 75 µl 12.5% H_2SO_4 to each well to fix the colour.

12. Repeat steps 10 and 11 for the **second strip**. Both strips must be incubated for exactly the same time.

13. Estimate the cotinine concentrations in sera A, B and C by eye by reference to the standards, then determine which of the samples represent the smoker, passive smoker and non-smoker. Complete the table below. (In the clinical laboratory, a special colorimeter called a "**plate reader**" would be used to give a quantitative estimate of cotinine concentration.

Sample	**A**	**B**	**C**
Cotinine (µg/l)			
Diagnosis			

Questions:

1. If the molecular weight of cotinine is 176, what are the molar concentrations equivalent to 450 and 20 µg/l serum cotinine?

2. What is the purpose of well 1 (no immobilized cotinine)?