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Special section on techniques:

Yeast Two Hybrid Assay: A Fishing Tale

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Purpose

The yeast "two hybrid" or "interaction trap" assay, is a technique used to study protein-protein interactions, which are critical in virtually all cellular processes. The study of protein-protein interactions can be divided into three major parts:

- identification of binding proteins
- the characterization of known interactions
- the potential to manipulate such interactions¹

Theory

The yeast two hybrid assay, developed by Fields and Colleagues over a decade ago², has since become one of the most popular tools used in molecular biology. In order to understand how the two hybrid system was first developed, it is useful to understand the process of galactose metabolism in yeast.

Galactose metabolism

Galactose is imported into the cell and converted to galactose-6-phosphate by six enzymes (GAL1, GAL2, PGM2, GAL7, GAL10, MEL1) which are transcriptionally regulated by the proteins Gal80, Gal3, and Gal4, the latter of which plays the central role of DNA-binding transactivator (Figure 1). Gal80 binds Gal4 and inhibits its transcriptional ability. Gal3, in the presence of galactose, binds and causes a conformational change in Gal80, which then allows Gal4 to function as a transcriptional activator

Gal4, like other transcriptional activators, is a modular protein that requires both DNA-binding (BD) and activation domains (AD). The "two hybrid" technique exploits the fact that Gal4 cannot function as a transcriptional activator unless physically bound to an activation domain. Furthermore, it has been demonstrated that this interaction does not need to

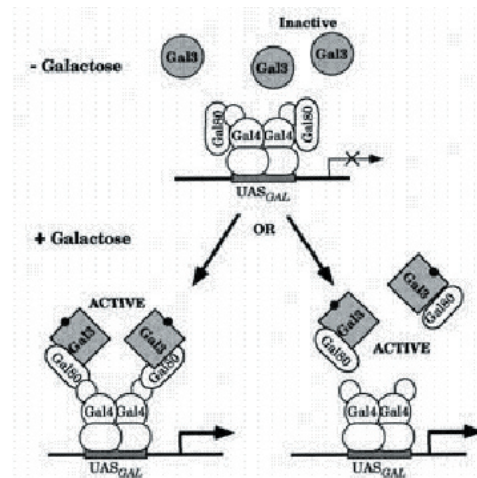


Figure 1 Galactose metabolism

be covalent: an experiment was performed where the negative regulatory protein, Gal80, was fused with an activation domain to produce Gal80-AD, and was able to act as a transcriptional activator through its natural binding interaction with a Gal4 protein that was missing its own activation domain.

Based on the above observations, a two hybrid assay is performed by expressing two fusion proteins in yeast, the "hunter" and the "bait", where the "hunter" protein is the potential binding partner fused to a yeast activation domain, and the bait is your protein of interest is fused to a yeast binding domain.

The hunter and bait constructs are co-transfected into the yeast strain, containing the appropriate "Upstream Activating Sequence" (UAS) proximal to a reporter gene, which is expressed if a binding interaction occurs between the hunter and the bait (Figure 3).

To identify proteins that may interact with the bait, a plasmid library, expressing cDNA-encoded AD-fusion proteins, can be screened by introduction into a yeast

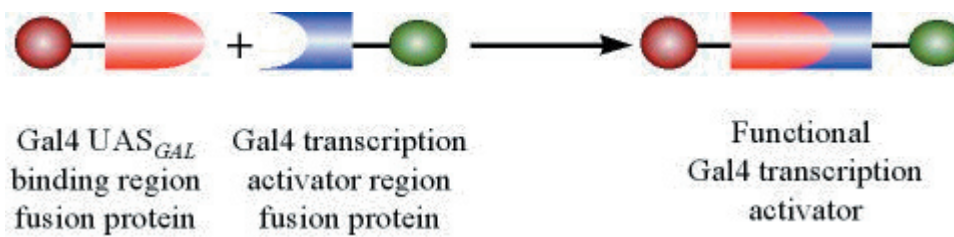


Figure 2 Gal4 transcriptional activator exploited for yeast two hybrid assay

strain. The interaction of the potential hunter proteins with the bait results in the activation of the reporter gene, allowing for the identification of cells containing the interacting proteins.

