

# ELISA Optimization Protocol

## 1.0 Checkerboard Titrations (CBT)

The many systems described previously all require that the reagents used are optimized. In other words, the working concentration of each component of the test has to be assessed.

A key feature in helping this process is through the use of chessboard or checkerboard titrations (henceforth abbreviated to CBT).

The use of microtitre plates is a key feature of ELISAs and this type of format is described in this chapter. CBTs can be accomplished in any format whereby reagents can be diluted, but the microtitre plate, with its associated equipment for ease of pipetting, is ideal.

It will become clear that CBT is not the only method for optimizing reagents and that there is often some adjustment to be made to concentrations with reference to actual test conditions.

The process of CBT involves the dilution of two reagents against each other to examine the activities inherent at all the resulting combinations. The maximum number of reagents that can be titrated on a plate is two and this is illustrated in the Direct ELISA shown in 1.1.

The use of CBT in some other systems with more than 2 reagents, is also illustrated. The descriptions of pipetting and diluting techniques are also fundamental to performance of ELISAs in general.

The amount of detail needed to describe the tests will reduce as the reader becomes familiar with the methods.

**Table 1.**  
**Basic ELISA system components requiring titration**

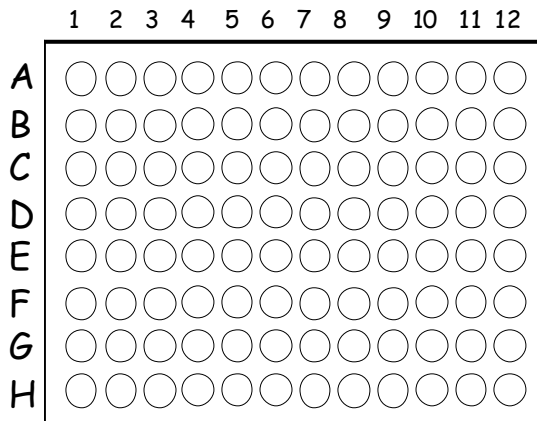
| <b>ELISA titrated</b>    | <b>Reagents Involved</b>   | <b>Number titrations</b> |
|--------------------------|--|--------------------------|
| <b>Direct</b>            | 1. Antigen<br>2. Antibody conjugated to enzyme   | 2                        |
| <b>Indirect</b>          | 1. Antigen.<br>2. Antibody.<br>3. Anti-species conjugate.  | 3                        |
| <b>Sandwich Direct</b>   | 1. Capture antibody.<br>2. Antigen.<br>3. Conjugated second antibody.  | 3                        |
| <b>Sandwich Indirect</b> | 1. Capture antibody<br>2. Antigen<br>3. Second antibody<br>4. Anti-species conjugate against second antibody | 4                        |

Table 1, gives a simplified overview of the systems indicating the number of reagents needed to be optimized, as a reminder.

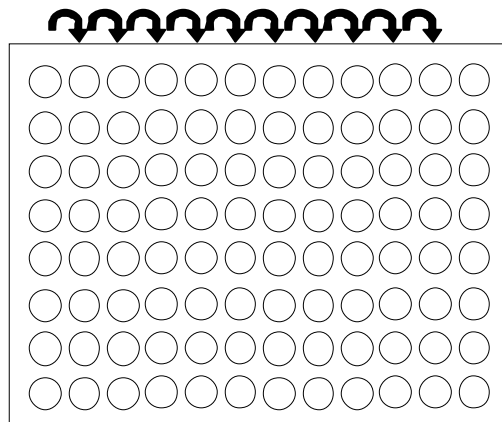
## 1.1 Direct ELISA CBT

**Fig 1. Direct ELISA scheme**

The numbers 1 to 12 denote columns, A-H denote rows.



Stage i) Dilute the antigen from column 1 to column 11. Column 12 receives diluent only. Incubate. Wash wells

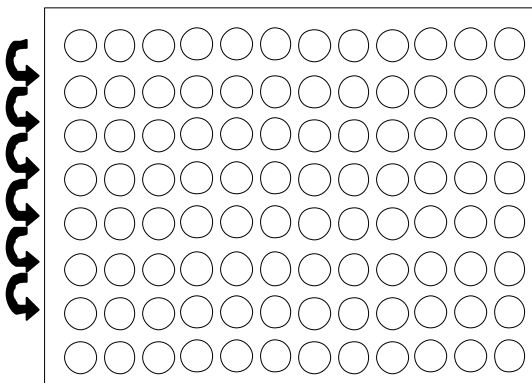


Stage ii)

Add the labeled antibodies, dilute from row A to row G. Row H receives diluent only. Antibody is diluted in blocking buffer (containing inert protein and or detergent to prevent non-specific adsorption of protein. Incubate. Wash.

Add chromophore/substrate.

Read OD.



The first part of the figure illustrates the typical numbering and lettering associated with microtiter plates. Thus, columns are labeled 1 to 12 and rows are labeled A to H. This nomenclature will be used from now on to identify locations on the plates.

## Stage One of Direct ELISA CBT

This involves diluting the antigen in a coating buffer. The volumes usually used in ELISA are 50 or 100  $\mu\text{L}$ .

A 2 fold dilution range is usual at this stage e.g., one volume taken from one well and added to the same volume in the next well, and so on.

The most practical way of performing the test is to:

1. Add the diluent (in this stage the coating buffer) in 50  $\mu\text{L}$  volumes to all wells of the plate using a multichannel pipette.
2. Add 50  $\mu\text{L}$  of a dilution of the antigen to all wells in column 1. The initial dilution can be made in a small bottle to result in a volume necessary for addition to column 1, i.e., you will need 8 wells  $\times$  50  $\mu\text{L}$  = 400  $\mu\text{L}$  of antigen dilution.

It is advisable to make slightly more of the initially diluted antigen than is theoretically needed to allow for material adhering to bottles etc., in this case 500  $\mu\text{L}$  (0.5 mL) should be made.

Assessment of the initial dilution is based on any knowledge of the antigen concentration likely (e.g., as assessed from other tests). The point about the CBT is that there is going to be a direct assessment of activity in the ELISA at a range of concentrations, therefore if there is a gross under or over estimate of antigen, then another CBT can be made accounting for such problems.

Some considerations have to be taken of: the likely purity of the antigen (concentration of specific antigens as compared to contaminants) and, the availability of antigen. A useful starting dilution for all antigens might be 1/10-20 in coating buffer. Let us assume that we add 1/20.

3. Add 50  $\mu\text{L}$  of the pre-diluted antigen to all wells in column A. Mix with multichannel pipet fitted with 8 tips. The mixing implies that the liquid in the well is pipeted up and down in the tip at least 5 times. This should not be done too vigorously.
4. After the final mixing take 50  $\mu\text{L}$  of the diluted antigen from the 8 wells in column A and transfer to column B. Mix as before.

Repeat the procedure until column 11. Note that this means that there is no antigen in column 12 and this will serve as one control (development of color with conjugate dilutions on wells containing no antigen).

5. After final mixing action in column 11, take out 50  $\mu\text{L}$  and discard. In the wells now we have created a 2-fold dilution series of antigen in coating buffer, beginning at 1/20 in column A and ending at 1/20,480 in column 11. For a full treatment of the use of different dilution ranges see later in this chapter.
5. Read the OD of color in a spectrophotometer.
6. The plate can now be incubated to allow time for the adsorption of antigen to the wells. The nature/time of the incubation should be that to be used in the test proper.

Most antigens will attach with incubation under stationary conditions at 37°C in 2 hours. However, it may be more convenient to allow overnight incubation at 4°C. Whatever conditions are applied they must be followed in the subsequent development of the test since alteration in times/temperatures/regimes of shaking or tapping plates, will alter the kinetics of adsorption.

7. The plate is now washed by flooding and emptying the wells with PBS.

## Stage Two of Direct ELISA CBT

1. This involves making a similar dilution range of the conjugated antibody made against the antigen. In this case the dilution range is made from row A to row G. The added buffer is blocking buffer (containing a relatively high concentration of inert protein to prevent non-specific binding of proteins (see Chapter 3). In this case the blocking buffer might be PBS (0.1M, pH 7.6, containing skimmed milk powder (5%) and 0.05% Tween 20.

The dilution range is made using the multichannel pipet with 12 tips, directly in the wells. Again there must be mixing between each addition. Note that there is no dilution of conjugate into row H, this acts as a control for substrate and antigen only (since the wells contain a dilution range of antigen. The initial dilution should be in the region of 1/50 for a direct conjugate. The plate is incubated under rotation (best) at 37°C for 1 hour or stationary at 37°C for 2 hour. Wash the wells.

