Procedure 7: Immunoblotting.

Techniques:

Immunological detection of antigens.
Handling of antibody and antibody-enzyme conjugate reagents.
Reagent preparation.

Principle:

This immunoassay procedure utilises membrane strips containing antigen. Antibodies present in the serum sample bind to the immobilised antigen during a defined incubation period. After a washing step (any unbound antibody is removed by washing), conjugate (anti-mouse IgG-horseradish peroxidase (HRP)) is added to the membrane (Fig. 1). The bound antibody-conjugate complex is detected by the addition of a substrate which precipitates due to the action of bound HRP (Fig. 1). Band intensity is determined visually and a positive result is indicated by the appearance of spots corresponding to the position of antigen.

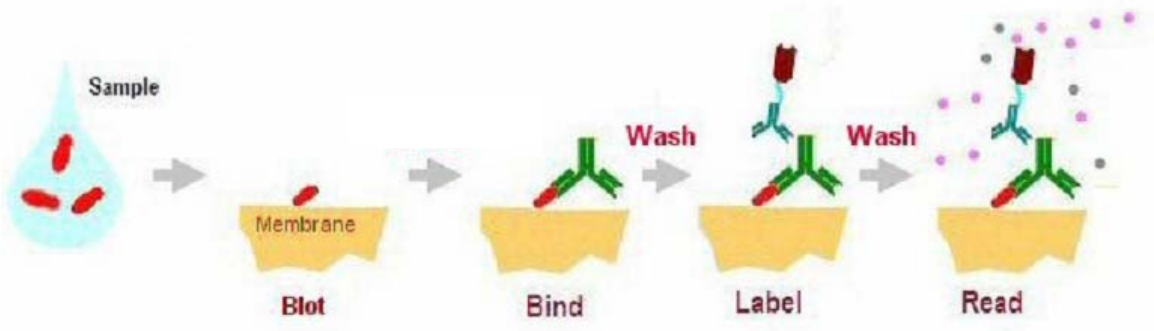

Figure 1: Immunoblot analysis. Antigens in the sample bind to the membrane. Following a 'blocking' step, primary antibody is added to the membrane. This antibody can bind to the antigen. After washing, to remove unwanted proteins/minimize non-specific binding, an anti-species-enzyme conjugate is added to the membrane. Bound enzyme is detected by a precipitating substrate or chemiluminescence.

Introduction :

Monoclonal antibodies (produced in vitro or in vivo (mice)) or purified IgG from animals (e.g., mice and rabbits) previously immunised with antigen, are significant raw materials for enzyme immunoassays as they are used for both microtiter platecoating and conjugation to enzymes (e.g., horseradish peroxidase). However, the monoclonal antibodies or primary antisera must be checked for both sensitivity and specificity towards the antigen prior to IgG purification or all subsequent uses. This practical session describes the use of species IgG (mouse) and anti-species conjugate for the detection of immobilised antigen. It should be noted that the following detection systems may also be used for antigen visualisation.

1. Direct conjugate (i.e., IgG [anti-antigen]- HRP)

2. Biotinylated IgG [anti-antigen] / Streptavidin - HRP

Furthermore, alternative enzymes may be used to identify bound IgG (e.g. alkaline phosphatase) along with different substrates (e.g. NBT / BCIP), and different species Ig may be detected by altering anti-species conjugate system. Antiserum and conjugate dilutions given are for guidance only and it is likely that you will have to optimise reagent dilutions for your particular application in the future.

Reagents:

1. Stock Antigens -Examples
   - *Aspergillus fumigatus* Gst3/GstA* (*E. coli* recombinant)- 500 µg/ml
   - Bovine serum albumin (BSA)- 500 µg/ml

2. Murine monoclonal IgG [anti-His_6]	Approx. 20µl
3. Phosphate Buffered Saline-Tween 20 (PBST)	Approx. 300ml
4. Anti-mouse IgG-Peroxidase conjugate	20µl
5. Marvel	Variable
6. DAB Tablets	1 Tablet
7. Hydrogen Peroxide	Approx. 10µl
8. Trizma Base	12.1g

*** Note:** gst = glutathione s-transferase, a detoxification enzyme.

Materials and Equipment:

Nitrocellulose paper
Balance

Micropipettes
Eppendorf tubes
Weighing Boats
Reciprocal Shaker
25ml Universals
10 and 50ml Graduated cylinder

Reagent Preparation:

1.1 Blocking Solution (PBST / 5%(w/v) Marvel)
Dissolve 5g Marvel in 100ml PBST

2.1 Antibody and Conjugate Buffer (PBST / 1%(w/v) Marvel)
Add 200ml PBST to 50ml Blocking Solution

3.1 Substrate Buffer:

Dissolve 12.1g Trizma base in 800ml deionised water. Adjust to pH 7.6 with 5M HCl and bring to a final volume of 1L. Store at room temperature for 6 months.

Procedure: Dot Blot Analysis

4.1 Obtain purified antigen from 2-8 ^{O}C or -20 ^{O}C storage. Allow to thaw if appropriate. Record protein concentration.

4.2 Label 8 Eppendorf tubes A- H, twice. Add 50 µl PBST to both sets of tubes A-H. Prepare the following dilutions from **each** of the stock antigens (GstA and BSA) as directed in **Table 1**.

Table 1: Preparation of **Antigen** solutions for Dot Blot Analysis as follows. Pre-label two sets of Eppendorf tubes A through H** for GstA and BSA, respectively:

Sample	Volume Stock (µl)	Volume PBST (µl)	Final Conc. (ng/µl)	Amount/dot* (ng)
A	50	50	250	500
B	50 **A**	50	125	250
C	50 **B**	50	62.5	125
D	50 **C**	50	31.3	62.5
E	50 **D**	50	15.6	31.3
F	50 **E**	50	7.8	15.6
G	50 **F**	50	3.9	7.8
H**	0	50	0	0

(* in 2 µl) * *Sample 'H' (Negative Buffer Control).

4.3 Spot 2 µl of Solutions A through H, for both proteins, onto a nitrocellulose membrane. Use a pencil to lightly mark the membrane at points of application. Each mark should be 0.5-1.0 cm apart in a vertical direction. Block the membrane in 5%(w/v) Marvel in PBST for 30min, shaking, at room temperature. Use sufficient blocker to cover the entire membrane. Cover and/or seal the container. Remove blocker prior to Step 4.4.

4.4 Prepare **Test Antibody** (Murine monoclonal IgG [anti-His_6]) at a dilution of 1/500 by adding 20 µl antiserum to 10 ml 1% (w/v) Marvel in PBST and incubate the membrane in this solution for 30 min, shaking at room temperature. Cover and/or seal the container.

4.5 Wash the membrane twice in PBST with shaking for 5 minutes on each occasion.

4.6 Prepare anti-mouse IgG-HRP conjugate (1/1000) by dissolving 10 µl conjugate in 10 ml 1%(w/v) Marvel in PBST and add to membrane. Incubate as in 4.4.

4.7 Repeat step 4.5

4.8 Prepare Substrate as described below and add to blot. Incubate for a defined time period not exceeding 10min. Do **NOT** shake.

Substrate:
Dissolve one DAB tablet in 15 ml 0.1 M Tris pH 7.6 (Substrate buffer) and add 7 µl H_2O_2 immediately prior to use. **(Caution: DAB is carcinogenic and H_2O_2 is corrosive).**

4.9 Wash the blot in copious amounts of deionised water, record results and store blot in tinfoil in a laboratory notebook.

Note: Mark signal strength as +1 to +5 and lightly circle the weakest signal using a pencil.