Lecture 10 - Screening cDNA libraries (AMG text pp. 128-134)
September 20, 2001

Using antibody probes to detect antigenic fusion proteins
Protein-specific antibodies can be used as "probes" to immunologically detect bacterial fusion proteins encoding the protein antigen. Antibody screening of cDNA libraries is facilitated by special lambda phage expression vectors that produce fusion proteins which can be detected on membrane filters. The most commonly used cDNA cloning vectors for antibody screening are the lambda gt11 and lambda Zap phage vectors which contain an E. coli transcriptional promoter linked to the beta-galactosidase (lacZ) coding sequences. The cDNA encoded protein is fused to the carboxy terminus of the beta-galactosidase enzyme (tetrameric complex would have four antigens), however, only one of three ligation products from a directional cloning strategy will be in the correct translational reading frame (only one of six is using EcoRI adaptors).

Antibodies recognize specific protein structures through noncovalent interactions. Immunoglobulins function as antibodies that bind with high affinity to foreign (non-self) molecules called antigens. Immunoglobulin G (IgG) class molecules are large tetrameric protein complexes found at high levels in the blood. Each IgG molecule contains two heavy chain polypeptides and two light chain polypeptides that are linked together by disulfide bridges and noncovalent subunit interactions. The amino terminal ends of the light and heavy chains are called the variable regions because they correspond to amino acids that impart antigen specificity and are derived from different IgG genes. Cleavage of the IgG molecule with the protease papain, releases two Fab domains that each contain antigen binding pockets, and one Fc domain encompassing the carboxy terminal ends of the two heavy chain polypeptides. All three of these subdomains are held together by disulfide bridges.

Structure of an isolated Fab domain bound to an antigen molecule. The space filling model of the antigen binding site illustrates the close contact made between the IgG residues in the light and heavy chains and the antigen. Notice how many contacts are made between the IgG molecule and the antigen.

Why is the filter soaked in IPTG before overlaying the plate, why not just include IPTG in the growth media?

What does a positive signal in an antibody screening strategy tell you about the fusion protein?

What two methods can be used to determine if the "antigen" cDNA corresponds to your gene of interest?

Screening cDNA libraries based on differential expression
In this screening strategy, mRNA is isolated from cells that express the phenotype (or protein) of interest (+), and from cells that do not (-). The (+)mRNA is converted to radioactive (+)cDNA using RTase and 32P-dNTPs, and then hybridized to a mass excess of (-)mRNA using solution hybridization. By removing the double-stranded
cDNA:mRNA heteroduplexes, it is possible to obtain an enriched probe containing (+)cDNA sequences.

Although subtraction hybridization screening has been useful in the isolation of a number of important cDNAs, this method has largely been superseded by PCR-dependent strategies and high-throughput screening using.

This example illustrates how salt-stress in plants can alter the expression of genes required for osmotic regulation. High specific activity cDNA probes, are hybridized to duplicate filter lifts to identify plaques that contain cDNA inserts corresponding to differentially expressed genes.

Cloned cDNA sequences have the potential to encode active proteins. The technique of cDNA "expression cloning" uses a functional assay to identify positive recombinants without prior knowledge of the gene product. Antibody screening of cDNA expression libraries is a type of functional screen, however it is in principle different than expression cloning since sufficient information about the protein antigen is already available.

Three types of cDNA screening methods based on functional assays have been exploited:

1. Protein activity assays (table 5.1); see HOT chili peppers.
2. Yeast two-hybrid system is based on protein-protein interactions.
3. cDNA phage display is a method utilizing solid support interactions.

Example of Protein Activity Assays used to isolate functional cDNA sequences.


Hot peppers and painful heat both activate sensory nerve fibres through an ion channel discovered by David Julius and colleagues. The channel, known as vanilloid receptor subtype 1 (VR1), is activated by binding of capsaicin -- the compound that makes chilli peppers hot. When activated, the channel opens, allowing an influx of calcium and sodium ions. The influx depolarizes neuronal pain fibres, initiating a nerve impulse through the dorsal root ganglion (DRG) to the brain. Noxious temperature uses the same elements, explaining why our mouths feel hot when we eat chilli peppers.

The capsaicin receptor was cloned by constructing a cDNA library using mRNA from dorsal root ganglion cells. Recombinant clones from the plasmid library were pooled (16,000 clones per pool) and transfected into a human cell line to screen for functional cDNAs that resulted in calcium uptake. Eventually a single plasmid cDNA clone was identified that encoded the desired activity. Subsequent characterization experiments demonstrated that they had indeed cloned the capsaicin receptor, interestingly, calcium uptake was also stimulated by heat through an unknown mechanism.
List 3 criteria that must be met for these protein activity assays to work for cDNA screening.

What property of the caspaicin receptor function made it possible to identify plasmids with the correct insert even when the clone was only one of 16,000 in the plasmid pool?

How does "sib selection" work in the context of plasmid pools and the Julius cloning strategy?

**Yeast Two-Hybrid screening**

Biological processes require protein-protein interactions that can be defined by non-covalent molecular interactions. The idea of using a cloned protein to screen cDNA expression libraries for interacting proteins, follows from the work of Stan Fields and colleagues. The yeast two-hybrid strategy was based on an earlier finding by Mark Ptashne and co-workers demonstrating that the yeast Gal4 transcription factor is a modular protein. The Ptashne lab showed that the transcriptional regulatory activity of Gal4 requires a sequence specific DNA binding domain (DBD), and a transcriptional activation domain (AD).

The yeast two-hybrid system is used to identify cDNA sequences encoding protein domains capable of interacting with a "bait" protein. The DBD-bait and AD-cDNA plasmids replicate episomally in yeast.

What are the three functional components required for the Two-Hybrid screening strategy to work?

What protein function cannot be present in the bait protein, i.e., what function would give high background?

Why would you want to have both the Gal4 dependent His3 and lacZ reporter genes in the host yeast strain? Which is a genetic selection and which is a genetic screen?

**cDNA Phage Display library screening**

The yeast two-hybrid system is well-suited for identifying protein interacting domains, but it is limited to bimolecular interactions involving two DNA encoded protein products. There are a number of important biological interactions that occur between a protein product and a small diffusible molecule, such as a ligand or antigen. The Phage Display strategy was designed to find functional proteins that bind small ligands.

Filamentous phage cDNA expression libraries can be screened for fusion proteins that bind to a solid support using the panning method. In-frame coding sequences have the potential to produce a fusion protein that is expressed on the phage surface.

Sequential panning and infection cycles are carried out to enrich for phage that bind to the "bait" attached to the solid support. The phagemids are rescued in E. coli and individual picks can be assayed by superinfection with M13 helper phage to produce phage for a 96-well ELISA (enzyme-linked immunosorbent assay).