The Pros and Cons of the Two hybrid Assay

Disadvantages and Trouble-Shooting:

- 1) Since the two hybrid assay measures reporter activity in response to transcriptional activation, an obvious problem would arise if your protein of interest were able to activate transcription on its own (auto-activation). It is, therefore, imperative that an initial experiment be done to test for the transcriptional activity of the protein of interest.
- 2) The extensive use of fusion proteins may change the conformation of the hunter or bait, which may alter activity or binding. Nevertheless, there have been shown to be few problems with tagged proteins, perhaps due to their modular nature, where domains can fold independently, often allowing the introduction of artificial modules.
One way to test whether the protein of interest is folded properly is to clone a positive interactor (a protein known to interact with the bait) into the vector and test for a two hybrid interaction, which will only result if both proteins are folded correctly. However, this technique only works for the domains involved in "positive" interactions and may not be conclusive for domains involved in novel interactions. Furthermore, an empirical way to circumvent this problem may be through the reciprocal transfer of proteins, involving the switching of a BD-fusion to an AD-fusion protein and vice et versa.
- 3) A major drawback of testing protein-protein interactions in a heterologous system such as the yeast is that interactions may depend on certain post-transcriptional modifications, such as disulfide bridge formation, glycosylation, or phosphorylation, which may not occur properly or at all in the yeast system¹.
This problem may be, in certain cases, circumvented perhaps by co-expressing the enzymes

necessary for such modifications.

- 4) Since the fusion proteins in the two hybrid system must be targeted to the nucleus, extracellular proteins or proteins with stronger targeting signals may be at a disadvantage.
- 5) In the classical two hybrid library preparations, only one sixth of the cDNA is in the correct frame. Nevertheless, this works out to over a million independent clones to be studied (which pushes feasibility), if a good representation is to be obtained.
A solution to this may be to make directional libraries of a relevant tissue or cell type, or if possible, to simply choose a less complex organism to study.
- 6) It has been shown that sub-domains of proteins may interact better than full length clones, perhaps due to the lack of certain folding restraints. Since screening of libraries selects for optimized reactions, one may obtain a false representation. This problem can be dealt with by very tediously ensuring that only full-length cDNAs in the correct reading frame are cloned.
- 7) Given that the two hybrid assay measures reporter activity, it cannot be excluded that a third protein may perhaps be bridging the hunter and the bait. This possibility is unlikely, but should always be kept in mind, since this is also a problem encountered with many other conventional biochemical binding assays.
- 8) Certain proteins, when expressed in the yeast system or targeted to the nucleus, may become toxic. Other proteins may degrade essential yeast proteins or proteins whose presence are required for the assay. Such genes may be counter-selected for during growth and may result in problems.
- 9) As typical with all exhaustive screening assays, the identification of false binding partners presents itself as a disadvantage in the two hybrid assay. Due to localization and time restraints, and cell context in different cell types, even though two proteins can interact, it is not certain that they will interact in real situations given the factors above. Therefore, the biological relevance of any two proteins found to interact is a great concern,

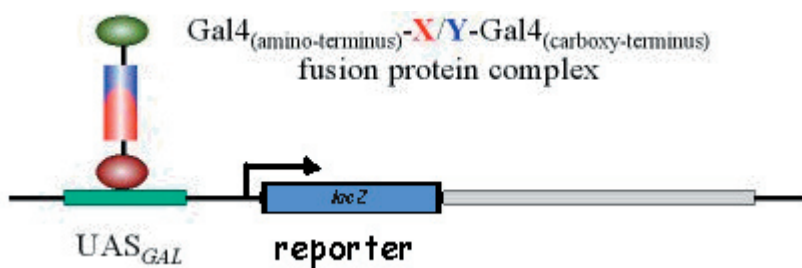


Figure 3 Gal4 binds to an upstream activating sequence

and must be kept in perspective.

