Enzyme-Linked Immunosorbent Assay (ELISA)

1.0 REAGENTS

1.1. PHOSPHATE-BUFFERED SALINE (PBS)

1.9m NaH_2PO_4

8.1mM Na_2HPO_4

150mM NaCl

pH to 7.2-7.4

*crystalline phosphate salts vary widely in formula weight/molar mass, depending on the amount of water of hydration. Check formula weight of each batch before use and adjust weights used accordingly

1.2. COATING BUFFER

15mM Na_2CO_3

35mM $NaHCO_3$

pH 9.3-9.6

(Pink or Red food colouring at 1:2000 can be added to this buffer to help visualize well coating – check compatibility with assay before use)

1.3. WASHING BUFFER (PBS-TWEEN-20)

PBS as above +0.05% Tween-20

1.4. BLOCKING BUFFER

1% [w/v] skimmed milk powder (or alternative) in washing buffer

1.5. SECONDARY ANTIBODY: HORSERADISH PEROXIDASE-CONJUGATED (ANTI-RABBIT IgG OR ANTI-MOUSE IgG OR ANTI-HUMAN IgG) ACCORDING as required).

Although data sheets give approximate dilutions, all new batches of secondary antibodies should be titrated for specificity and sensitivity before use. High quality and specificity conjugated antibodies are purchased from either Dako Ltd, High Wycombe, UK, or The Binding Site, Birmingham, UK.

1.6. STOP SOLUTION

2M H_2SO_4 *

* stocks of sulphuric acid are highly corrosive and produce violent reactions if diluted incorrectly – always add acid to large excess of water (NOT the other way round!) and exercise caution as the dilution is an exothermic reaction.

1.7. SUBSTRATE BUFFER

0.04mg ml^{-1} o-phenylenediamine [OPD, Sigma] + 0.012% H_2O_2 in development buffer (24.5mM citric acid monohydrate and 52mM Na_2HPO_4, pH 5.0). Add OPD tablets and H_2O_2 immediately before use – OPD substrate is light sensitive.*

* OPD is a known carcinogen and should be handled with care. Dispose of contaminated plasticware via the cytotoxic waste route.

Make stock solutions of :-

0.1M citric acid – this will be OK at 4˚C for 2 weeks
0.2M Na_2HPO_4 – this can be autoclaved and will last for months if kept free of bacteria

To make 25mL substrate buffer (immediately before use - or multiples thereof), combine the following in a clean beaker.

	6mL	0.1M citric acid
	6.4mL	0.2M Na_2HPO_4
	12.5mL	H_2O
	10mg	OPD
	10µL	H_2O_2 (30% solution)
Total	25mL	

OPD tablets are kept at 4˚C in foil packets – tablets come in 10mg, 20mg, 30mg and 60mg sizes and can be combined to produce any multiple of the 25mL recipe above.

2.0 EQUIPMENT

2.1. 96-WELL MICROTITRE PLATES

Immulon 4 HBX, (Dynex), Greiner Microlon or other high protein binding polystyrene ELISA plates

2.2. ELISA PLATE WASHER

Skatron 300 Skanstacker, Skatron 300/400 or similar

2.3. ELISA READER

Labsystems Multiskan Ascent or similar fitted with 450 and 492nm filters

3.0 METHOD

1. Design the layout of the experiment in the 96-well format. All test sera should appear twice on the same plate. Included on each plate should be duplicate wells containing a) reagents alone and b) known negative sera controls

2. Add 100 µL of antigen made up to the desired concentration (normally 0·5 µg/mL – Ag specific) in Coating Buffer. Add 100 µL per well to 96-well microtitre plates. Cover stacks of ~ 10 plates with a cover plate (old plates in lab drawer). Place stack of plates on two wetted tissues and wrap in clingfilm, then incubate at 4˚C overnight.

3. Remove unbound antigen by washing plates 4x with Washing buffer using the Skatron washer. Blot dry (gently) on paper towels (Kimberley Clark 3 ply hand towels, Cat. No. 6771) and add 200 µL of blocking buffer (1% non-fat milk powder* in Washing Buffer). Incubate at room temperature for 5 hours.

* we use a branded non-fat milk powder named Marvel in the UK. You will need to test batches of powder in advance to ensure background readings are not affected. Alternative sources of blocking protein are BSA (not always compatible), hydrolysed gelatin (fish or bovine) and casein. Milk is no good for biotinylated antibody ELISA assays as there is abundant biotin in the milk powder.

4. Make up serum dilutions in Blocking Buffer. After 5 hours, wash plates 4x with Washing buffer and add 100 µL of serum/antibody (diluted in Blocking Buffer) to each of duplicate wells and incubate (humidified as in 2) above) overnight at 4°C.

5. Wash plates 4x, blot dry and add 100 µl of a suitable dilution of secondary antibody in Washing Buffer (e.g. 1/5000 dilution of horseradish peroxidase conjugated rabbit anti-human IgG antibody; 1/1000 dilution of horseradish peroxidase conjugated rabbit anti-mouse IgG antibody (Dako Ltd) or 1/1000 rabbit anti human IgG1/IgG3 (Binding Site, UK) and incubate for 3 h at room temperature.
6. Remove unbound secondary antibody by washing plates 4x with Washing buffer. Blot dry.
7. Develop the reaction with 100 µl of substrate buffer at room temperature for 10-15 min. Read the unstopped positive control plate at 450nm – an A_{450nm} of 0.7-0.8 is equivalent to a stopped A_{492nm} of 2.5-3.0.
8. Stop the reaction by adding 25 µL of 2.0M H_2SO_4 per well.

9. Read the optical density (OD) at 492nm using a Labsystems Multiskan Ascent microtitre plate reader or similar suitable ELISA Plate Reader.