General Protocol for Sandwich-style Enzyme-Linked Immunosorbent Assay (ELISA)

Principle

Analytes in plasma and other fluids can be measured by capturing onto a microtitre plate coated with a capture antibody. After washing the plate to remove unbound proteins the captured analyte is detected by incubating with another antibody containing a reporter molecule, in this case the enzyme horseradish peroxidase. The unbound detecting antibody is washed away and the plate developed with a solution of peroxidase substrate which produces a coloured end product. After a fixed time the reaction is stopped and the adsorbance of each well in the microtitre plate is determined. As the concentrations of capture antibody and detecting antibody are fixed, the colour generated is proportional to the concentration of analyte present in the sample.

Materials Required but Not Provided

Coating Buffer: 50 mM Carbonate - 1.59g of Na_2CO_3 and 2.93g of $NaHCO_3$ up to 1 litre. The pH should be 9.6. Store at room temperature for no more than 2 weeks.

Blocking Buffer: 2.0 g NaCl and 0.29 g Na_2HPO_4, 0.05 g of KH_2PO_4, 0.05 g of KCl and 2.5g Bovine Serum Albumin (Sigma - RIA Grade) up to 250 ml. Readjust the pH to 7.4 with dilute NaOH or K_3PO_4 if necessary.

PBS-Tween: 8.0g NaCl and 1.15g Na_2HPO_4, 0.2g of KH_2PO_4, 0.2g of KCl and 1.0 ml of Tween-20, up to 1 litre. The pH should be 7.4.

Sample Diluent: (HBS-BSA-Tween): 5.95g HEPES, 1.46g NaCl, 2.5g Bovine Serum Albumin and 0.25 ml Tween-20, up to 250 ml. Adjust pH to 7.4 with NaOH.

Substrate Buffer: 2.6g Citric acid and 6.9g Na_2HPO4 up to 500 ml. Adjust pH to 5.0 if needed.

OPD Substrate: (O-Phenylenediamine) - Toxic! - available in 5 mg tablets: Sigma # P-6912. Dissolve one 5 mg OPD tablet in 12 ml substrate buffer then add 12 ul 30% H_2O_2. Make up immediately before use, do not store.

Stop Solution: (2.5 M H_2SO_4) Caution: very corrosive! Dilution of acid produces heat! Where stock sulphuric acid is 18 Molar, add 13.9 ml stock acid to 86 ml H_2O.

Microplates: EIA-grade microplates: Immulon 4HBX (www.labsystems.fi)

1395 Sandhill Drive. Ancaster, Ontario, Canada L9G 4V5
905·304·9896 • 800·903·6020 • fax 905·304·9897

Affinity Biologicals™ INC.

Procedure

Coating plates: Dilute the capture antibody in coating buffer in a polypropylene tube and immediately dispense 0.1 ml per well. Incubate 2 hours at ambient temperature or overnight @ 4°C. Optimal coating concentrations differ with each antibody and should be determined empirically by titration. Our experience has shown that the following general guidelines can be used to indicate a range of concentrations to start with:

Whole IgG polyclonal antibodies from rabbit, goat or sheep: 10-25 ug/ml
Crude ascites as a source of murine monoclonal antibodies: 10-25 ug/ml
Affinity-purified polyclonal antibodies: 2.5-10 ug/ml
Purified murine monoclonal antibodies: 2.5-10 ug/ml

Blocking: Empty contents of plate and add 0.15 ml of blocking buffer to every well and incubate for 60 minutes at ambient temperature. Wash plate X3 with PBS-Tween.

Samples: Standard reference plasma is diluted in sample diluent to achieve a final analyte concentration of about 50 ng/ml for use as the high point on the standard curve. Further dilutions are made in sample diluent to obtain concentrations of 25, 12.5, 6.25, 3.1 and 1.56 ng/ml. Test samples are usually diluted an extra factor of two relative to the standard. For example, where the dilutions used for the reference plasma are 1/100 to 1/3200, dilutions of 1/200 and 1/400 would be used for the unknown test samples. Apply all reference and test sample dilutions 0.1 ml per well (in duplicate) and incubate at ambient temperature for 60 minutes. Wash X 3 with PBS-Tween.

Detecting Antibody:

Dilute the detecting antibody in sample diluent and apply 0.1 ml per well. Incubate plate at ambient temperature for 60 minutes. Wash X 3 with PBS-Tween. Optimal detecting antibody concentrations differ with each antibody conjugate and should be determined empirically by titration. Our experience with peroxidase labelled antibodies has shown that the following general guidelines can be used to indicate a range of concentrations to start with.

Peroxidase **- Whole IgG** antibody conjugates: 5-10 ug/ml
Peroxidase **- Affinity-purified** antibody conjugates: 0.5-5ug/ml

OPD Substrate:

Apply 0.1 ml of freshly prepared OPD substrate to every well. Allow colour to develop for a fixed time (usually 5-15 minutes) then stop colour reaction with the addition of 50 ul/well of 2.5 M H_2SO_4 stop solution. The plate can be read at a wavelength of 490 nm. The plate should be read within 1 hour of adding the acid.

1395 Sandhill Drive. Ancaster, Ontario, Canada L9G 4V5
905·304·9896 • 800·903·6020 • fax 905·304·9897

Affinity Biologicals™ INC.

Calculation of Results**:**

The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit [1,2]. The coefficient of determination (R^2) is a valuable indicator of the overall fit, but should not be used as the only criteria in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone [1,2]. In general, the simplest model that defines the concentration-response relationship should be used [3].
The "back-fit" test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits[3]. Different curve fitting methods may be employed but we recommend that the back-fit test be applied as evidence that the fitting method is appropriate.

References**:**

1. Nix,B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.
2. NCCLS. Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guidline – Second Edition. NCCLS Document EP6-P2 (ISBN 1-56238-446-5, NCCLS, Wayne, Pennsylvania USA, 2001
3. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available on the internet: www.fda.gov/cder/guidance/index.htm

1395 Sandhill Drive. Ancaster, Ontario, Canada L9G 4V5
905·304·9896 • 800·903·6020 • fax 905·304·9897