Advantages:

- 1) The yeast two hybrid system has a clear advantage over classical biochemical or genetic methods, in that it is an *in vivo* technique that uses the yeast cell as a living test-tube.
- 2) The use of the yeast host can be considered an advantage since it bears a greater resemblance to higher eukaryotic systems than a system based on a bacterial host.
- 3) With regards to classical biochemical approaches, which can require high quantities of purified proteins or good quality anti-bodies, the two hybrid system has minimal requirements to initiate screening, since only the cDNA of the gene of interest is needed.
- 4) In signaling cascades, weak and transient interactions are often very important. Such interactions are more readily detected with the two hybrid system since the reporter gene response often leads to significant amplification. However, one must keep in mind that there are trade-offs between detecting weak signal and obtaining false-positives in screening procedures.
- 5) The two hybrid assay is also useful for analysis of known interactions, which can be achieved by modifying important residues or modules and observing this effect on binding.
- 6) Interactions can be measured semi-quantitatively using the two hybrid system, allowing discrimination between high, intermediate, and low-affinity bindings, the power of which correlates with that of *in vitro* approaches.
- 7) Although the two hybrid assay was predicted to be limited to the study of cellular proteins, given that extracellular proteins often undergo modifications such as glycosylation or disulfide cross-links that are not expected to occur in the yeast nucleus, there have been various reported successes with extracellular receptor/ligand complexes.
- 8) Two hybrid screens are sometimes termed "functional screens", since if at least one of the proteins screened has a known function in a well-defined

pathway, it might provide a functional hint in the current interaction.

- 9) Although there are certain disadvantages involving the two hybrid assay, the most convincing argument for its use is the speed and ease by which the molecular mechanisms of many signaling cascades have been defined using this technique.

Methods and Protocols

There are numerous two hybrid systems that can be used to detect protein-protein interactions for different purposes. Here are listed some of the more common methods used for such studies, as well as a brief outline of practical steps to help maximize experimental success. Some protocols for these methods are also provided in the links provided.

Standard Yeast Two hybrid Systems

- 1) The Gal4 System identifies the interaction between two proteins by reconstituting active Gal4 protein. The two proteins involved are expressed as fusion proteins with the Gal4 BD and AD. The two plasmids containing these constructs are co-transfected into a strain of yeast containing the upstream activation sequences from the GAL1-GAL10 regions, which promote transcription of the *E. coli* LacZ gene. If interaction occurs, LacZ is transcribed, resulting in the turning blue of the strain when placed in medium containing X-gal (a chromogenic substrate). Note that when using the Gal4 system, to avoid interference by endogenous Gal4 and Gal80 proteins, the yeast host must carry deletions in the GAL4 GAL8 genes, and due to the deletion of these genes, the yeast cells will grow more slowly than wild type⁷.
- 2) The LexA system is another version of the two hybrid system that uses the operator sequence and the BD from the *E. coli* *lexA* repressor protein. In this system, the AD comes from a segment of *E. coli* DNA that expresses an acidic peptide, acting as transcriptional activator in yeast when fused with a DNA binding domain. These constructs are then co-transfected into yeast strains containing the *lexA* operators upstream of either the *E. coli*

Table 1: Overview of the most commonly used two-hybrid vectors. The last column describes the origin of the promoter and the accession number in EMBL (AC)

Name	Selection-marker	Functional domain	promoter, AC
GAL4-based			
pMA424	<i>HIS3</i>	GAL4DB	Original vector, 12 kb
pGBT9	<i>TRP1</i>	GAL4DB	ADH1(truncated) AC: U07646
pAS1	<i>TRP1</i>	GAL4DB+HA	ADH1(full length), CYH2
pAS2	<i>TRP1</i>	GAL4DB+HA	ADH1(full length) CYH2
pAS2-1	<i>TRP1</i>	GAL4DB	AC: U30496 ADH1(full length) CYH2
pGAD2F	<i>LEU2</i>	GAL4AD	AC: U30497
pGAD424	<i>LEU2</i>	GAL4AD	Original vector, 13 kb ADH1(truncated)
pGAD10	<i>LEU2</i>	GAL4AD	AC: U07647 ADH1(truncated)
pGAD-GL	<i>LEU2</i>	GAL4AD	AC: U13188 ADH1(truncated)
pGAD-GH	<i>LEU2</i>	GAL4AD	ADH1(full length)
pGAD1318	<i>LEU2</i>	GAL4AD	ADH1(full length)
pSE1107	<i>LEU2</i>	GAL4AD	
pSD-10	<i>URA3</i>	VP16AD	
pACT1	<i>LEU2</i>	GAL4AD	
pACT2	<i>LEU2</i>	GAL4AD+HA	ADH1(truncated), medium expression AC: U29899
LexA-based			
pBTM116	<i>TRP1</i>	LexA	ADH1(truncated)
pLexA	<i>HIS3</i>	LexA	ADH1(full length) - pEG202
pB42AD	<i>TRP1</i>	B42+ SV40 NLS + HA	GAL1 (full length), inducible promoter
pHybLex/Zeo	<i>Zeo^o</i>	LexA	- pJG4-5 ADH1(truncated)
pYESTrp	<i>TRP1</i>	V5 epitope + SV40 NLS + B42	GAL1 (full length), inducible promoter
pGilda	<i>HIS3</i>	LexA	GAL1 (full length), inducible promoter centromeric vector