2. Add chromophore/substrate. This could be any of the ones described in Chapter 3 added with due care as to accuracy and checks on the pHs of the buffers involved. In this example we shall assume we add  $\text{H}_2\text{O}_2/\text{OPD}$  at 50  $\mu\text{L}$  per well (in every well of plate).
3. Leave plate stationary for 15 minutes to allow color to develop. The exact timing of color development and conditions should be adhered to in subsequent assays. At this stage it is good practice to observe the plate for the rate of color development.

4. Stop (depending on system).
5. Read the OD of color in a spectrophotometer.

## Results

Table 2 shows stylized results which might be obtained.

**Table 2**  
**Results of CBT (OD data)**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2.1 | 2.2 | 2.1 | 2.1 | 2.0 | 2.1 | 1.9 | 1.7 | 1.5 | 1.3 | 1.0 | 0.6 |
| B | 2.1 | 2.0 | 1.9 | 2.0 | 1.9 | 1.8 | 1.7 | 1.5 | 1.3 | 0.9 | 0.5 | 0.3 |
| C | 2.1 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.7 | 1.5 | 1.2 | 0.9 | 0.5 | 0.3 |
| D | 1.8 | 1.8 | 1.7 | 1.8 | 1.5 | 1.2 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| E | 1.8 | 1.8 | 1.7 | 1.5 | 1.3 | 1.1 | 0.9 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| F | 1.5 | 1.5 | 1.4 | 1.3 | 1.1 | 0.9 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 |
| G | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Note that rows D and E are highlighted and column 12 rows A, B, C.

We are attempting to assess the optimal dilutions of antigen to coat the wells and the interaction of the conjugate. Fig. 2. shows the data plotted.

## Analysis of Data

Each of the columns contains a constant but different dilution of antigen. Differences in color between the columns, where there is a constant addition of labelled antibody, reflects the effect of altering the concentration of antigen.

The rows can be assessed for a maximum color where we have a range of values which are similar. This can be regarded as a plateau and reflects areas where the antibody is in excess. In this area, where there is no decrease in color on dilution of the antigen this indicates that a maximum saturating level of antigen is coated to the plates. Thus, we can identify regions of antigen/antibody excess.

Taking row A

| 1 |     | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2.1 | 2.2 | 2.1 | 2.1 | 2.0 | 2.1 | 1.9 | 1.7 | 1.5 | 1.3 | 1.0 | 0.6 |

The shaded values are similar, giving a plateau maximum value of around 2.0 OD units. There is no effect on color where antigen is coated at 1/20, 1/40, 1/80, 1/160, 1/320 or 1/640, (wells 1-6). This indicates that in the presence of a constant dilution of antibody there is a similar amount of antigen coating the wells to a dilution of 1/640. Following further dilution there is a decrease in OD values on dilution of the antigen. Note that rows B and C give similar results showing a plateau from rows 1-6. Fig. 2 shows graphically that the curves are similar, although there are slight reductions on dilution of the conjugate. Again this indicates that antigen is in excess, certainly at the dilution added to column 5. Thus, increasing the concentration of antigen above that contained in dilution at 1/320 is a waste of antigen.

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2.1 | 2.2 | 2.1 | 2.1 | 2.0 | 2.1 | 1.9 | 1.7 | 1.5 | 1.3 | 1.0 | 0.6 |
| B | 2.1 | 2.0 | 1.9 | 2.0 | 1.9 | 1.8 | 1.7 | 1.5 | 1.3 | 0.9 | 0.5 | 0.3 |
| C | 2.1 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.7 | 1.5 | 1.2 | 0.9 | 0.5 | 0.3 |

Note should be taken of the OD values in column 12 since this represents the color developing where there is no antigen. The values are high for A, B and C, relative to color for D, E, F and G, which are low and the same. This color can be presumed to be the result of non-specific attachment of the conjugate to the wells. The higher the amount of protein added, the greater the chance on non-specific events.

Note that there is a reduction in color down each of the columns on dilution of conjugate even where we have shown there to be excess antigen (columns 1-5/6). This is due to the reduction in concentration of the conjugate (diluting out of reactive enzyme-labeled antibodies).

To obtain the most "controllable" results in ELISA, it is advised that maximum OD values are around 1.5. to 1.7 OD units. Values above this are inaccurate on instrumental grounds. Thus, optimization involves assessment of assays where plateau maxima are around these OD values.

This is based on OPD/H<sub>2</sub>O<sub>2</sub> and other systems have their own optima, but these should never approach very high ODs with respect to each substrate/chromophore system. We should now concentrate on rows D and E where we have a plateau height of 1.8 in the region where antigen is in excess.

|          |     |     |     |     |     |     |     |     |     |     |     |     |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <b>D</b> | 1.8 | 1.8 | 1.7 | 1.8 | 1.5 | 1.2 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| <b>E</b> | 1.8 | 1.8 | 1.7 | 1.5 | 1.3 | 1.1 | 0.9 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |

For row D we have a titration of antigen (decrease in color from the maximum on dilution of antigen), beginning at column 5, for row E starting at column 4. The titration curves for each seen in Fig. 2 are not as "high" as for those in A, B and C however the background values for both are low (0.1OD) and identical with controls where there is no antigen or conjugate addition (e.g. H-12).

On further dilution of conjugate (rows F, G and H), there is a distinct decrease in the color obtained where antigen has been shown to be in excess and on dilution of the antigen. Here there is a drop in the potential "analytical sensitivity" of the assay, (ability to detect antigen), reflected in the decrease in plateau height OD and the area under the titration curves seen in Fig. 2

This has been a rather long description of a relatively simple operation. However, remember that at the beginning of the test we had no idea as to whether antigen bound to a plate or whether the labeled antibody would bind to antigen. We can view the results of the plate "by-eye" very quickly and almost instantly see that the test has worked and what the optimal areas are, without reference to the actual OD results. This can be important when performing initial experiments where a relatively large amount of work is made to assess a more complex situation. The ability to assess tests very rapidly by eye is a distinct plus factor over assays where quantification relies on instrumentation alone.



## Summarizing the results

1. The antigen coats plate to give maximum reaction where antibody conjugate is in excess to 1/320-1/640.
2. Conjugate dilutions in rows A, B and C are too "strong" and give too high plateau maxima.
3. Rows D and E give good titration curves for antigen and have "ideal" plateau height maxima.
4. There is a loss in analytical sensitivity if the conjugate is diluted as in rows F and G.

We have now completed the first "sighting" experiment evaluating the Direct ELISA. At this stage we can repeat the experiment with some alterations of reagent dilutions or conditions, depending on the results obtained. In the above example we have ideal results with good activity for antigen and antibody conjugate. This need not be the case and below there are two examples of poor results necessitating alterations and reassessment.

## Poor Results

**Table 3**

**Results of chessboard titration (OD data) where the antigen is limiting reaction**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1.7 | 1.3 | 0.7 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| B | 1.7 | 1.3 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C | 1.7 | 1.2 | 0.4 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| D | 1.6 | 1.8 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| E | 1.5 | 1.8 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 1.4 | 1.5 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.9 | 0.7 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

This example shows the results of an identical experiment to that described in 1.1.

We now see that there is a good result in rows A and B and that after this dilution of antigen, very little color is produced. This indicates that there is little antigen attaching on dilutions greater than that used in row B.

The conjugate appears to be usable to detect antigen until rows D and E since we obtain similar OD values where there is enough antigen coating the wells. In this case the CBT could be repeated with a different dilution range of antigen to possibly increase the plateau maximum area in the presence of excess conjugate.

The next example, results of which are shown in Table 4. shows what may happen where the conjugate is of a low reactivity.

**Table 4**  
**Results of chessboard titration (OD data) where the labeled antibody is limiting reaction**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1.0 | 1.0 | 1.0 | 0.9 | 0.8 | 0.4 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| B | 0.6 | 0.6 | 0.5 | 0.5 | 0.5 | 0.4 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| D | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| E | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

The results show that we have low color in the test. There is a rapid decrease in color on dilution of the labeled serum , rows A to B to C, etc.

There is however, a plateau from columns 1 -5 (A, B, C) indicating that there is antigen attaching at a similar level in these wells. In this case, one variation suggested from the initial CBT would be to coat plates with antigen at the dilution used in row 3 (the last dilution showing a plateau maximum value), and titrate the conjugate, beginning at a higher concentration. In this way a better estimate of conjugate activity would be obtained.

This situation is common to Direct ELISA systems since production of "good" conjugates depends on the specific activity of the antibodies labeled which is a function of the weight of enzyme attached to antigen specific antibodies. Conjugation of polyclonal sera usually results in the specific attachment of enzyme to the relatively small percentage of the total protein content of the sample which is specific antibody, the rest of the protein is labeled leading to problems with high backgrounds.

