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## How to Set Up an ELISA

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### 1. Introduction

The enzyme-linked immunosorbent assay (ELISA) represents a simple and sensitive technique for specific quantitative detection of molecules to which an antibody is available (1,2). Although there are a huge number of variations based on the original ELISA principle, this chapter will focus on the two perhaps most useful and routinely performed: the indirect sandwich ELISA, providing high sensitivity and specificity; and the basic direct ELISA, useful when only one antibody to the sample antigen is available.

During the indirect sandwich ELISA (Fig. 1A), an antibody specific for the substance to be measured is first coated onto a high capacity protein-binding microtiter plate. Any vacant binding sites on the plate are then blocked with the use of an irrelevant protein, such as fetal calf serum (FCS) or bovine serum albumin (BSA). The samples, standards, and controls are then incubated on the plate, binding to the capture antibody. The bound sample can be detected using a secondary antibody recognizing a different epitope on the sample molecule, thus creating the "sandwich." The detection antibody is commonly directly conjugated to biotin, allowing an amplification procedure to be carried out with the use of streptavidin bound to the enzyme horse-radish peroxidase (HRP). Because streptavidin is a tetrameric protein, binding four biotin molecules, the threshold of detection is greatly enhanced. The addition of a suitable substrate, such as 3,3',5,5'-tetramethylbenzidine (TMB), allows a colorimetric reaction to occur in the presence of the HRP that can be read on a spectrophotometer, with the resulting optical density (OD) relating directly to the amount of antigen present within the sample.

In some cases, however, only one specific antibody may be available, and in such a small quantity that directly conjugating it to biotin would be impractical.

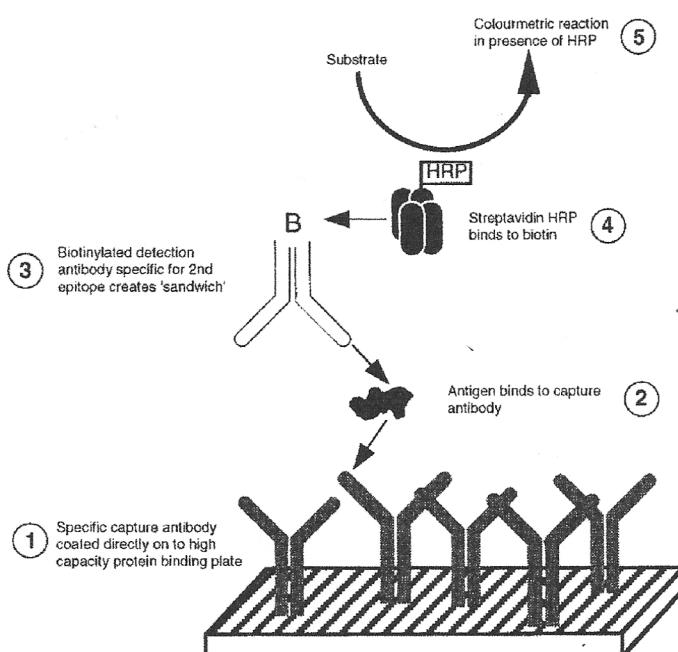
**A**

Fig. 1. Diagrammatic representation of (A) the indirect sandwich ELISA, and (B) [facing page] the basic direct ELISA.

cal. In this situation a direct ELISA should be used. During the direct ELISA the sample itself is coated directly onto the microtiter plate and is then detected using the specific antibody. If this antibody is biotinylated, the procedure can proceed as in the indirect ELISA; if not, then a secondary biotinylated antibody directed against the species of the detecting antibody itself can be used. **Figure 1B** demonstrates this technique.

To set up a reliable and durable ELISA it is essential to first optimize a number of the parameters mentioned above. The level of optimization will, of course, depend on exactly what is required from the assay. In some cases a simple "yes or no" answer is desired and a simple standard procedure may be sufficient. If, however, high sensitivity is the aim with accurate quantitation of the molecule in question, then carefully setting up the optimal conditions in advance will pay dividends and save a great deal of time in the long term.