lacZ or yeast Leu2 reporter genes. Since LexA, unlike Gal4, is not endogenous to yeast, this system is preferred in certain situations because it is less likely to produce false-positives through auto-activation (due to the presence of the bait alone).

Useful Things to know in setting up a Two hybrid Assay⁷

1) Choosing a Vector

The first step in a two hybrid-assay is to choose an appropriate vector system. As mentioned before, both the Gal4 and LexA are popular systems, each having different properties, so that interactions may be detected differently in each. Also a number of different BD and AD containing vectors are commercially available besides the two mentioned above, and trying a combination of vectors may increase the chance of success.

2) Choosing a Promoter

The expression level of your target protein will depend on the promoter that regulates it. The full-length ADH1 promoter, which normally drives the expression of alcohol dehydrogenase, produces high level expression of its downstream sequence. However, depending on the nature of the protein being expressed (ie if the protein interferes with yeast metabolism in high concentrations), a truncated version of the ADH1 promoter may be used to achieve a lower expression. Nevertheless, the weak expressing plasmids are many folds less sensitive, so that whenever possible, a higher expression vector should be used. Note that the ADH1 promoter is most active in exponentially growing yeast and is inhibited in the presence of non-fermentable carbon sources. Other promoter sequences are also commercially available.

For an overview of the most commonly used two hybrid vectors, refer to Table 1. (Biological

Procedures Online-vol.2 no.1-Oct 4, 1999-www.biologicalprocedures.com)

3) Verifying the Expression of your Fusion Protein

Before proceeding with the two hybrid experiment, it is first necessary to test whether the fusion protein is being expressed inside the yeast, which can be done by western blot analysis

Procedure described in: Protocol 1. SDS and Western Blot Analysis (Biological Procedures Online-vol.2 no.1-Oct 4, 1999-www.biologicalprocedures.com)

4) Testing for Auto-activation

As mentioned previously, since the two hybrid assay is dependent on the reconstitution of a functional transcription factor, autoactivation would give rise to large amount of false positives. Therefore, it is crucial to test for the auto-activation capacity of your target protein, and this can be tested for in a single transformation and reporter gene assay.

Procedure described in: Protocol 1. Testing whether a bait activates transcription (Russ Finley, April 1997, <http://cmmg.biosci.wayne.edu/finlab/Update.html>)

5) Testing for Localization to the Yeast Nucleus

Since transcription is a nuclear event, it is essential to ensure that the fusion proteins localize to the nucleus. With the Gal4 system, the Gal4 BD already has a nuclear localization signal (NLS) and so the problem in this case is trivial and can be tested for with using a "positive control", which will be discussed below. With the LexA system, however, problems may arise. LexA is a bacterial protein and as such does not contain an NLS, and so is made in fusion with the NLS of SV40 large T. The localization of the LexA fusion can be assayed using a "Repression" or "Blocking" assay, which is based on the ability of most transcriptionally inert LexA fusions to inhibit transcription of a reporter gene when bound to the operator located between the UAS and the tatabox. The repression assay uses the LacZ reporter which is activated at high levels by the GAL1 UAS in the presence of galactose and at low levels in the presence of glucose. Thus any repression of GAL1 UAS in the presence of either substrate demonstrates that LexA has entered the nucleus and occupies the LexA operator.