## Plateau OD Values

The maximum color obtained in an assay where reagents are in excess results from:

1. The amount of antigen that can passively attach to a well
2. The number of antigenic sites available for antibody binding
3. The specific activity of the conjugate in terms of how much enzyme is attached to particular
4. The density of the antigenic components on the wells antibody species in the whole serum and their respective affinities

These factors will be examined later, but the CBT allows a rapid estimate of the feasibility of assays whose results may indicate problems associated with the factors above.

## More Complicated Systems

After the simplest case of the Direct ELISA we have to consider situations where there are three or more components to titrate. These are shown in Table 5.

It must be remembered that only two components can be varied by dilution in any test. The criteria for assessment of each of the stages in CBT are similar to those described extensively for the Direct ELISA.

Remember also that the CBT aims to indicate optimal conditions and is very useful in arriving at a feasible concentration range for all components in the desired assay in a rapid way. The objective is to examine reagents as to their usefulness and lead to conditions for a fully defined test to perform a specific task.

**Table 5**  
**Possible combinations for titrations**

|   |           |            |                          |
|---|-----------|------------|--------------------------|
| <b>D = dilution series made</b>                                   |           |            |                          |
| <b>C = constant (no dilution)</b>                                 |           |            |                          |
| <b>Indirect ELISA-Antigen + Antibody + Anti-species conjugate</b> |           |            |                          |
| <b>Phase</b>  |           |            |                          |
| <b>1</b>  | Antigen D | Antibody D | Anti-species conjugate C |
| <b>2</b>  | Antigen C | Antibody D | Anti-species conjugate D |
| <i>Optimized reagents in 1 and 2 checked in 3.</i>                |           |            |                          |
| <b>3</b>  | Antigen C | Antibody C | Anti-species conjugate D |

|   |            |           |                     |
|---|------------|-----------|---------------------|
|   |            |           |                     |
| <b>Sandwich ELISA Direct (3 components) Antibody + Antigen + Labeled Antibody</b> |            |           |                     |
| <b>1</b>  | Antibody D | Antigen D | Labelled antibody C |
| <b>2</b>  | Antibody C | Antigen D | Labelled antibody D |
| <i>Optimized reagents in 1 and 2 checked in 3.</i>                                |            |           |                     |
| <b>3</b>  | Antibody C | Antigen C | Labelled antibody D |

|  |            |           |            |                 |
|--|------------|-----------|------------|-----------------|
| +  |            |           |            |                 |
| <b>Sandwich ELISA Indirect (4 components) \Antibody + Antigen ANTIBODY + Anti-ANTIBODY Conjugate</b> |            |           |            |                 |
| <b>1</b>   | Antibody D | Antigen D | ANTIBODY C | Anti-ANTIBODY C |
| <b>2</b>   | Antibody C | Antigen C | ANTIBODY D | Anti-ANTIBODY D |
| <i>Optimized reagents in C antibody</i>  |            |           |            |                 |
| <b>3</b>   | Antibody C | Antigen C | ANTIBODY D | Anti-ANTIBODY C |
| <b>4</b>   | Antibody C | Antigen C | ANTIBODY C | Anti-ANTIBODY D |

Competition assays involve the interruption of these systems these they have to be titrated in the same way to allow competitive or inhibition techniques.

## 1.2 Indirect ELISA

The indirect assay is used mainly to measure antibodies against a specific antigen either through the full titration of a sample or as a single dilution.

We need a test with an optimal amount of antigen coated to wells which will successfully bind to antibodies which in turn can be detected with an optimal amount of anti-species conjugate.

We can only titrate two of these variables in one assay.

The most important aspects to consider are:

- **That enough antigen is available for antibody binding. We do not want to waste antigen by adding concentrations which are too high such that the wells receive large excess of antigen compared to that amount which is needed to "fill" available plastic sites**
- **That we have optimal amounts of conjugate to avoid high non-specific backgrounds and to allow the detection of all bound antibody molecules to give the required analytical sensitivity to be achieved**
- **We also need to assess the effect of diluting negative sera (from same species as test samples) on an assay to obtain an idea as to the possible backgrounds of such sera at various dilutions**

Thus, we can modify the needs for setting up this assay:

- 1) We must have antigen coating to wells sufficient to capture antibodies
- 2) We need at least one serum positive for the antigen
- 3) We need at least one negative serum from the same species as the test samples
- 4) We need an anti-species conjugate

## Initial Chessboard Titration

The initial test should be a CBT relating antigen dilutions to the positive and negative sera, using a commercial conjugate diluted to the recommended level.

However, the individual laboratory estimation of optimal titres of anti-species conjugates is relatively easily made using the Direct ELISA format. Either whole serum from the particular animal species target or a fraction (IgG, etc.) from the serum can be used to coat plates in stage one of the direct assay.

- **Where whole serum is used, 1/200 should be the initial dilution from rows 1-11**
- **Where IgG is prepared the weight can be measured spectrophotometrically and a starting concentration of 10 µg/mL used**

The second stage involves dilution of the conjugate in blocking buffer.

Estimation of the optimal amount of conjugate is as already described. A dilution yielding a plateau maximum or around 1.8 OD units with a "good" titration curve should be used in Indirect ELISA assessment.

The dilution used can be altered later as a result of examination of results from the titration. This may be necessary since the exact nature and concentration of the specific immunoglobulins binding in the Indirect ELISA to the specific antigen(s) may differ from those in the serum or serum fraction preparations.

## Stage One of Indirect ELISA CBT

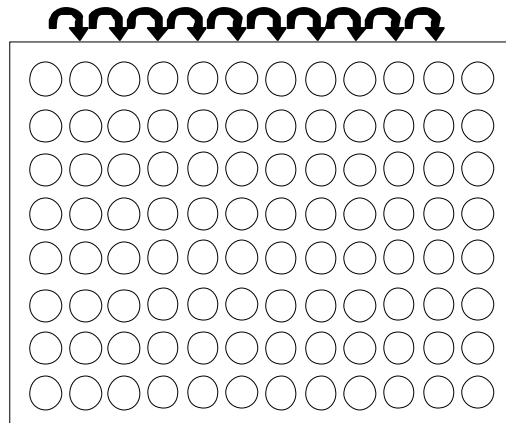
Figure 3 shows the stage 1 CBT for the Indirect ELISA. This titrates the antigen against the positive and negative sera. This can be regarded as a "sighting" exercise, remember that conditions can be changed in a repeat chessboard titration. Typical results might be those in Table 6.

**Fig. 3 Indirect ELISA scheme**

### Stage i)

Dilute the antigen from column 1 to column 11. Column 12 receives diluent only. Incubate.

Wash wells



### Stage ii)

Add the serum containing antibodies against antigen (one plate) or negative serum (second plate), dilute from row A to row G. Row H receives diluent only. Antibody is diluted in blocking buffer (containing inert protein and or detergent to prevent non-specific adsorption of protein.

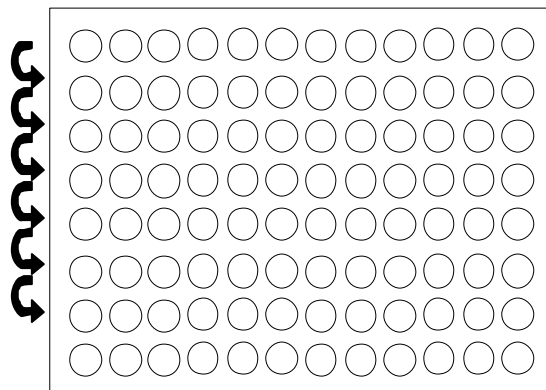
Incubate. Wash.

Add anti-species conjugate (given or pre-titrated) at single dilution in blocking buffer. Incubate. Wash.

Add chromophore / substrate.

Incubate. Stop reaction.

Read OD.



**Table 6**  
**Results of Stage 1 titration of antigen and positive antibody**  
**containing samples for Indirect ELISA**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2.2 | 2.3 | 2.2 | 2.1 | 2.0 | 2.0 | 1.9 | 1.6 | 1.5 | 1.3 | 0.8 | 0.6 |
| B | 2.2 | 2.0 | 1.9 | 2.0 | 1.9 | 1.8 | 1.7 | 1.5 | 1.3 | 0.8 | 0.5 | 0.3 |
| C | 2.1 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.6 | 1.5 | 1.2 | 0.7 | 0.5 | 0.3 |
| D | 1.9 | 1.9 | 1.7 | 1.8 | 1.5 | 1.2 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| E | 1.8 | 1.8 | 1.7 | 1.5 | 1.3 | 1.1 | 0.8 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 |
| F | 1.3 | 1.3 | 1.2 | 1.2 | 1.1 | 0.8 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 |
| G | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

In this example, we have used a titration of antigen from 1/50, 2 fold, and a titration of serum from 1/50, 2 fold.

