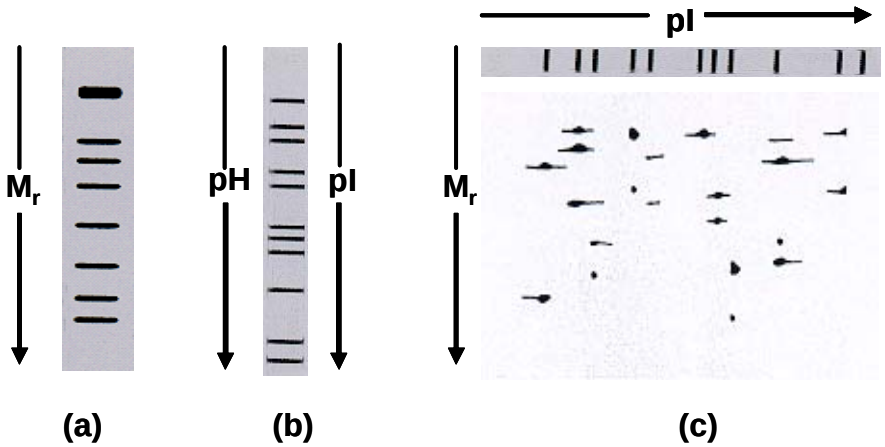
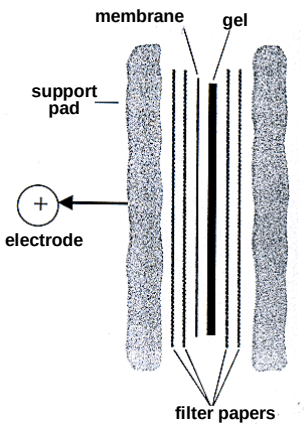
Immunoblotting of proteins: Western blot

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. Immunoblotting can be used to determine a number of important characteristics of protein antigens such as: (1) the presence and quantity of an antigen; (2) the relative molecular mass of the polypeptide chain; (3) the efficiency of extraction of the antigen. The latter is particularly useful when dealing with antigens that are insoluble, difficult to label, or easily degraded, and thus less amenable to analysis by immunoprecipitation. Immunoblotting can be combined with immunoprecipitation to allow very sensitive detection of minor antigens and to study specific interactions between the antigen and other proteins (protein-protein interactions). It is also a powerful technique for assaying the presence, quantity, and specificity of antibodies from different samples of polyclonal sera. Finally, it can be used to purify specific antibodies from polyclonal sera. The immunoblotting procedure can be divided into six steps: (1) Preparation of the antigen sample: antigens may be purified or partially purified proteins, but often they are included in lysates or extracts of cells or tissues. The antigen sample is prepared in a gel electrophoresis sample buffer to denature proteins and disrupt protein-protein interactions; (2) Resolution of the sample by gel electrophoresis: this is usually carried out on SDS-polyacrylamide gel, or by isoelectric focusing, or the combination of these on two-dimensional gels;



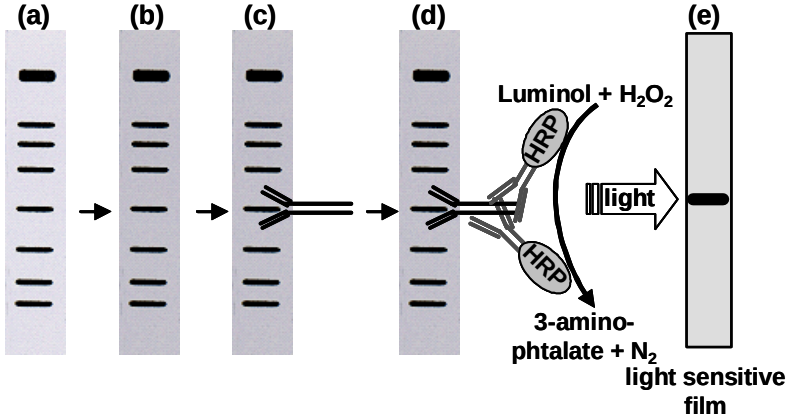
Separation of proteins for immunoblotting (Western blot): (a) according to molecular mass by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); (b) according to isoelectric points (pI) along a pH gradient; (c) by two-dimensional gel electrophoresis according to isoelectric points in the first dimension, then according to molecular size in the second dimension.



The scheme of a transfer unit for immunoblotting

(3) Transfer of the separated polypeptides to a membrane support: the separated proteins are transferred to a membrane (e.g. nitrocellulose, polyvinyl difluoride) that binds the proteins nonspecifically. Transfer usually is achieved by placing the membrane in direct contact with the gel as a "sandwhich" in a transfer unit (see below), then this unit is immersed into a transfer buffer and an electric field is applied to drive the proteins from the gel onto the membrane;

(4) Blocking nonspecific bindings sites on the membrane: remaining binding sites on the membrane are blocked with indifferent proteins (e.g. nonfat dried milk, bovine serum albumin, etc.) to eliminate any further reaction with the membrane; (5) Addition of the antibody to form specific antigen-antibody complex; (6) Detection: the location of specific antigens is determined using a labeled primary antibody or an unlabeled primary antibody, followed by a labeled secondary antibody. A very popular way of the detection is based on the reaction of luminol with H_2O_2 catalyzed by horseradish peroxidase (HRP) resulting in chemiluminescence, thus the location of antigen-antibody interaction could be identified on light sensitive film. Alternatively, a substrate resulting in a colored precipitate could also be used in the reaction.



Identification of an antigen-antibody interaction by Western blot: (a) membrane with transferred proteins including the antigen; (b) membrane after blocking the nonspecific binding sites with an inert protein; (c) interaction of the antigen with the primary antibody; (d) identification of the binding of primary antibody by incubation with horseradish-peroxidase (HRP) labeled secondary antibody; (e) localization of the site of antigen-antibody interaction on a light sensitive film using a HRP catalyzed reaction resulting in chemiluminescence.

The major factor that will determine the success of an immunoblotting procedure is the nature of the epitopes recognized by the antibodies. High-resolution gel electrophoresis techniques involve denaturation of the antigen sample, so only antibodies that recognize denaturation-resistant epitopes will bind. Most polyclonal sera contain at least some antibodies of this type, but many monoclonal antibodies will not react with denatured antigens. The sensitivitv of immunoblotting is also determined by the detection method. For most detection systems, this limit will be about 20 femtomoles. For a 50 000 dalton protein, this is approximately 1 ng. Because the loading capacities of gels are limited, an antigen usually cannot be detected when its concentration falls below 1 ng/sample.