

Technical tips

Session 11

Far-Western blotting: Far-Western blotting was originally developed to screen protein expression libraries with ^{32}P -labeled glutathione S-transferase (GST)-fusion protein. Far-Western blotting is now used to identify protein:protein interactions. In recent years, far-Western blotting has been used to determine receptor:ligand interactions and to screen libraries for interacting proteins. With this method of analysis it is possible to study the effect of post-translational modifications on protein:protein interactions, examine interaction sequences using synthetic peptides as probes, and identify protein:protein interactions without using antigen-specific antibodies.

Far-Western Blotting vs. Western Blotting

The far-Western blotting technique is quite similar to Western blotting. In a Western blot, an antibody is used to detect the corresponding antigen on a membrane. In a classical far-Western analysis, a labeled or antibody-detectable "bait" protein is used to probe and detect the target "prey" protein on the membrane. The sample (usually a lysate) containing the unknown prey protein is separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or native PAGE and then transferred to a membrane. When attached to the surface of the membrane, the prey protein becomes accessible to probing. After transfer, the membrane is blocked and then probed with a known bait protein, which usually is applied in pure form. Following reaction of the bait protein with the prey protein, a detection system specific for the bait protein is used to identify the corresponding band.

Thin-layer chromatography (TLC): TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate).

A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.

As the solvent moves past the spot that was applied, an equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will

be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates. (The plate itself contains a fluor which fluoresces everywhere *except* where an organic compound is on the plate.)

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Ninhydrin reagent is very useful for amino acid analysis. You can separate the amino acids on a mixture by TLC, in basis of their different polarities and therefore more or less affinity for a determined solvent. **Ninhydrin** is a selective oxidizing agent which causes oxidative decarboxylation of α -amino acids, producing CO_2 , NH_3 , and an aldehyde with one less carbon atom than the parent amino acid. The reduced ninhydrin then reacts with the liberated Ammonia to form Ruhemann's Purple, a complex which maximally absorbs light at 570 nm. Secondary amines, Proline and 4-Hydroxyproline, react via a different path and form a yellow distinctive derivative with an optimal absorbance at 440 nm.

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2-D Gel electrophoresis and isoelectric focusing (IEF):

The technique of two-dimensional electrophoresis involves separating proteins in the **first dimension** according to **charge** (isoelectric focusing, IEF), followed by separating the focused proteins in the **second dimension** according to **molecular weight** by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

Isoelectric focusing gel electrophoresis (**first dimension**) separates proteins according to their **net charge**. At a given pH, a protein's net charge will depend on its relative number of positive and negative charges (which depend both on the amino-, carboxy- terminals and the net charge on the side chains of its amino acid residues). During isoelectric focusing, separation is accomplished by placing the protein in a pH gradient generated by an electric field. Under these conditions the protein migrates until it reaches the position in the pH gradient at which its net charge, or **isoelectric point**, is zero.

The **second dimension** of two-dimensional gel electrophoresis simply consists of SDS-PAGE in which either a strip or a tube gel from isoelectric focusing is fitted over an SDS-polyacrylamide gel and the proteins are separated according to molecular weight by electrophoresis.

The proteins may then be visualized by staining with Coomassie Brilliant Blue R250, silver stain, fluorescent dyes or through radioisotope detection (phosphorescence) after the proteins are metabolically labeled with ^3H , ^{14}C , or ^{35}S labeled amino acids. This two-dimensional array will produce spots that correspond to a single protein species in the sample. Using this technique combined with the 2D gel analysis system, thousands of different protein can be separated and information such as pI, molecular weight and protein amount can be determined. These spots can be excised for further analysis or the 2-D array can be analyzed for differences in protein quantity or in proteins present in the gel. 2-D gels can also be electroblotted to PVDF or nitrocellulose membranes for further analysis.

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Fluorescence In Situ Hybridization (FISH):

What is FISH?

Fluorescence in situ hybridization (FISH) uses fluorescent molecules to vividly paint genes or chromosomes. This technique is particularly useful for gene mapping and for identifying chromosomal abnormalities.

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How does FISH work?

FISH involves the preparation of short sequences of single-stranded DNA, called probes, which are complementary to the DNA sequences the researchers wish to paint and examine. These probes hybridize, or bind, to the complementary DNA and, because they are labeled with fluorescent tags, allow researchers to see the location of those sequences of DNA. Unlike most other techniques used to study chromosomes, which require that the cells be actively dividing, FISH can also be performed on nondividing cells, making it a highly versatile procedure.

What is FISH used for?

Scientists use three different types of FISH probes, each of which has a different application:

Locus specific probes hybridize to a particular region of a chromosome. This type of probe is useful when scientists have isolated a small portion of a gene and wish to determine which chromosome that gene is located on. They prepare a probe from the piece of the gene and observe which chromosome the probe hybridizes to.

Alphoid or centromeric repeat probes are generated from repetitive sequences found at the centromeres of chromosomes. Because each chromosome can be painted in a different color, researchers use this technique to determine whether an individual has the correct number of chromosomes or, for example, whether a person has an extra copy of a chromosome.

Whole chromosome probes are actually collections of smaller probes, each of which hybridizes to a different sequence along the length of the same chromosome. Using these libraries of probes, scientists are able to paint an entire chromosome and generate a spectral karyotype. This full color image of the chromosomes allows scientists to distinguish between the chromosomes based on their colors, rather than based on their dark and light banding patterns, viewed in black and white through traditional karyotyping. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.