Procedure described in: Protocol 2. Repression Assay (Russ Finley, April 1997, <http://cmmg.biosci.wayne.edu/finlab/Update.html>)

6) Positive Control¹⁵

In using the two hybrid method, one can avoid most pitfalls involving bait construction by first conducting a positive control experiment. This is done by cloning a protein sequence known to interact with your bait, and assaying this interaction in the two hybrid system. If the interaction is detected, it likely implies that the bait is properly folded and is localized to the nucleus.

Procedure described in: Protocol 1. Testing whether a bait activates transcription (Russ Finley, April 1997, <http://cmmg.biosci.wayne.edu/finlab/Update.html>)

8) Choosing a Library

In constructing your cDNA library, it is important to choose a tissue where your protein of interest may have a relevant biological role, since the whole mammalian cDNA library will produce more than 10 million transformants which will need to be screened. In choosing a vector for you library, you must consider the number of independent clones before and after amplification, the mean length of the insert, and the number of clones containing the insert. In addition, it may be beneficial to use a vector with multiple cloning sites so that the insert can be subcloned in later stages for other experiments.

Furthermore, the relative strength of the AD must also be considered depending on your experiment. Strong activator such as the AD of Gal4 and VP16 make the system more sensitive, whereas the use of B42AD, having intermediate transactivation ability, makes the system less sensitive.

9) Choosing a Host

A number of yeast strains are commercially available for the two hybrid assay, defined by the reporter genes they contain and the type and copy number of their promoters. The appropriate strain of yeast should be chosen based upon which reporter was used in the fusion constructs, and the desired sensitivity.

A list of different strains is provided in: Table 3: Survey of commonly used yeast strains

(Biological Procedures Online-vol.2 no.1-Oct 4, 1999-www.biologicalprocedures.com)

Methods and Protocols

Useful protocols and approaches can be obtained through the following links:

<http://cmmg.biosci.wayne.edu/finlab/YTHprotocols.htm>

http://www.protocol-online.org/prot/Molecular_Biology/Protein/Protein_Interactions/

http://www.ebioinfogen.com/yeast_two.htm

<http://www.clontech.com/archive/JUL96UPD/>

Novel Applications

The yeast two hybrid assay has, since its discovery, evolved past solely the identification and characterization of interactions, and on to very new and exciting applications. Some of these are discussed below.

Interaction Suppression

The interaction suppression assay allows one to evaluate the biological significance of protein interactions. First a screen is performed for mutations which might affect the interaction of the protein of interest (the bait) with its partner. Once a mutant is identified, one can study its manifestation on phenotype. To ensure that the interaction is abolished due to the disruption in binding between the mutant bait and its partner (the hunter) and not some third protein, the second step is to construct a mutant versions of the hunter, able interact with the mutant bait and restore the altered phenotype,

Protease Trap

In the protease trap technique, a functional transcription factor is fused to a domain which prevents it from entering the nucleus, and an orphan protease site is cloned in between the two. In such a way, one can screen for a protease that can cleave the anti-nuclear localization and thus enable nuclear localization, by monitoring the transcription of the reporter gene. This technique may also be applied in screening for target sequences for a known protease, by cloning the random sequences in between the two domains.

Whole Genome Approaches

The two hybrid assay can be used to establish protein linkage maps (PLM) which include all possible protein interactions that occur in the life-time of a cell. In this approach, initially random libraries are fused both to the BD and AD and exhaustively screened for all possible interactions. Nevertheless, problems such as auto-activation become significant when looking at all genomic proteins, and given that false positives or negatives can greatly hinder subsequent analyses, instead of using random exhaustive screens a more laborious route must be taken, whereby double-PCR-in-vivo-cloning is used to ensure that all clones contain full-length ORFs in the correct frame (this process can be automatised to some extent).

Overall

The yeast two hybrid assay is a relatively quick, easy, and flexible technique, which provides a straightforward approach to the study of protein interactions. Furthermore, the article from Fields et al., which first

described the technique, has been cited nearly 2,500 times in magazines such as "Nature" and "Cell", demonstrating its high publication value. Depending on the nature of the proteins and interactions wished to be explored, the two hybrid technique may be an appropriate choice.

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