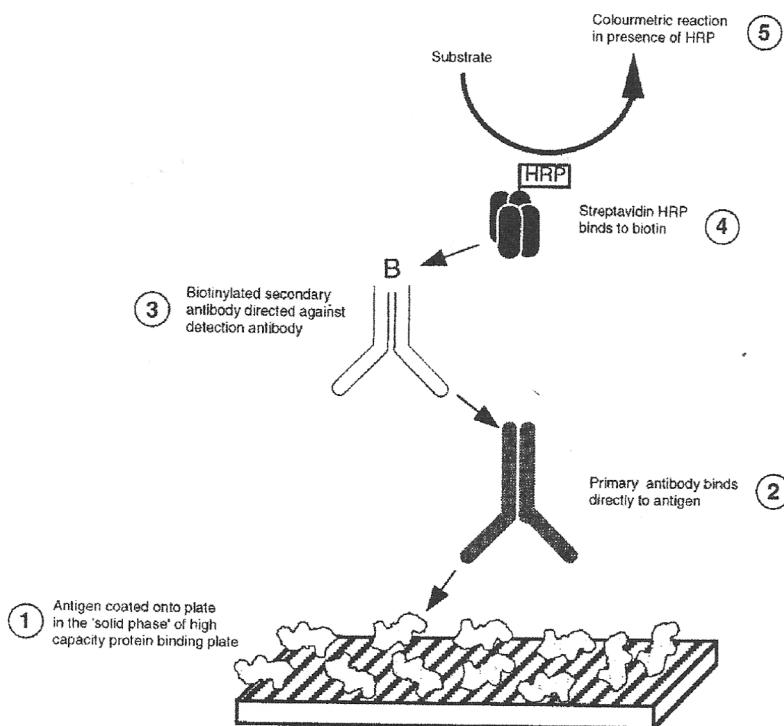
**B**

Fig. 1B.

## 2. Materials

1. **Antibodies:** For the indirect ELISA, antibody pairs can often be bought commercially and consist of a capture antibody and a biotinylated detection antibody. Both antibodies are specific for the molecule in question, with the detection antibody being directly biotinylated and able to recognize the sample molecule when it is bound to the capture antibody on the plate. For the direct ELISA, one specific detection antibody is required, preferably biotinylated, but if not a secondary biotinylated antibody specific for the detection antibody is also needed. Aliquot and freeze at -20°C or lower in small, usable quantities with a carrier protein, at such as 10% FCS or 1% BSA (*see Note 1*).
2. **Blocking buffer:** Phosphate-buffered saline (PBS), pH 7.4, supplemented with either 1% fatty-acid-free BSA or filtered 10% FCS. If measuring in tissue culture samples, use protein representing that within the culture conditions, i.e., 10% FCS; in effect the sample will have been preabsorbed with this protein during culture and thus any nonspecific binding to the blocking buffer is avoided.

3. Carbonate coating buffer: 8.41g Na<sub>2</sub>HCO<sub>3</sub> in 1 L freshly made PBS. Dissolve and adjust to desired pH with HCl or NaOH. Store for no more than 1 mo at 4°C (*see Note 2*).
4. High-capacity protein-binding 96-well microtiter plates: There are a large number of suitable makes including Maxisorp (Nunc, Roskilde, Denmark), Immunoware (Pierce, Chester, UK), Immunlon II (Dynex, Middlesex, UK), and Costar (*see Note 3*).
5. PBS/Tween/10% FCS: Add 0.05 mL Tween 20 to 90 mL PBS, pH 7.4, and 10 mL filtered FCS. Make up fresh as required.
6. Plate sealers or plastic wrap.
7. Plate-washing apparatus: Adequate washing is a vital element of achieving a successful ELISA. Although a number of automatic plate washers are available, they are expensive and the use of a wash bottle with good pressure is perfectly suitable, though a little more time consuming.
8. Samples/standards: Standards of known amounts are required for positive controls, and for estimation of levels within samples (via comparison to a titration of known amounts). All standards should be diluted in medium as near as, or identical to, that of the sample solution. Standards are best obtained from a reliable commercial source that has already mass calibrated them and should be frozen in small, concentrated aliquots at -20°C or lower. Repeated freeze-thaw cycles must be avoided.
9. Spectrophotometer: Any suitable microplate reader able to measure absorbance at the appropriate wavelength.
10. Stop solution: 0.5 M H<sub>2</sub>SO<sub>4</sub>.
11. Streptavidin HRP: Use in accordance with the manufacturer's instructions. Sources of TMB include Sigma (St. Louis, MO), Pierce, and Zymed.
12. Substrate: One-step TMB (Zymed). Although many other substrates are available for HRP, TMB has high sensitivity with a quick development time. OD can be monitored at 650 nm while the color develops, then at 450 nm when the reaction is stopped with H<sub>2</sub>SO<sub>4</sub>. TMB may also be obtained in a lyophilized state and made up fresh with hydrogen peroxidase, however, the pre-prepared solution is quick, easy, and, more importantly, extremely consistent (*see Note 4*).
13. Washing buffer: Add 0.5 mL Tween-20 to 1 L PBS, pH 7.4. Make up fresh as required.

### 3. Methods

#### 3.1. Basic Sandwich ELISA Protocol

1. Dilute the capture antibody to 1 µg/mL in coating buffer, pH 9.5. Add 100 µL to each well of a high-capacity protein-binding 96-well microtiter plate.
2. Seal the plate to avoid evaporation and incubate overnight (12–18 h) at 2–8°C.
3. Wash plate: Discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with 300 µL washing buffer and leave for at least 10 s before discarding once more. Ensure all liquid has been removed between each wash by repeatedly tapping the plate onto clean paper towels. Repeat three times.

