

## Technical tips. Session 3

**Dominant negative allele:** a mutant version of a gene (*allele*) whose phenotypic effect dominates that of the wild type (functional) version of the gene. A dominant negative allele makes an inactive protein that disrupts function even in the presence of a normal allele (and normal protein). Note: This is not the usual case -- most negative or "lack of function" alleles (or mutations) are recessive.

**High-copy suppressor:** Gene that when over-expressed (for instance, because is placed in a vector that can exist in multiple copies inside cells) suppresses the phenotypic effect of another mutated gene. For example, if such mutated gene caused lethality under certain conditions (i.e. growth at high temperature) the high-copy suppressor would now restore viability under the same conditions.

**Promoter "shut-off" techniques:** You want to control when to express your protein of interest. Then you place the coding sequence for the protein (ORF, see tech tips for Session 2) downstream from a promoter from which gene expression can be activated/inactivated by changing certain conditions. For example, in the yeast *Saccharomyces* there are sets of genes that can specifically activated/repressed depending on the carbon source you add into the medium. *GAL* genes are repressed when in the presence of glucose, unrepressed expressing at a constitutive low level in the presence of non-fermentable carbon sources (such as raffinose) and activated (expressing up to 10-fold over the unrepressed constitutive levels) in the presence of galactose. Molecular biologists have made use of this property these genes have to subject the expression of any protein of interest to the same kind of regulation. All you have to do is place the ORF under the control of a *GAL* promoter and control the expression by controlling the carbon sources you add in the cells environment.

Other examples using this system are for instance the use of *CUP* promoters (induction of expression in the presence of copper), *MET* promoters (expression shut-off by addition of methionine), etc.

**Cycloheximide:** inhibitor of translation. It inhibits the peptidyltransferase activity of the eukaryotic ribosome.

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**Northern blot analysis:** Method used to quantify gene expression based on the detection of the corresponding messenger RNA (mRNA) from cell extracts. Total RNA is extracted and run by electrophoresis in agarose gel. Then is transferred from the gel onto a membrane. The membrane is exposed to a small sequence of DNA (called DNA probe) that is complementary to your mRNA of interest and it will only bind to it by forming a double-strand heteroduplex. The probe is either radiolabeled or it can have coupled a substrate for a colorimetric, or most commonly fluorescent or luminescent reaction. When you expose the membrane to the enzyme and components necessary for the reaction you will be able to detect the light or radiation produced in specific places of the membrane where your mRNA of interest is, by autoradiography (see tech. tips Session 2).

**Western blot analysis:** Similar basics such as those for Northern but in this case with proteins. The proteins are separated by PAGE. Then, they are transferred from the gel to a membrane. They are next exposed to a primary antibody (Ab that specifically recognizes your protein of interest). Your protein can be epitope-tagged and then you will use a primary Ab that recognizes the tag. Usually, to enhance the signal detected, a secondary antibody is used which recognizes the first one. It has coupled an enzyme

(such as horseradish peroxidase) able to carry out a luminescent reaction in the presence of the proper substrate.

One of the most common luminescent reactions utilized to detect antibodies bound to a protein is the one below. The enzyme HRP can be coupled to an antibody and when exposed to its substrates it will carry out the reaction:

(Image removed due to copyright reasons.)

The detection will also be carried out by autorradiography.

**Membrane fractionation:** To discern whether a given protein is or not associated to membranes cells are usually lysed and the corresponding crude extract is centrifuged at high speed (i.e. 55,000 r.p.m.). The pellet will contain the membranous fraction and the supernatant the soluble fraction. Sometimes a protein is associated peripherally to membranes. If so, treatment of the extract prior centrifugation with salts (NaCl), high pH ( $\text{Na}_2\text{CO}_3$ ), or urea will usually disrupt the association to membranes and now the protein will appear in the soluble fraction. When the protein is an integral membrane protein only prior treatment with detergents (such as SDS or Triton X-100) will remove the protein from the membranous fraction.

**Proteinase K treatment:** Is normally used to know whether the C-t and/or N-t of a membrane protein are facing the cytosol or the lumen of a membranous compartment or vesicle (such as the ER). If you attach an

epitope tag to an extreme of the protein you can find to which side is that extreme facing to, because the proteinase K will only destroy the part of the protein that is facing outside the vesicle. If you can still detect the protein with antibodies is because the epitope-tagged side was protected. However, if you add a detergent such as SDS that disrupts the membranes, now that side is no longer protected and you won't detect signal.

**Two-hybrid analysis:** You want to know which proteins interact with your protein of interest. You express your protein fused to the DNA-binding domain of a transcriptional factor (i.e. Gal4p for activation of *GAL* genes). This construct will be the '*bait hybrid*' (the DNA-binding domain will be called '*binding domain*', BD). BD alone can bind to its specific promoter but cannot activate gene expression because it still lacks its activation domain.

Then you have a library of plasmids that contain another different kind of construct: each of them has a different cDNA (encoding for a different cellular protein) fused to the sequence encoding for the activating domain of the same transcription factor). This is what is called '*prey hybrid*' (the activation domain alone will be referred to as '*activation domain*' or AD).

Now you co-express together both kinds of constructions for instance by co-transformation. Only those cells that inherit a prey hybrid construct that expresses a protein that interact with your protein of interest will be able to turn on the expression of the corresponding genes that are activated by the transcriptional factor. AD and BD cannot interact unless the two proteins to which they are associated do.

In the case Gal4p and *GAL* promoters are used, usually the selection is for growth in a medium lacking one amino acid or nitrogen base (i.e. *HIS3*, *ADE2*). The strain utilized lacks a gene encoding for an enzyme necessary to synthesize that molecule and instead has the gene under the *GAL* promoter so only when activated it will express the enzyme and the cells will survive.

Secondary assays are performed to make sure the interaction is consistent and is activating the genes placed under *GAL* promoters. For instance, the coding sequence for an enzyme that breaks the toxic compound 3-aminotriazol (3-AT) and that encoding for a  $\beta$ -galactosidase able to break a galactoside compound giving a blue colored-product (which enables to quantify the activation of the expression) are also used.

Example of a 2-Hybrid experiment carried out in yeast cells:

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**Immunofluorescence microscopy analysis:** The subcellular localization and relative abundance of a given protein is detected with antibodies recognizing the protein or epitope tags on them. It is also useful to check whether two proteins co-localize in specific parts of the cell.

The antibody is covalently attached to a fluorescent dye (such as Cy3) so it can be visualized under UV light in a fluorescent microscope. When the light illuminates the fluorescent dye, it absorbs the light and emits a different color light, which is visible and can be photographed.

Cells are fixed with formaldehyde to retain shape and location of all cellular proteins. The cell is treated with a mild detergent to dissolve small holes in the membranes so the antibodies can access all compartments in the cells.