The controls for the test are shown in column 12, which contains no antigen, but contains a dilution range of antibody; and row H which contains a dilution range of antigen, but no antibody.

The results can be regarded as "good", since we have color development at high levels and a corresponding titration as we reduce antigen or antibody. Table 7 shows the area in grey, where there is an optimal amount of antigen coating allowing antibodies to be titrated maximally.



**Table 7**  
**Optimal coating region for titrating antibodies**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2.2 | 2.3 | 2.2 | 2.1 | 2.0 | 2.0 | 1.9 | 1.6 | 1.5 | 1.3 | 0.8 | 0.6 |
| B | 2.2 | 2.0 | 1.9 | 2.0 | 1.9 | 1.8 | 1.7 | 1.5 | 1.3 | 0.8 | 0.5 | 0.3 |
| C | 2.1 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.6 | 1.5 | 1.2 | 0.7 | 0.5 | 0.3 |
| D | 1.9 | 1.9 | 1.7 | 1.8 | 1.5 | 1.2 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| E | 1.8 | 1.8 | 1.7 | 1.5 | 1.3 | 1.1 | 0.8 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 |
| F | 1.3 | 1.3 | 1.2 | 1.2 | 1.1 | 0.8 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 |
| G | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Thus, antigen can be diluted to the levels in column 5-6 before there is a loss in color. Notice that the plateau height maxima do decrease particularly after row E indicating that there is a reduction in antibodies binding due to the decrease in amount of antigen coating the wells.

Examination of column 12 indicates that in the absence of antigen, the positive serum at dilutions of 1/50, 1/100 and 1/200 does bind to the plate producing background.

This is typical of serum in the Indirect assay and such non-specific backgrounds have to be considered carefully when adapting the Indirect ELISA for screening of test samples at single dilutions, since they influence the effective analytical sensitivity of assays.

The same color develops in the titration of the negative serum (Table 8) indicating that this background stems from an interaction of the serum proteins contained in the serum.

Table 8 shows the results of titrating the negative serum. There is generally much lower color development as to be expected if there is no antibody binding to the antigen. There is however, some color at various combinations particularly where the concentrations of the negative serum are high. This color is due to non-specific attachment of the serum components from the species of animal being tested. Thus, the background needs to be examined and can be reassessed when re-titrating the conjugate as described in Stage 2. As already indicated there is color in A12 which is

almost as high as that seen in A1. The latter well contains antigen indicating that there is a slight increase in binding here, although this not great. The serum dilutions of 1/50 and 1/100 do show some background and as already indicated this is mainly due to interaction of the serum proteins with the plate non-specifically.

**Table 8**  
**Chessboard titration of antigen and negative serum**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 0.7 | 0.7 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.6 |
| B | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| C | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| D | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| E | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

### Stage Two of Indirect ELISA CBT - Titration of Sera and Conjugate

From the first CBT, we can estimate an antigen dilution where there is good color development as a result of the binding with antibodies.

Since we do not want to waste antigen by adding excess (which is washed away in coating phase), we can select the last dilution of antigen which gives a good titration curve for the antiserum (i.e., high plateau height maxima and high end point). Reference to Table 7 indicates that column 5/6 has enough antigen to fulfil these criteria. Thus, wells could be coated at this single dilution and it would be expected that the positive serum would titrate with a maximum OD (where antibodies were in excess) of approximately 2.0; and that antibodies would still be detected (on dilution) to row G.

### Titration of Sera

In this example the positive serum dilution range is not extensive enough to allow titration of antibodies to an end-point (where OD in presence of antibodies equals background OD).

This can be addressed by altering the dilution range of the positive antiserum in stage two. Assuming we have taken the antigen dilution as that in column 5 then the first operation in stage two involves:

1. Coat wells of two plates with antigen at a dilution equivalent to that in column 5 (1/800). Incubate for same times as in stage one. Wash wells.
2. Add dilution range of positive or negative antisera as used in stage one. Incubate and wash wells.

Here, as indicated above, we can alter the range since it was indicated in stage (i) that we did not find the end-point of the positive serum because we used a too-limited range of dilutions. We can increase the range in any of three ways namely:

1. Dilution the serum beginning at column 1 and diluting to column 11
2. Starting at a higher initial dilution, e.g., 1/200
3. Altering the dilution range to a three-fold (rather than two-fold)

The best method has to be assessed with reference to the initial CBT. Where the dilution range was far too low and high color obtained across the plate with indication of a titration only in the last 2 rows, then use of a three fold range is recommended.

In the present example, the dilution of positive serum began at 1/50 and was diluted 2 -fold, in a seven well series.

Thus, we obtained dilutions: 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200.

At the last dilution we had not obtained an end-point and by examination of the curves we can predict that at least another four similar dilution steps are needed before the color would be reduced to background (as the antibodies were diluted out). Thus, in stage (ii) we can use the coated plate and dilute the positive serum from 1/50 (as in stage (i)) by eleven steps to 1/51,200, using columns 1 to 11.

## Titration of Anti-species Conjugate

In stage (i) we estimated the appropriate conjugate dilution either from information given by the producers or from a preliminary CBT of the conjugate against serum coated to wells.

Titration of the conjugate at this point offers an examination of it's activity under the Indirect Assay conditions proper, allowing refinement of the dilution to maximise analytical sensitivity as a result of identifying areas where excess conjugate produces high backgrounds.

1. Add 2 fold dilution range of conjugate from row A to row H. Incubate. Wash

Again, we have the opportunity of adjusting the starting dilution based on Stage (i) results. Where the commercial company recommendation was used then it is good practice to begin the conjugate dilution approximately 4-fold higher than recommended and dilute to at least 4 fold lower. As an example, if the recommended dilution was 1/2000, then the conjugate should be titrated from 1/500 in 2-fold steps to 32,000. Where there was an initial CBT against relevant serum, then the same procedure should be adopted around the initially found "optimum".

2. Add relevant chromophore/substrate system. Incubate.

3. Stop reaction as in stage (i) and read OD in spectrophotometer.

## Results

Table 9 shows that there is a high background of 0.5 in row A, column 12. In this row 1/500 conjugate was used which indicates that this is binding non-specifically with the antigen coated plates. This is confirmed in Table 10, where the background value is maintained on dilution of the negative serum from A2 to A12. Although these are idealized results, they do illustrate a common phenomenon. This background is reduced in row B (dilution of conjugate 1/1000 with reference to the value in column 12 of Table 9, but Table 10 shows that there is still this higher background in B3 to B12, indicating some non-specific complications.

**Table 9**  
**Results of CBT for positive serum**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1.8 | 1.8 | 1.8 | 1.8 | 1.6 | 1.2 | 1.1 | 0.9 | 0.6 | 0.5 | 0.5 | 0.5 |
| B | 1.8 | 1.8 | 1.8 | 1.8 | 1.6 | 1.2 | 1.1 | 0.9 | 0.6 | 0.5 | 0.3 | 0.2 |
| C | 1.7 | 1.7 | 1.7 | 1.7 | 1.6 | 1.1 | 0.9 | 0.7 | 0.5 | 0.4 | 0.2 | 0.1 |
| D | 1.6 | 1.6 | 1.5 | 1.3 | 1.1 | 0.9 | 0.7 | 0.5 | 0.3 | 0.2 | 0.2 | 0.1 |
| E | 1.2 | 1.1 | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 |
| F | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.5 | 0.5 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Antigen constant, Serum diluted 1/50, 2 fold, from column 1 to 11. Conjugate diluted 1/500, 2 fold, rows A to G

**Table 10**  
**Results of CBT for negative serum**

|   | 1 2 |     | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 0.6 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| B | 0.4 | 0.3 | 0.2 | 1.8 | 1.6 | 1.2 | 1.1 | 0.9 | 0.6 | 0.5 | 0.3 | 0.2 |
| C | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| D | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| E | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Antigen constant Serum diluted 1/50, 2 fold, from column 1 to 11. Conjugate diluted 1/500, 2 fold, rows A to G

In row C for both plates, the background in column 12 is low (0.1) and remains constant for the rest of the dilutions of the conjugate. This can be regarded as the minimum background for the test (plate background).

Table 9 indicates that there is a "good" titration of serum in row C, where we have a plateau (region of maximum OD), indicating a region of antibody excess. Thus, the conjugate at the dilution in row C (1/2000) can detect the bound antibodies and an optimal OD reading be obtained. The end-point (with reference to the OD obtained in the absence of serum (C12) has not been obtained but there is a gradual reduction in OD as the serum is diluted (titration curve).

This can be contrasted to the idealized results for row C in Table 10. Here no titration is observed at any dilution of the negative serum.