4. Add 200 µL blocking buffer to each well. Seal the plate and incubate for at least 2 h at room temperature.
5. Discard blocking buffer. Wash the plate three times as in **step 3**.
6. Prepare a titration series of known standards (for example, in 1.5 mL Eppendorf tubes) diluted in a matrix representing that of the samples (e.g., culture medium or human serum). Include a negative control (i.e., culture medium only). Transfer samples and antigen standards to the ELISA plate in duplicate at 100 µL per well. Seal the plate and incubate at room temperature for at least 2 h, or overnight at 4°C for increased sensitivity.
7. Wash plate three times as in **step 3**.
8. Dilute biotinylated detection antibody to 1 µg/mL in PBS, pH 7.4. Add 50 µL per well. Incubate at room temperature for 2 h. If problems with nonspecific binding of the biotinylated antibody to the plate occur, dilute the antibody in PBS/Tween/10% FCS rather than just PBS (*see Note 5*).
9. Wash plate three times as in **step 3**.
10. Dilute streptavidin HRP according to the manufacturer's instructions. Add 100 µL/well. Incubate at room temperature for 30 min.
11. Wash plate four times as in **step 3**.
12. Add 100 µL/well of "one step" TMB. Allow color to develop for 10–60 min (20 min is usually sufficient). OD may be monitored at this stage at 650 nm as the color develops (*see Note 6*).
13. Add 100 µL 0.5 M H<sub>2</sub>SO<sub>4</sub> to each well to stop the reaction. Read OD at 450 nm.
14. Estimate the amount of antigen within the samples by comparing ODs to those of known standards.

### 3.2. Direct ELISA Protocol

1. Make serial dilutions of samples from neat to 1:16 in coating buffer, pH 9.5. Add 100 µL to each well of a high-capacity protein-binding microtiter plate. Also set up wells with antigen standards and a negative control diluted in the same coating buffer.
2. Seal the plate with an acetate plate sealer or cling film wrap to avoid evaporation and incubate overnight (12–18 h) at 2–8°C.
3. Wash plate: Discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with 300 µL washing buffer, leave for at least 10 s, and discard once more. Ensure all liquid has been removed between each wash by repeatedly tapping the plate onto clean paper towels. Repeat three times.
4. Add 200 µL blocking buffer to each well. Seal the plate and incubate for at least 2 h at room temperature.
5. Discard blocking buffer. Wash plate three times as in **step 3**.
6. Dilute detection antibody to 2 µg/mL in PBS. Add 50 µL/well. Incubate at room temperature for 2 h. If the antibody is biotinylated proceed to **step 9**.
7. Wash plate three times as in **step 3**.
8. Add secondary antibody (100 µL, 2 µg/µL) specific for the detection antibody (i.e., if the detection antibody is mouse, use biotinylated rabbit antimouse Ig) (*see Note 5*).

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11. Wash plate four times as in **step 3**.
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13. Add 100 µL 0.5 M H<sub>2</sub>SO<sub>4</sub> to each well to stop the reaction. Read OD at 450 nm.
14. Estimate amount of antigen within samples by comparing ODs to those of known standards of sample.

### **3.3. Optimization of ELISA**

At this stage the ELISA may be more than adequate, although optimization of the following parameters is usually required (*see Note 7*):

1. pH of carbonate coating buffer (pH 7.0–10.0): Also try PBS at pH 7.4.
2. Concentration of capture antibody (0.5–10.0 µg/mL).
3. Concentration of secondary antibody (indirect ELISA, 0.5–4.0 µg/mL).
4. Concentration of biotinylated detection antibody (0.1–2.0 µg/mL).
5. Concentration of streptavidin-HRP conjugate (usually between 1:1000 and 1:10,000).

### **4. Notes**

1. The quality of the antibodies used is perhaps the most important aspect in setting up a good ELISA. Antibodies need to have a high affinity for the sample to be measured, reducing the chance of being "washed off" during the assay. The indirect ELISA relies on two specific antibodies and thus the increased specificity increases sensitivity of the assay by reducing background.
2. The pH of the coating buffer can have a huge effect on the amount of antibody that will bind to the plate and thus to the ELISA as a whole. Basically, a higher pH will result in more antibody binding but may have a detrimental effect on its immunoreactivity. Thus, a pH must be found that is suitable for the antibody in question; this can vary dramatically. When beginning optimization of the assay, test a range of carbonate buffers from pH 7.0 to 10.0 as well as PBS, pH 7.4. We usually find a carbonate buffer pH of 9.5 gives good results. In some cases we have found commercially available coating antibodies recommended by the manufacturer to be adsorbed onto the plate at pH 7.4 to be far more effective at higher pHs, improving the lower detection limit of sensitivity by up to 1000%, and thus allowing the coating concentration to be vastly reduced and creating an extremely cost-effective assay.
3. There can be a significant difference in the protein binding capabilities of different makes, and even batches of microtiter plates. Some appear to be extremely good for binding antibodies, whereas others more useful for other proteins. The only real way to choose a suitable plate is by trial and error, or using a recommended make known to be good for the protein you intend to coat.

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