KONERU LAKSHMAIAH COLLEGE OF ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

Guest Lecture &Workshop

Mr .Sankar ,Technical person from 'HELINI BIOMOLECULES' Guntur has delivered lecture and conducted work shop on **Molecular Biology Techniques**.The details are given below on 12th August 2009

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of Proteins and Nucleic Acids

Proteins are amphoteric compounds; their nett charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a nett negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The nett charge carried by a protein is in addition independent of its size - ie: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of the molecules.

Nucleic acids however, remain negative at any pH used for electrophoresis and in addition carry a fixed negative charge per unit length of molecule, provided by the PO4 group of each nucleotide of the the nucleic acid. Electrophoretic separation of nucleic acids therefore is strictly according to size.

SDS-PAGE of Proteins

Separation of Proteins under Denaturing conditions

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - ie: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

Western" or Immunoelectroblotting of Proteins

Immunoassay

The reason for transferring proteins to membranes from gels is so as to be able to get at them more efficiently with various probes, as polyacrylamide is not particularly amenable to the diffusion of large molecules. The most popular type of probe of immobilised proteins is an antibody of one type or another: the attachment of specific antibodies to specific immobilised antigens can be readily visualised by indirect enzyme immunoassay techniques, usually using a chromogenic substrate which produces an insoluble product. Lately chemiluminescent substrates have begun to be used because of their greater detection sensitivity. Other possibilities for probing include the use of fluorescent or radioisotope labels (fluorescein, 125I). Probes for the detection of antibody binding can be conjugated anti-immunoglobulins (eg: goat-anti-rabbit / human); conjugated staphylococcal Protein A (which binds IgG of various species of animal); or probes to biotinylated / digoxigeninylated primary antibodies (eg: conjugated avidin / streptavidin / antibody).

The immunoassay is normally done by blocking the transfer membrane with a concentrated protein solution (eg: 10% foetal calf serum, 5% non-fat milk powder) to prevent further non-specific binding of proteins; this is followed by incubation of the membrane in a diluted antiserum / antibody solution, washing of the membrane, incubation in diluted conjugated probe antibody or other detecting reagent, further washing, and the colorimetric / autoradiographic / chemiluminescent detection.

The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass: proteins are first separated by mass in the SDS-PAGE, then specifically detected in the immunoassay step.

It is also possible to use a similar technique to elute specific antibodies from specific proteins resolved out of a complex mixture, many of whose components react with a given antiserum: one can electrophorese a mixture of proteins, cut out a specific band from a gel or membrane, and use this to fish out specific antibodies from a serum.

- **Immunoabsorption Techniques**

Immunoassay techniques

Antibody preparations may be tested for specific activity by indirect ELISA (eg: Rybicki and von Wechmar 1981) and western blotting (eg: Rybicki and von Wechmar 1982). An example of a blot - taken from the source reference below - is shown here:

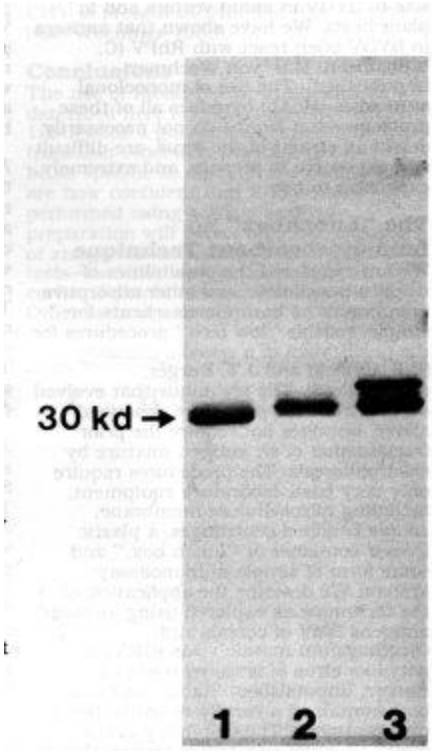

Figure 2. Western blot of three isolates of *Ornithogalum* mosaic virus probed with monospecific antibodies purified from virus adsorbed onto a nitrocellulose sheet.

Crudely purified virus isolates: left to right, OMV; OMV ex *Lachenalia* sp.; OMV 'wild' isolate. Antibodies eluted from virus (OMV) adsorbed onto nitrocellulose as described in the text, and detected using alkaline phosphatase-labelled goat-anti-rabbit antibodies and Nitro blue tetrazolium complex substrate.

Discussion

As mentioned above, 1 mg or more antibody can be prepared from a single elution of a single BMV-infected sap extract-coated membrane; this is sufficient to prepare enzyme conjugates for DAS-ELISA, and would provide materials for many assays. In any case, re-use of the antigen-coated membrane enables accumulation of enough antibody to allow many serological tests. If antisera are absorbed with host plant antigens prior to application to blots, eluted antibodies react almost exclusively with virus coat protein, with very little "background" reaction with other polypeptides in Western blot tests (see Figure). Eluted anti-BMV antibody could be diluted by a factor of 1/50 for use in Western blots, and up to 1/500 for indirect ELISA. This is a far better yield than can be obtained with individual excised polypeptides.

