

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_2HPO_4 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)

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 H₂SO₄ 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.

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 used³.

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 limits³. 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 Guideline – 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

**Immuno-Blotting (Western Blotting):
Enhanced Chemiluminescent Detection using Peroxidase Labelled Primary Antibody
Conjugate**

Solutions Required

- **Electroblot Transfer Buffer:** 0.025M Tris, 0.192M Glycine, 20% (v/v) Methanol, pH 8.3. Add 18.2 g Tris, 86.4 g Glycine, 1200 ml Methanol, up to a total volume of 6 litres.
- **PBS:** 8.0g NaCl and 1.15g Na₂HPO₄, 0.2g of KH₂PO₄, and 0.2g of KCl, up to 1 litre. Adjust pH to 7.4 if necessary.
- **PBS-Tween (0.1% (v/v) Tween 20):** 0.5 ml Tween 20 to 500 ml PBS.
- **PBS-2% (w/v) BSA (Blocker):** 10 g BSA to 500 ml PBS, adjust pH to 7.4 with 1 M NaOH. Freeze in 50 ml tubes.
- **Probing Buffer:** 5% (w/v) Carnation Skim Milk Powder in PBS + 0.1% (w/v) Tween 20. Adjust pH to 6.5 with 1 M Phosphoric Acid. Centrifuge at 3500 X g for 30 minutes just before addition of antibody.
- **Bromophenol Blue:** 0.1% (w/v) Bromophenol blue in water.
- **Amido Black:** 100 mg Amido Black, 45 ml Methanol, 10 ml Acetic Acid, 45 ml water.
- **Destain Solution:** 10% (w/v) Acetic Acid, 25% (w/v) Methanol in water.
- **ECL Western Blotting Detection System:** Amersham #RPN-2106

Method

A. SDS PAGE:

For precast 10 x 8 cm gels: Prepare samples and load 0.5 ul plasma or 50 ng purified protein per well. Electrophorese until the dye front is at the edge of the gel.

B. Electroblotting onto membrane:

1. When tracking dye has reached bottom of gel, turn off power supply.
2. Hydrate membrane (nitrocellulose or Immobilon-P PVDF) as per manufacturer's instructions. For Immobilon this consists of a 10-second wash in 100% MeOH followed by 60 seconds in distilled water then several minutes in the transfer buffer. The cassette is then assembled with sponge on bottom, followed by 3MM filter paper, gel, membrane, filter paper and top sponge. Each item should be soaked by dipping in transfer buffer prior to assembly of the cassette, and kept wet during the assembly. Do not allow air bubbles to become trapped between the gel and membrane. The cassette is closed and placed in the Transphor unit with the membrane on the cathode side (red) of the gel.
3. Transfer of proteins is performed at 500 mAmps for 1 hour at RT.

C. Probing and detection:

1. Turn off power and remove the cassette from the Transphor unit. Trim the membrane to the same size as gel. Put a small nick in the membrane to mark the point of application of the first sample. Cut off molecular weight standard lane and stain in Amido Black for 5 minutes, and then destain. Place remaining membrane in a plastic dish containing 50 ml of PBS-2% (w/v) BSA (blocking solution).
2. Block membrane for 2 hours at ambient temp., or overnight at 4°C on a shaking platform.
3. Rinse briefly with PBS-Tween.
4. Incubate membrane with 50 ml probing buffer containing peroxidase conjugated antibody for 2 hours at ambient temperature on a rocker. The appropriate dilution for each species should be determined by titration.
5. Wash membrane with PBS-Tween three times, 15 minutes each.
6. Develop as per instructions for the ECL Western Blotting Detection System, starting at step #12 on page #12 in the ECL Instruction Manual. Exposure times using Kodak XAR-5 film is typically between 2 and 20 minutes at room temperature.

Reference

Towbin, H., Gordon, J.: Immunoblotting and Dot Immunobinding - Current Status and Outlook. J. Immunol. Meth., 72:313 (1984).

