Indirect ELISA Protocol

Author : Dan Souw

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This is the third post in our series of posts and protocols on Enzyme Linked Immunosorbent Assays – ELISAs. The first was a detailed ELISA protocol, the second on direct ELISA and this time, the focus is on *indirect* ELISAs.

What makes an indirect ELISA different to a direct one? Well, in a direct ELISA your primary antibody, the one which binds to your antigen, is also your detection antibody – it's labelled with an enzyme or some other method of visualising it.

Whereas in an *indirect* ELISA, your primary antibody is unlabelled, and a second 'detection' antibody is directed against this. This extra step means you are *indirectly* detecting the antigen, based on how much primary antibody has bound to the plate.

So, basically, the analyte you're interested in is coated, or immobilised somehow, onto a microtitre plate. You then add you're primary antibody which binds to any of the analyte, followed by your detection antibody which binds to the primary antibody and develops a measurable signal in proportion to the amount of bound analyte.

This is kind of a half-way point between a direct ELISA and a sandwich ELISA. A sandwich ELISA 'sandwiches' your antigen between capture and primary antibodies. It's also the subject of a future post, so we won't go into any more detail on that for now.

So, you may be asking, what's the benefit of an indirect ELISA? Well, there's a few.

Firstly, you could use an 'off the shelf' ready labelled detection antibody in the event the primary antibody you're using isn't available in that format.

Secondly, compared to direct ELISAs, indirect ELISAs can offer greater sensitivity since more than one detection antibody can bind to a single primary antibody. Thus, you can detect smaller amounts of antigen.

Thirdly, it can be cheaper and more flexible – if you're running lots of ELISAs you could use a single detection antibody, an HRP conjugated anti-mouse IgG for example – with a wide range of primary antibody targets.

Below is a basic protocol for performing an indirect ELISA – again with the usual qualification that the specific concentrations you might need to use will need to be optimised for your analyte and reagents, but this guide should get you on the right track.

Reagents:

96-well microplate

Coating buffer: PBS (pH 7.4) or carbonate/bicarbonate (pH9.6) for coating

Washing buffer: PBS + 0.05% Tween-20

Blocking buffer: PBS + 1% BSA

General tip: If adding the same reagent *to all wells of a plate, a multichannel pipette can save a huge amount of time.*

Plate preparation

1. Dilute the analyte to a final concentration of 20 ?g/mL in coating buffer and add 50 ?L to each well of a microtitre plate.

Note: If your assay is comparing samples with unknown concentrations of the analyte *to known concentrations of analyte, then at this step you would want to prepare a dilution series of you analyte, for example from 20 ?g/mL, serially diluted 1 in 2 with coating buffer.*

2. Cover with a plate sealer and incubate overnight at +4 °C. Alternatively, incubate for 2 hours at room temperature.
3. Aspirate each well, and wash with 200 ?L of wash buffer. Repeat twice more for a total of three washes.

Tip: rap the plate smartly on some paper towels after washing to ensure all washing buffer is removed.

Blocking

4. Add 200 ?L of blocking buffer to all wells of the plate, cover with a plate sealer and incubate at room temp for at least an hour. Ideally 2-3 hours.

This step uses the BSA in solution to bind to any remaining free binding sites in each microwell, 'blocking' the detection antibodies, antigen or other samples components from binding non-specifically to the plate.

5. Aspirate each well, and wash with 200 ?L of wash buffer. Repeat twice more for a total of three washes.

Incubation with primary antibody

6. Add 100 ?L per well of the primary antibody prepared at a concentration according to the manufacturer's instructions (this could vary greatly).

Tip: dilute in blocking buffer if there is no other suggestion.

7. Cover with a plate sealer and incubate for 2 hours at room temperature.

Note: This step may need optimisation. Generally, 2 hours should be enough to see a good signal, but in the event of weak signal, you may even need to incubate overnight at +4 °C.

8. Aspirate each well, and wash with 200 ?L of wash buffer. Repeat twice more for a total of three washes.

Incubation with detection antibody

9. Add 100 ?L per well of the conjugated detection antibody prepared at a concentration according to the manufacturer's instructions (this could vary greatly).

Tip: dilute in blocking buffer if there is no other suggestion and prepare this immediately before use.

10. Cover with a plate sealer and incubate for 1-2 hours at room temperature.

Note: Again, this step may need optimisation.

11. Aspirate each well, and wash with 200 ?L of wash buffer. Repeat twice more for a total of three washes.

Detection

12. Add 100 ?L of substrate solution to each well and incubate for 20 minutes at room temperature. After 20 minutes, or sufficient color development, add stop solution if required. (see below).

Note: The substrate will depend on your detection system/antibody. This may entail an additional step, such as incubating the detection antibody with strep-HRP before adding the detection solution. If using HRP, most assays use TMB as the substrate, in which case 50 ?L of 0.16M sulfuric acid would be added to each well.

13. Immediately use a plate reader (set to 450 nm minus 570 nm, for wavelength correction, for TMB) to determine the optical density (OD) of each well.

Data Analysis

How you perform this will very much depend on what you are trying to measure. If it is a qualitative assay, then a simple spreadsheet can be used to give you a ‘+ve’ or ‘-ve’ result for sample wells based on their OD. If quantitative.

If your assay is quantitative, use the values from the standards to make a calibration curve, plotting concentration (x-axis) against OD (y-axis). Most commonly, a four parameter logistic curve fit (4-PL) can be used, though sometimes with a direct ELISA a straight line fit performed in a spreadsheet may work equally well.

Use this curve to interpolate sample concentrations. Don’t forget to multiply the results of any diluted samples read from the calibration curve by the dilution factor to get the ‘original’ concentration.

We hope this general indirect ELISA protocol will be useful. As ever, specifics will depend on the reagents, samples and antigen of interest, so where possible follow the manufacturer's guidance.