Reduction in the concentration of the anti-species conjugate has two effects. One is to reduce the plateau maximum color, and the second is to effectively reduce the end-point of the titration. Thus, in Table 9 we see a small reduction in plateau in row D which becomes marked when we further dilute. By row G we have a low OD even where we know that there is enough antibody binding to give a strong signal in the presence of excess antibody (as seen in A, B, C and D, columns 1 to 4/5). The optimal dilution of the conjugate at this stage is therefore taken from assessing the plateau maximum color and the titration end-point with reference to the backgrounds in the controls. In this case a dilution of conjugate of 1/2000 to 1/4000 appears optimal with the serum dilutions used. At this dilution there is no OD measured in the negative serum.

## Binding Ratios

Another way of examining the results is to calculate the binding ratios relating the positive and negative titrations. This is simply the OD value at a given dilution for the positive serum divided by that of the negative serum. Table 11 shows the binding ratios (BR) for above example. This process gives a clearer picture of the best conditions for setting up assays and is a feature used when tests are used for diagnostic purposes.

**Table 11**  
**Binding ratios of positive and negative sera from Tables 9 and 10**

|   | 1    | 2    | 3    | 4    | 5    | 6    | 7   | 8   | 9   | 10  | 11  | 12  |
|---|------|------|------|------|------|------|-----|-----|-----|-----|-----|-----|
| A | 3.0  | 3.6  | 3.6  | 3.6  | 3.2  | 2.4  | 2.2 | 1.8 | 1.2 | 1.0 | 1.0 | 1.0 |
| B | 4.5  | 6.0  | 9.0  | 9.0  | 8.0  | 6.0  | 5.5 | 4.5 | 3.0 | 2.5 | 1.5 | 1.0 |
| C | 17.0 | 17.0 | 17.0 | 17.0 | 16.0 | 11.0 | 9.0 | 7.0 | 5.0 | 4.0 | 2.0 | 1.0 |
| D | 16.0 | 16.0 | 15.0 | 13.0 | 11.0 | 9.0  | 7.0 | 5.0 | 3.0 | 2.0 | 2.0 | 1.0 |
| E | 12.0 | 11.0 | 9.0  | 8.0  | 7.0  | 6.0  | 5.0 | 4.0 | 3.0 | 2.0 | 1.0 | 1.0 |
| F | 8.0  | 7.0  | 6.0  | 5.0  | 4.0  | 3.0  | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| G | 5.0  | 5.0  | 4.0  | 3.0  | 2.0  | 1.0  | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| H | 3.0  | 2.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

The table illustrates the highest binding ratios in rows C and D, despite the OD values for the positive serum being higher in A and B. This results from the relatively low OD values for the negative serum at higher dilutions of conjugate. Note that the BR at 1/50 and 1/100 positive serum in A1 and B1 and 2 are lower than in subsequent wells. This is typical and results from the effect of high non-specific binding at these dilutions with negative sera. In fact it is usual for this effect to be more exaggerated in practice.

Some care is needed in interpreting "best" conditions by this method since where there are extremely low OD values for binding with negative sera, even low OD values for positive sera can appear to give "best" results.

However, the lower the OD values being examined, the higher the potential variation in results and a compromise between what seem to be highest BR values and obtaining a reasonable OD value in the positive sample is required.

**Table 12**  
**Endpoints of serum titrations**

|   | 1    | 2    | 3    | 4    | 5    | 6    | 7   | 8   | 9   | 10  | 11  | 12  |
|---|------|------|------|------|------|------|-----|-----|-----|-----|-----|-----|
| A | 3.0  | 3.6  | 3.6  | 3.6  | 3.2  | 2.4  | 2.2 | 1.8 | 1.2 | 1.0 | 1.0 | 1.0 |
| B | 4.5  | 6.0  | 9.0  | 9.0  | 8.0  | 6.0  | 5.5 | 4.5 | 3.0 | 2.5 | 1.5 | 1.0 |
| C | 17.0 | 17.0 | 17.0 | 17.0 | 16.0 | 11.0 | 9.0 | 7.0 | 5.0 | 4.0 | 2.0 | 1.0 |
| D | 16.0 | 16.0 | 15.0 | 13.0 | 11.0 | 9.0  | 7.0 | 5.0 | 3.0 | 2.0 | 2.0 | 1.0 |
| E | 12.0 | 11.0 | 9.0  | 8.0  | 7.0  | 6.0  | 5.0 | 4.0 | 3.0 | 2.0 | 1.0 | 1.0 |
| F | 8.0  | 7.0  | 6.0  | 5.0  | 4.0  | 3.0  | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| G | 5.0  | 5.0  | 4.0  | 3.0  | 2.0  | 1.0  | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| H | 3.0  | 2.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

The assessment of "end-points" can also be made using this method. The last dilution of serum which gives a BR of over 1.0 can be judged as the end-point. In the above idealized example, the end points for the various dilutions of conjugate are shown as a line in Table 12. Here we can see that the effect of diluting conjugate is to reduce the end points (E, F and G). In B, C and D the final end point has not been found, although the indication from examination of the BR data is that the conjugate dilution in C gives the highest potential analytical sensitivity since it has a BR of 4.0 at the dilution in column 10, as compared to the other results. The only controls not discussed are those in row H. These are antigen coated wells (constant), antibody dilutions 1/50 2 fold, but no conjugate. In this example there is some color in the 1/50 and 1/100 positive serum wells despite there being no conjugate. This can affect the estimation of which serum dilution should be used in an Indirect assay involving testing of samples at a single dilution. This effect disappears at 1/200 positive serum and is not observed for the negative serum.

## Conclusions

Although this may seem a laborious process, the principles are easy. Initially the antigen was titrated against the antisera using an estimate of the conjugate dilution. This indicated that there was a significant difference between the two sera in the OD values obtained. The approximate antigen concentration which coated the plates was then taken and used to relate the antisera and conjugate dilutions. Thus, we can estimate that:

- The antigen can be used at 1/800.
- The conjugate can be used at 1/2000 to 1/4000.
- There is a good discrimination of positive serum from negative serum using these conditions, and a dilution of serum at 1/400 could be suggested for a test involving single dilutions of sample.



## Developing Indirect ELISAs

The tests just described could be made in two days. Further refinements could be made using the reagents in further tests where smaller changes in dilutions could be assessed. However, as with all ELISAs, it is imperative that the ultimate purpose of the test be addressed as early as possible.

Where the test is to be used to screen hundreds or thousands of sera according to positivity, based on a single dilution (in duplicate or triplicate), then the titration phase must include a reasonable amount of work to recognize the factors inherent in examination of a varied population of antisera.

The example centered on the use of a single positive and negative serum. This is patently not going to reflect differences in the population of sera to be examined. Thus, at the stage where we have a working dilution of the antigen and conjugate we must now include more positive and negative sera (where possible) to further test the parameters for optimizing analytical sensitivity. The problems then is to assess:

1. Whether the optimal antigen holds for a number of negative and positive sera
2. The optimal antiserum dilution to use for single sample screening. This is a balance between achieving maximum analytical sensitivity with maximal specificity
3. What is the mean OD value of a negative population (and its variability). This allows designation of positivity at different confidence levels and its variation

The Indirect assay may be used in a competitive or inhibition assay. In this case the problem is to screen positive sera for characteristics which best match those for field or experimental sera in terms of antibody populations reacting with specific determinants on the antigen used. In this case it may be necessary to screen a number of positive sera under test conditions to identify a single serum with the best properties.

Indirect assays also offer a relatively easy and rapid method of end point titration of many sera. Relatively simple titrations can give confidence in the properties of sera to be used in other tests i.e. they can confirm positivity or negativity. Again this is examined below in the worked examples of the use of Indirect ELISAs.

## 1.3 Sandwich ELISA-Direct

This is a three component assay. We have to titrate:

- 1. The capture antibody**
- 2. The antigen which is captured**
- 3. The detecting conjugate**

Only two components can be varied in any one test, so the same criteria as indicated in the Indirect ELISA apply.

Probably the most variable area in this assay is the activity of the labelled conjugate, which is usually produced in the laboratory developing the assay. Such conjugates can have very different properties due to the intrinsic amount of enzyme which is attached to specific antibodies within a polyclonal serum produced against the antigen.

Thus, no assumptions as to the activity can be made as in the case of anti-species conjugates from commercial sources, and the initial titrations require possibly more cycles to fine tune concentrations.