Immunohistochemical Staining for von-Willebrand Factor (vWF) Using Affinity Purified Goat antibody and Vectastain-Elite ABC Kit

Introduction

von Willebrand Factor (vWF) is an adhesive protein that circulates in plasma at about 10 ug/ml and is also found in platelets and endothelial cells. vWF is found as multimers of disulphide-linked 220,000 Dalton subunits and its molecular weight ranges from 0.5-20 million Daltons. vWF is involved in the transport of Factor VIII (antihemophilic factor) and plays an important role in platelet adhesion and aggregation. von Willebrand Disease (vWD) can be due to a quantitative deficiency of vWF (vWD Types I & III) or to qualitative disorders resulting from the production of functionally abnormal protein (vWD Type II). The procedure outlined here was developed for immunohistochemical staining of vWF in endothelial cells at the light microscopy level and uses Product # GAVWF-AP as the primary antibody, Biotinylated rabbit anti-goat IgG (Product # BA-5000, Vector Laboratories, Burlingame, CA) as the second antibody. Detection of biotinylated antibody was performed using the VectaStain-Elite detection kit from Vector Labs.

Method

1. Paraformaldehyde fixed tissues samples were embedded in paraffin.
2. Remove paraffin in toluene (2 X 20 min).
3. Put through graded alcohols (100% - 25% (w/v), 5 min each), then rinse in water.
4. Block with methanol-peroxide (0.3% H₂O₂ in MeOH) for 30 minutes.
5. Sections were washed in PBS (10 mM NaHPO₄, pH 7.4, 0.15 M NaCl) for 20 min.
6. Layer on PBS containing a 1/100 dilution of normal rabbit serum for 20 min.
7. Rinse and layer on the primary antibody diluted in PBS containing 1% (w/v) Bovine Serum Albumin and place in a humidity chamber overnight at 4°C.
8. **Note:** For vWF staining we recommend affinity-purified goat anti-vWF (GAVWF-AP) diluted to 0.5 ug/ml. In general, affinity-purified polyclonal antibodies or purified monoclonal antibodies may be used in a concentration range of 0.5 to 5 ug/ml, while whole IgG antibodies or ascites may be used in a concentration range of 5 to 25 ug/ml. Optimal concentrations of each different primary and secondary antibody should always be determined empirically by titration.
9. Wash in PBS for 10 minutes then layer on secondary antibody (biotinylated Rabbit anti-Goat IgG) diluted approximately 1/100 in PBS for 30 minutes at ambient temperature.
10. Wash in PBS for 10 minutes, then apply the VectaStain ABC reagent mixture as per the manufacturer's instructions.
11. Develop in 0.05% (w/v) DAB in 0.05 M Tris-HCl, pH 7.6, containing 0.075% H₂O₂ for 15 minutes at ambient temperature.
12. Rinse in cold water, then counterstain in hematoxylin for 3-5 minutes.
13. Rinse in water, then graded alcohols (25% - 100% (w/v), 5 minutes each).
14. Rinse in 3 changes of toluene then coverslip with permount.

Working concentrations for ELISA, immunoblotting and immunohistochemistry applications.

The optimal concentration of antibody for any particular application will vary depending on the antibody format as well as the detection method used. ABI strongly suggests that each antibody be titrated by the end user to determine the optimal working concentration for each particular application. However, based on our experience we can make some general recommendations regarding antibody concentration ranges that can be used as a starting point in optimizing assay conditions.

The following chart contains **general recommendations only** and may not apply to all our products.

Format	Immunoblotting (ECL detection)	ELISA (capture)	ELISA (detecting)
Whole IgG	5-25 ug/ml	20-50 ug/ml	NA
Affinity-Purified (AP-IgG)	0.5-5 ug/ml	10 ug/ml	NA
IgG Peroxidase (HRP)	5 ug/ml	NA	10 ug/ml
AP-IgG Peroxidase (AP-HRP)	1 ug/ml	NA	2 ug/ml