An important consideration is that cheap materials may be used. Nitrocellulose or other membrane is the only expensive component, and that is far cheaper, far easier to use, and far better as an adsorbent than CNBr- Sepharose, or other comparable column chromatography materials commonly used as immunoadsorbents. Up to 100 ug protein/cm2 may be adsorbed onto nitrocellulose; polyvinyllidene fluoride's capacity is even higher.

The use of crude extracts to purify antibodies is important when no facilities exist for purification of low-yielding viruses: clarified sap extracts could be concentrated by PEG treatment; non-infected extracts could be used to absorb antisera; and infected extracts to purify monospecific antibodies. Even if antibody preparations are not exactly monospecific, their preparation in this way represents an important purification and concentration step, as relatively specific antibodies are purified in one step from raw serum, with a consequent increase in activity of the preparation once most of the extraneous serum proteins and immunoglobulins are removed.

This has worked very sucessfully with detection of plant virus coat protein bands against a background of whole plant, using antisera that reacted with EVERYTHING before absorption; also with antisera raised against purified proteins either from, or cloned into, E coli: in latter case, as anyone who has done it knows, when you do a Western, ALL the bands light up, as rabbits are immune to E coli and related gut microflora - and no-one thought to tell you...!

You can use the same technique to mass-absorb / elute Ab to a particular purified protein, without having to go to the trouble of making up an expensive column immunoabsorbent: soak NC or other memb in protein of interest, wash, block, soak in AS. Wash thoroughly, then elute Ab with preferred elution mix (I use 0.1M Glycine/HCl/0.15M NaCl pH 2.9). You can repeat the absorption/elution several times, and yield is quite high - certainly enough for labelling specific Ab for immunofluorescence, ELISA, etc. We have used it in our labs to make monospecific Ab to plant viruses, and to E coli proteins or proteins cloned in E coli, as long as one has a background free of the protein of interest.

You can also combine two techniques: pre-absorb antisera with membrane with complex mixture NOT containing protein of interest, then pour off antisera onto memb with complex mixture CONTAINING protein of interest, preferably at highish concentration. First absorption takes out Ab reacting with "host protein", in second, what is left is hopefully relatively monospecific, and can be eluted as above, for labelling, etc.

- **Polymerase Chain Reaction**
    - PCR Primer Design and Reaction Optimisation
    - Standard PCR Protocol
    - Reverse Transcription PCR
    - Core Sample PCR (for bands in gels)
    - Calculating Concentrations for PCR Reactions

Factors Affecting the PCR:

Denaturing Temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing - if it is not single-stranded already, like most RNA viruses - by heating it to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100oC - which is why PCR works with denaturing temperatures of 91-97^{o}C.

A more detailed treatment of annealing / hybridisation is given in an accompanying page, together with explanations of calculations of complexity, conditions for annealing / hybridisation, etc.

Taq polymerase is given as having a half-life of 30 min at 95^{o}C, which is partly why one should not do more than about 30 amplification cycles: however, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased: for templates of 300bp or less, denaturation temperature may be

reduced to as low as 88^{o}C for 50% (G+C) templates (Yap and McGee, 1991), which means one may do as many as 40 cycles without much decrease in enzyme efficiency.

"Time at temperature" is the main reason for denaturation / loss of activity of Taq: thus, if one reduces this, one will increase the number of cycles that are possible, whether the temperature is reduced or not. Normally the denaturation time is 1 min at 94^{o}C: it is possible, for short template sequences, to reduce this to 30 sec or less. Increase in denaturation temperature and decrease in time may also work: Innis and Gelfand (1990) recommend 96^{o}C for 15 sec.

Annealing Temperature and Primer Design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature of a NA duplex increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the Tm is

$Tm = 4(G + C) + 2(A + T)^{o}C.$

Thus, the annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). One should aim at using an annealing temperature (Ta) about 5^{o}C below the lowest Tm of ther pair of primers to be used (Innis and Gelfand, 1990). A more rigorous treatment of Ta is given by Rychlik et al. (1990): they maintain that if the Ta is increased by 1^{o}C every other cycle, specificity of amplification and yield of products <1kb in length are both increased. One consequence of having too low a Ta is that one or both primers will anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated: this is fine if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target.

A consequence of too high a Ta is that too little product will be made, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different Tas may never give appreciable yields of a unique product, and may also result in inadvertent "asymmetric" or single-strand amplification of the most efficiently primed product strand.

Annealing does not take long: most primers will anneal efficiently in 30 sec or less, unless the Ta is too close to the Tm, or unless they are unusually long.

Detection of Nucleic Acids by Hybridisation

Hybridisation is a term used to describe the specific complementary association due to hydrogen bonding, under experimental conditions, of single-stranded nucleic acids. It should more properly be referred to as "annealing", as this is the physical process responsible for the association: two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-

stranded, anti-parallel "hybrid" helical molecule. One may make ones nucleic acid single-stranded for the purpose of annealing - if it is not single-stranded already, like most RNA viruses - by heating it in 0.01M NaCl to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling to $\pm$0oC: this ensures the "denatured" or separated strands do not re-anneal.

Alternatively, one may denature DNA reversibly by treatment with 0.5M NaOH: this does not work for RNA, as this hydrolyses under these conditions.

Thus the lecture & workshop cleared all sorts of practical doubts raised by students.