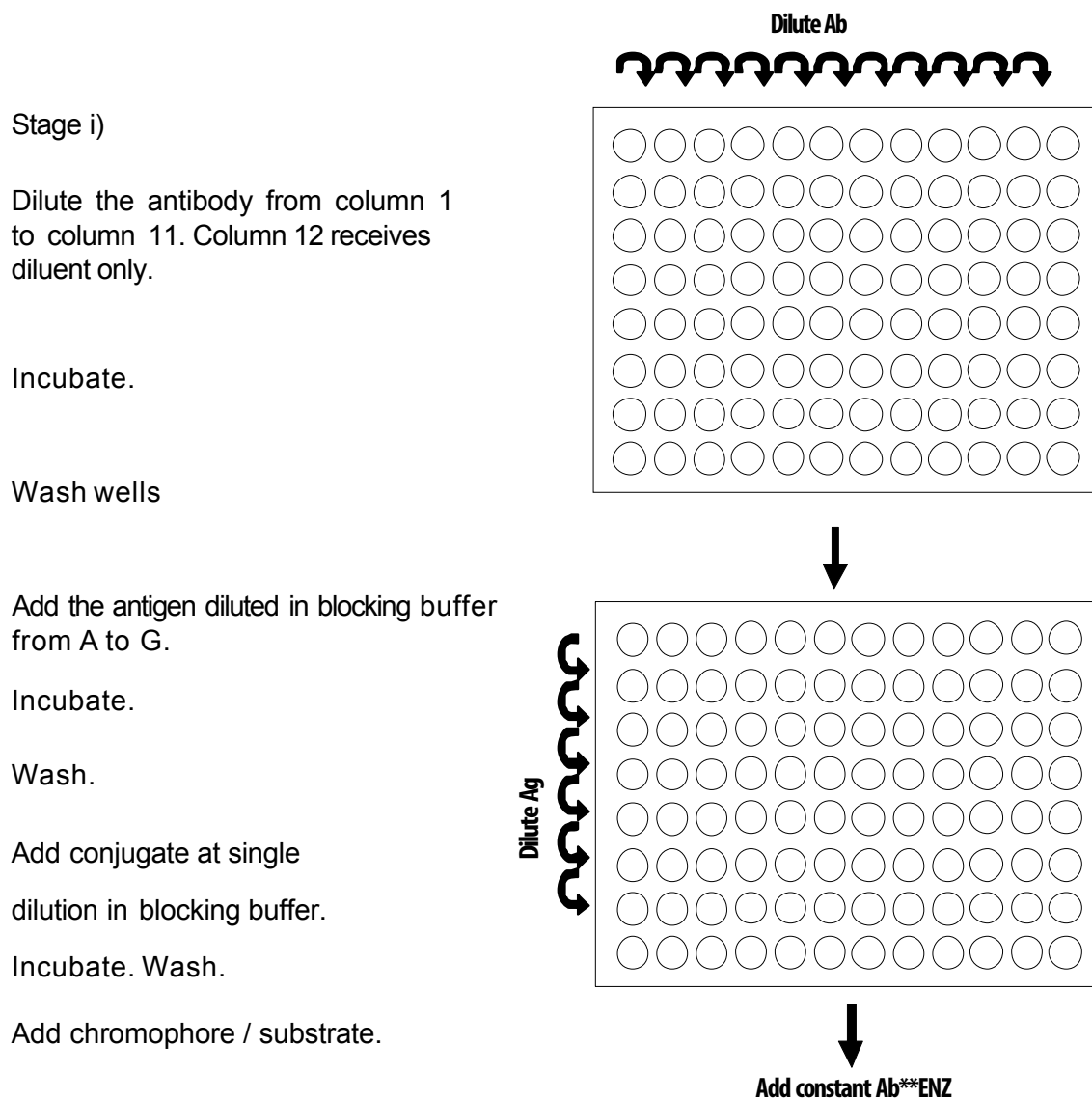
### Aids in Developing Assays

One aid to developing such assays can be the use of Indirect ELISAs to assess the relationship of antigen and antibody binding. However, the need to develop a capture ELISA, (antibody on wells as reagent to capture antigen), usually stems from the need to concentrate a weak antigen (unsuitable for Indirect ELISA), or need to capture a specific component of an antigenic mixture. The selection of an antiserum for labeling and conjugation, in the Sandwich ELISA, could be based on estimation of the titre of a number of sera by the Indirect ELISA where the antigen can be coated in a sufficient quantity. Thus, high titre sera can be identified. Such titrations can be made by other method, leading to the selection of sera with the highest activities.

## Stage One Titration of Capture Antibody and Antigen

These stages can be described more briefly than in the previous examples, since by now the reader should be getting familiar with the principles involved in CBTs. Fig. 4 shows the scheme.

**Fig. 4. Chessboard titration of capture antibody against antigen for Direct-Sandwich ELISA.**



Where whole serum is used as the capture reagent, then a dilution of 1/100 should be used in column 1.

Where immunoglobulin (e.g., IgG), has been prepared from the antiserum, the concentration of protein can be easily found through reading absorbance in a UV spectrophotometer. In this case a starting concentration of 10 µg/mL should be used, (we are still using a 50 µL volume as constant in this example).

At this level of protein, the wells will be saturated so that the activity of the capture antibody relies on the relative concentration of the specific antibodies in the IgG fraction as compared to the other IgG molecules in the serum. Addition of any higher concentration is a "waste" and will not improve the capturing ability of the coating reagent. The dilution range should be 2-fold and serum diluted to column 11. The usual diluents for antibodies are PBS, pH 7.2 or carbonate/bicarbonate buffer, pH 9.6.

After incubation (e.g., 1-2 hours, 37°C), the wells are washed. Next the antigen is diluted from row A to row G. Where there is no indication from other assays as to the likely concentration of the antigen, then begin dilution range at 1/50.

This makes the assumption that there is a sufficient volume of antigen to allow such a dilution. Where there are very small volumes of antigen of unknown concentration, then one has to decide whether there is enough antigen to develop any test or that it is of a high concentration and that 1/50 will be enough to titrate originally. The results of stage (i) will indicate whether the antigen is of a high or low concentration. For a 1/50 dilution in a 50µL volume test we will require 12 wells at 1/50 for row A plus 12 wells at 1/50 for dilution in row B (a total of 1200 µL (1.2ml) for test.

This equals 24 µL of undiluted antigen for a single stage (i) CBT. Remember to allow a little extra volume than that exactly required. Thus 30 µL will be a more realistic volume diluted to 1500 µL. The antibody is diluted in a suitable blocking buffer to prevent non-specific attachment of proteins to the wells. After incubation and washing a constant dilution of the labeled detecting serum is added. Since we have no idea as to the effective activity then add conjugate diluted to 1/200. Thus, we need a volume of 96 wells x 50µL = 4800 µL (4.8 mL); say 5000 µL (5.0 mL) to allow for losses. This means that we need 5000/200 µL of undiluted conjugate for a plate in stage (i) = 25 µL. The calculations are included here to remind operators to pay attention to the availability of reagents. After incubation, the relevant chromophore/substrate solution is added and the color development read with or without stopping (depending on system).

## Results of stage one

A good result would be that shown in Table 13.

**Table 13**  
**Example of good test results from Stage (i)**

|   | 1   | 2   | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|---|-----|-----|------|------|------|------|------|------|------|------|------|------|
| A | 1.9 | 1.9 | 1.9  | 1.9  | 1.8  | 1.3  | 1.1  | 0.9  | 0.6  | 0.5  | 0.3  | 0.1  |
| B | 1.9 | 1.9 | 1.9  | 1.9  | 1.8  | 1.3  | 1.1  | 0.9  | 0.6  | 0.5  | 0.3  | 0.1  |
| C | 1.8 | 1.8 | 1.8  | 1.8  | 1.6  | 1.1  | 0.9  | 0.7  | 0.5  | 0.4  | 0.2  | 0.05 |
| D | 1.6 | 1.6 | 1.6  | 1.5  | 1.2  | 1.0  | 0.9  | 0.7  | 0.5  | 0.3  | 0.1  | 0.05 |
| E | 1.2 | 1.1 | 0.9  | 0.8  | 0.7  | 0.6  | 0.5  | 0.4  | 0.3  | 0.2  | 0.05 | 0.05 |
| F | 0.8 | 0.7 | 0.6  | 0.5  | 0.4  | 0.3  | 0.2  | 0.2  | 0.05 | 0.05 | 0.05 | 0.05 |
| G | 0.5 | 0.5 | 0.5  | 0.4  | 0.3  | 0.2  | 0.1  | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| H | 0.2 | 0.1 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

The columns contain dilutions of capture serum, the rows contain dilutions of captured antigen. Row A contains the highest amount of antigen and examination of the OD values shows that there is a plateau of OD values from column 1 to 4. This indicates that detecting serum is in excess and that there is enough antigen to allow for a significant signal (1.9 OD units). A similar plateau is observed in row B, indicating that dilution of antigen has no significant effect on the titration of the serum. The data in A and B are identical indicating that there is the same amount of captured antigen present in the rows.

Row C shows a slightly reduced plateau OD value but the extent of the plateau (to column 4) is the same as for A and B. This indicates that there is a slight reduction in the amount of captured antigen. This is also reflected by examination of the full titration range values, however the background OD (column 12) is lower in C (0.05) than in A and B (0.1).

The continued reduction in antigen concentration (D to G) exaggerates the loss in ability of the detecting serum to be titrated where both the plateau height maxima and the end-points are reduced significantly. This data can be expressed as binding ratios, relating the values in the presence of serum to the control values in column .

12. Table 14 shows these values.

**Table 14**  
**Binding Ratios of serum**

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|---|----|----|----|----|----|----|----|----|----|----|----|----|
| A | 19 | 19 | 19 | 19 | 18 | 13 | 11 | 9  | 6  | 5  | 3  | 1  |
| B | 19 | 19 | 19 | 19 | 18 | 13 | 11 | 9  | 6  | 5  | 3  | 1  |
| C | 36 | 36 | 36 | 36 | 32 | 22 | 18 | 14 | 10 | 8  | 4  | 1  |
| D | 32 | 32 | 32 | 30 | 24 | 20 | 18 | 14 | 10 | 6  | 2  | 1  |
| E | 24 | 22 | 18 | 16 | 14 | 12 | 10 | 8  | 6  | 4  | 1  | 1  |
| F | 16 | 14 | 12 | 10 | 8  | 6  | 4  | 4  | 1  | 1  | 1  | 1  |
| G | 10 | 10 | 10 | 8  | 6  | 4  | 2  | 1  | 1  | 1  | 1  | 1  |
| H | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |

This exaggerates the advantage of using the antigen at lower concentrations than those used to obtain maximum OD, since the background OD values (antigen plus conjugate only) are lower. From the data in Tables 13 and 14, we can estimate an "optimal" dilution of antigen to be that based in row C and that the optimal dilution of capture antibody to be that used in column 4. This is highlighted on Table 14. Assuming we started the dilutions of capture serum at 1/200 and antigen at 1/50, we have optimal values for each of 1/1600 (serum) and 1/200 (antigen) respectively.

### Stage Two Titration of Antigen and Labeled Antibody

We can now fix the concentration of one of the reactants.

Stage one used a constant dilution of labeled detecting serum (1/200) which gave successful results, however, we need to know the optimal titration of this reagent so as not to waste a valuable resource (by under-estimation of the concentration) and to examine the effects on the ultimate analytical sensitivity of the assay. The idea is to optimize the dilution of conjugate in detecting the captured antigen.

Stage three involves coating plates with a constant amount of capture serum as determined above (equivalent to dilution in column 4 = 1/1600. After incubation and washing, the antigen is added as a 2 fold dilution range from a value 2-4 fold higher than found to be optimal in stage (i) from row A to row G. After incubation and washing, the detecting conjugate is diluted from column 1 to column 11 starting at a dilution 2-4 fold higher than that used in stage (i) i.e. 1/50-100. After incubation and washing and addition of substrate/chromophore the test is read. Table 15 shows idealized data.

**Table 15**  
**Titration of antigen and detecting labeled serum with**  
**constant capture antibody.**

|   | 1   | 2   | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   |
|---|-----|-----|------|------|------|------|------|------|------|------|------|------|
| A | 2.1 | 2.1 | 2.1  | 2.1  | 2.0  | 1.9  | 1.8  | 1.7  | 1.2  | 0.6  | 0.4  | 0.4  |
| B | 1.9 | 1.9 | 1.9  | 1.9  | 1.8  | 1.3  | 1.1  | 0.9  | 0.6  | 0.5  | 0.3  | 0.2  |
| C | 1.9 | 1.9 | 1.9  | 1.9  | 1.8  | 1.3  | 1.1  | 0.9  | 0.6  | 0.5  | 0.3  | 0.1  |
| D | 1.9 | 1.9 | 1.9  | 1.9  | 1.8  | 1.3  | 1.1  | 0.9  | 0.6  | 0.5  | 0.3  | 0.05 |
| E | 1.8 | 1.8 | 1.8  | 1.7  | 1.5  | 1.3  | 1.1  | 0.8  | 0.5  | 0.3  | 0.2  | 0.05 |
| F | 0.8 | 0.7 | 0.6  | 0.5  | 0.4  | 0.3  | 0.2  | 0.2  | 0.05 | 0.05 | 0.05 | 0.05 |
| G | 0.5 | 0.5 | 0.5  | 0.4  | 0.3  | 0.2  | 0.1  | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| H | 0.2 | 0.1 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

Constant capture antibody 1/1600

Antigen diluted from 1-11, beginning 1/50, 2 fold Labeled antibody diluted from A to G, beginning 1/50, 2 fold

Reference to the rows shows that at 1/50 conjugate we have a good titration range of antigen but there is a high background (0.4).

On dilution, we obtain similar results in rows B, C and D indicating that the detecting conjugate is in excess until the dilution used in row D. Notice also that there is a significantly lower background (column D12) than in C12 and B12.

On further dilution (rows E to G) we lose the plateau height and end points for titrating the antigen. Again the BRs can be plotted relating the antigen concentration to conjugate concentration to clarify the impact of the observed differences in background (Table 16). The data indicates that the optimal conjugate dilution for use in detecting available antigen is that observed in row D (1/400). The dilution in row E (1/800) gives very similar results and could be used to detect the antigen where the availability of the conjugate is a strong consideration.

**Table 16**  
**Binding ratios for data in Table 15**

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|---|----|----|----|----|----|----|----|----|----|----|----|----|
| A | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 4  | 3  | 2  | 1  | 1  |
| B | 10 | 10 | 10 | 10 | 9  | 7  | 6  | 5  | 3  | 3  | 2  | 1  |
| C | 10 | 10 | 10 | 10 | 9  | 7  | 6  | 5  | 3  | 3  | 2  | 1  |
| D | 38 | 38 | 38 | 38 | 36 | 26 | 22 | 18 | 12 | 10 | 6  | 1  |
| E | 16 | 14 | 12 | 10 | 8  | 6  | 4  | 4  | 1  | 1  | 1  | 1  |
| F | 16 | 14 | 12 | 10 | 8  | 6  | 4  | 4  | 1  | 1  | 1  | 1  |
| G | 10 | 10 | 10 | 8  | 6  | 4  | 2  | 1  | 1  | 1  | 1  | 1  |
| H | 4  | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |

Data rounded up to 1 decimal place

## Further Refinement

These two stages enable a good estimate of the concentrations of each reactant to be made. The idealized example is where the tests work "well". Even here, we may require further CBTs to establish more precise conditions e.g., a CBT of dilutions of capture antibody against constant antigen and dilutions of conjugate can be examined.

The initial CBTs also give an opportunity to set up limited studies on field samples. Thus, where one wishes to titrate antigen in samples, one could coat plates with antibody, add serial dilutions of test antigens and then detect these with the conjugate. This would investigate how "proper" field samples behave in a test and possibly give clues as to the need to modify conditions.

The titration of all three reactants also allows them to be used in similar assays with other reagents. Thus, we may wish to examine another antigen in the assay. The capture serum and detecting conjugate can be used at dilutions found above, but the "new" antigen titrated. Similarly, other capture antibody preparations can be used in tests involving the antigen and conjugate used at the optimal dilutions.



As for the description of the Direct ELISA, the purpose for which the assay is being developed should always be the strongest factor in test reagent optimization. Ultimately, the test will have to be proved to perform on particular samples and under specific conditions and validation of ELISAs has to meet such conditions.

## Bad Results

The idealized example is typical of good results where all reactants perform well i.e. can be used at high dilution and give OD values which are relatively high. Where one or more of the reactants is at a low concentration or has poor binding characteristics in the assay then, the CBT soon indicates where the problems reside.

Although the number of examples cannot be exhaustive, demonstration of a few bad results is probably far more informative than giving the ideal situation. Since we have the principles of CBTs well established through the rather lengthy treatment of the Direct, Indirect and Direct Sandwich ELISAs, we can indulge in some more rapid treatment of bad (and unwanted) results.

## Low color generally over plate

Table 17 shows the results of a similar CBT for stage one of the Sandwich ELISA-Direct (results shown in Table 13).

**Table 17**  
**CBT, low OD data, stage (i) Sandwich ELISA-Direct**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| B | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| C | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| D | 0.4 | 0.3 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.1 |
| E | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 0.4 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Capture serum diluted 1-11, antigen diluted A-H, constant conjugate

Generally there is low color. There is a plateau corresponding to a maximum value of 0.4 OD units, from column 1 to 5 (highest concentrations of capture antibody) which indicates that the antigen is being captured. There are several reasons for the low OD value in this region.

1. The capture antibodies specific for the antigen are at low concentration with respect to other serum proteins or do not bind as well as other proteins. Thus, the amount of antigen captured is limited. In this situation there is no observed increase in OD on increasing the capture antibody concentration
2. The amount of antigen available for capture is low. This is unlikely since dilution of antigen (from A to B to C etc.) does not decrease the OD observed in columns 1-4, indicating that there is an excess of antigen to row G (where we observe a reduction in OD)
3. The activity of the conjugate is low

One can repeat the CBT using increased starting concentrations of the titrated components. Thus, we could begin the capture antibody concentration at 10 x that used in the first CBT. It is unlikely, however, that this will increase the OD values since we did observe that there was an extensive (columns 1-5) plateau maximum indicating that there was maximal activity being measured which did not alter on dilution of the antigen.

Where there is an observed increase in OD on increasing capture antibody then the CBT can be reassessed and a stage (ii) CBT be performed.

Where there is no increase in OD values with increased concentrations of antigen or conjugate then the strongest candidate for replacement is the capture antibody. Where this antibody is the same used for conjugation, both should be replaced.

## Extremes of Color

Where there is a very high color in the majority of the wells, then the CBT must be repeated with lower concentrations of reactants. Table 18 shows data from such a plate. The reagent (reagents) responsible for the high readings may be directly identified from the CBT. Close examination of background values is also necessary since the results may be due to a very high non-specific binding of one of the reactants.

**Table 18**  
**High color in CBT**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2*5 | 2*6 | 2*5 | 2*7 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.6 | 1.6 |
| B | 2*8 | 2*6 | 2*5 | 2*8 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.6 | 1.6 |
| C | 2*7 | 2*5 | 2*4 | 2*6 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.6 | 1.6 |
| D | 2*6 | 2*7 | 2*4 | 2*6 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.5 | 1.4 |
| E | 2*2 | 2*3 | 2*3 | 2*6 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.5 | 1.3 |
| F | 2*1 | 2*2 | 2*2 | 2*6 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.5 | 1.0 |
| G | 2.0 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.4 | 1.3 | 1.2 | 1.6 | 1.3 | 0.9 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Capture antibody titrated 1-11, constant antigen A12 to H12, conjugate diluted A to G. \* denotes readings are out of accurate range for reader.

This data shows a high background for the conjugate in the absence of capture serum (column 12).

Row H indicated that there is no color obtained where there is antigen and capture antibody in the absence of conjugate. Thus, there is unwanted non-specific color through attachment of the enzyme conjugate to the wells. This could indicate that the conjugate is being used at far too high a concentration so that the blocking buffer conditions are not preventing non-specific adsorption of the enzyme labeled proteins. There is a titration of antigen on diluting the capture serum, indicated with reference to the backgrounds in column 12, e.g., in row G which has a background of 0.9, here a plateau of approximately 2.0 is observed with an OD above background observed in column 11 and a reduction in color gradually from columns 3 and 4 to column 11.

A further CBT can be made using the conjugate beginning at that used in row F in the first attempt. Table 19 shows idealized results. Here the background is eliminated by row D (results are the same in D12, E12, F12 and G12). The effect of diluting the capture antibody is to titrate the antigen after an initial plateau (region of capture antibody/antigen) excess). The conjugate dilution up to D is not suitable due to the high backgrounds obtained. Thus, a dilution of conjugate at around that in rows D/E can be assessed in the second stage of the CBT where the capture antibody and antigen can be varied.

**Table 19**

**Repeat of CBT where high color was obtained using more dilute conjugate.**

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 2*1 | 2*2 | 2*2 | 2*6 | 2*7 | 2*1 | 2.0 | 1.9 | 1.7 | 1.6 | 1.5 | 1.0 |
| B | 2.0 | 2.0 | 1.9 | 1.9 | 1.8 | 1.7 | 1.4 | 1.3 | 1.2 | 1.6 | 1.3 | 0.9 |
| C | 1.9 | 1.9 | 1.9 | 1.9 | 1.8 | 1.6 | 1.3 | 1.1 | 0.9 | 0.5 | 0.3 | 0.3 |
| D | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.5 | 1.1 | 0.8 | 0.6 | 0.4 | 0.2 | 0.1 |
| E | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.3 | 1.1 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 |
| F | 1.2 | 1.1 | 1.1 | 1.1 | 1.1 | 1.0 | 0.9 | 0.6 | 0.4 | 0.2 | 0.1 | 0.1 |
| G | 0.9 | 0.9 | 0.8 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Capture antibody titrated 1-11, constant antigen A12 to H12, conjugate diluted A to G (beginning at dilution used in row F in initial CBT).

### Very Weak Reactions

Where there is little color observed we run into obviously more difficulties since there is no obvious indicator of whether one or all the reagents are not functioning.

A special case is where there is no color development even after a significant time of incubation of the substrate/chromophore, then the most likely culprit is the operator forgetting to add substrate to the reaction mixture. This can be tested by dipping a microtip into a conjugate and putting the tip into the remaining substrate/chromophore solution. This should show a rapid color change. If not then repeat the test making sure of a proper substrate/chromophore mixture.

Where there is color, then the CBT can be repeated. Should very low color then be obtained, the initial CBT should be repeated beginning with much higher concentrations of the two reagents being titrated. This can be a relatively futile operation since we know the dilutions of reactants and have usually added these at a high concentration in the initial CBT. Obviously the third reactant (conjugate) can be critical, so that a higher concentration can be added. Once again, where conjugates are not reacting at dilutions from 1/100 and below, there is little practical value in pursuing their use.

Thus, low color can come from a mistake (failure to add proper reagents, or making a mistake in the original dilution or as a result of all reactants being of inadequate 'strength'.

It is easier to assess the reason for low color where there is an indication that one of the reagents is active. This is illustrated in Fig. 20, where there is some color development in one area of the plate associated with column 1 and rows A to E. Since we have a constant amount of antigen and 1.5 OD units in column 1 and is observed, this indicates that both the antigen and conjugate can function although the activity of the conjugate is also rapidly diminished after row C. The fault here lays with the capture antibody which rapidly dilutes out its activity by column

3. The CBT could be repeated with higher concentrations in column 1. As indicated above, since the capture antibodies are present as a small component of the total serum proteins, and its capture activity resides in the ability of these specific antibodies to bind to the wells, it may be impossible to achieve a better capture reagent with this serum.

**Table 20**  
**Results where one or more reagents are "weak"**

|   |     |     |     |     |     |     |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1.5 | 0.5 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| B | 1.5 | 0.4 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C | 1.2 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| D | 0.8 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| E | 0.4 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| F | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| G | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| H | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Capture antibody titrated 1-11, constant antigen A12 to H12, conjugate diluted A to G.

## CBT for Other Systems

The last examples are meant to indicate the first developmental steps in analysing the suitability of available reagents. In all cases there is always going to be the need to make adjustments to allow establishment of a defined test protocols. The CBT allows a rough estimate of activities only.

More complicated systems (e.g., those relying on 4 reactants), rely on establishing rough parameters for 2 of the reagents and examining the affect of diluting the other two. Such assays can be greatly helped where there has been some developmental work with other ELISA systems using the same reagents. Some examples are shown below.

### Developing a Sandwich ELISA Indirect

We may have titrated capture antibodies for use in a Direct Sandwich ELISA. Thus the effective concentrations of capture antibodies, antigen and conjugate are known. We may now wish to develop an Sandwich ELISA-Indirect replacing the labelled antibodies directly prepared against the target antigen by a non-labeled detecting serum and an anti species conjugate. A good reason to do this would be to allow use of many different animal sera for examination.

A good starting point would be to perform a CBT using constant capture antibodies and antigen and titration of the detecting serum and conjugate. The species of the detecting serum would have to be different to that of the capture antibodies since we are adopting an anti-species conjugate (the anti-species conjugate has to be tested for non-ELISA activity against the coating antibodies). Conditions for the optimal coating and antigen concentrations can be used initially. If the test is successful then adjustments can be made by altering any one of the reactants concentrations.

The establishment of the concentrations of reagents in tests other than those finally used is not uncommon. In fact, pre-titration in other systems could be used as a deliberate tool. In the majority of cases laboratories are working with a limited range of antigens and antibodies and their exploitation in different systems often results from the need to improve methods defined by a specific task at hand. The "reagent" link extends to developments in monoclonal antibodies (MAbs) where polyclonal antibodies may be used at some stage to help production of a more specific or sensitive test. As an example, a Sandwich ELISA Indirect based on polyclonal sera is available, and we are investigating the use of a detecting MAb (possibly to see whether we can increase specificity for detecting captured antigen).

Here the initial CBT should involve constant capture antibodies and antigen, and titrate the MAb and anti-mouse conjugate. We know for certain (based on original polyclonal based ELISA) that we can provide enough captured antigenic target for the MAb. Similarly the activity of the MAb as a capture reagent can be assessed using the "constant" components of antigen, polyclonal detecting serum and conjugate.