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A comparison of two hybrid approaches for detecting protein-protein interactions

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Abstract

Two hybrid systems are one of the most popular, preferred, cost effective and scalable *in vivo* genetic approaches for screening protein-protein interactions. A number of variants of yeast and bacterial two hybrid systems exist, rendering them ideal for modern, flexible proteomics-driven studies. For mapping protein interactions at genome scales (that is, constructing an interactome), the yeast two hybrid system has been extensively tested and is preferred over bacterial two hybrid systems, given that users have created more resources such as a variety of vectors and other modifications. Each system has its own advantages and limitations and thus needs to be compared directly. For instance, the bacterial two hybrid method seems a better fit than the yeast two hybrid system to screen membrane associated proteins. In this chapter, we provide detailed protocols for yeast and bacterial two hybrid systems as well as a comparison of outcomes for each approach using our own and published data.

1. Introduction

Most of the key biological processes of the cellular machinery, including transcription, translation, protein quality control, transport, and signaling are dependent on protein-protein interactions (PPIs) that occur either individually or in multiprotein complexes. For example, the 26 S proteasome requires interactions among more than thirty different proteins organized in three sub-complexes. Together, these proteins serve a common set of functions.

The mapping of protein-proteins interactions on a genome or proteome scale remains challenging and has been comprehensively explored only in a few model organisms such as *Escherichia coli*, budding yeast, and humans. Numerous biochemical and genetic approaches have been developed to study protein-protein interactions. This review focuses on The Yeast (Y2H) and Bacterial (B2H) Two Hybrid methods which are among the most explored *in vivo* genetic tools to map PPIs. The full extent of biochemical and genetic approaches in use is beyond the scope of this chapter but several detailed surveys have been published (e.g. PMID: [24393018](#), PMID: [22688816](#), PMID: [24693427](#)).

Both Y2H and B2H are methods that have been used to map and identify PPIs networks in many organisms (Mehla et al., 2015 JB, Yi Wang, 2010). They can be used on a small scale or as high-throughput screening approaches. Two-hybrid methods are based on the principle of restoring protein activity upon non-covalent reconstitution of split protein fragments. One fragment of a modular protein is fused to a protein X, and the other fragment is fused to a

protein Y. The resulting fusion proteins may be referred to as a bait and prey. If the proteins X and Y interact upon co-expression, the modular protein is reconstituted, regains its activity, and its activity is detected through a reporter gene. Although both the B2H and Y2H work on similar principles, there are fundamental differences and both have distinct advantages and limitations. Different users may choose one over another depending on their needs, available resources, properties of the proteins to be screened, and ease of screening. This chapter describes a detailed protocol for high-throughput yeast and bacterial two hybrid methods and a comparison of their use and some typical results.

2. The bacterial two hybrid system

2.1. Overview of the bacterial two hybrid system

The B2H system may be used as an alternative to Y2H screening. The B2H system has been applied to screening of proteins from different sources (bacterial, mammalian, viral), of different size and cellular location (cytoplasmic, peripheral, inner membrane) and of different functions (transporters, enzymes, regulatory proteins, *etc.*). Although we do not know of any systematic comparison of the two systems, the B2H may be preferable to Y2H when screening bacterial proteins as the screening conditions are more biologically similar to the proteins' natural environment.

The most commonly used B2H system is the Bacterial Adenylate Cyclase-based Two-Hybrid System (BACTH) which is based on the reconstitution of adenylate cyclase activity in *Escherichia coli* (Fig 1). The catalytic domain of bacterial adenylate cyclase has two sub-domains: a 25 kDa fragment (T25, or *cyaAT25*) with a catalytic site and a 18 kDa fragment (T18, or *cyaAT18*) with a binding site for calmodulin. These domains are not forming a functional protein when they are physically separated. The test proteins (X and Y) are fused to the T25 and T18 domains, respectively, allowing interactions between them to bring the domains in close proximity and – in the presence of calmodulin – restore adenylate cyclase activity. The restored activity leads to cyclic adenosine monophosphate (cAMP) synthesis. The cAMP produced by the reconstituted adenylate cyclase forms a complex with catabolite activator protein (CAP), inducing the transcriptional activation of catabolic operons such as lactose or maltose and yielding a characteristic phenotype in a *cya- E. coli* strain (usually BTH101/DHM1) on selective or indicator media plates.

2.2. Bacterial two hybrid protocol

2.2.1. Bacterial strains and vectors—Bacterial two hybrid screens as described by Karimova et al. (1998) frequently employ *E. coli* BTH101 and/or DHM1 as reporter strains. Both strains are adenylate cyclase deficient but only the latter of these two strains contains a *recA1* mutation, potentially improving plasmid stability. Store these stocks at -80°C .

Genotypes: BTH101: *F⁻, cya-99, araD139, galE15, galK16, rpsL1, hsdR2, mcrA1, mcrB1*

DHM1: *F⁻, cya-854, recA1, endA1, gyrA96, thi1, hsdR17, spoT1, rfbD1, glnV44 (AS)*

As described above, bacterial two hybrid screens use vectors allowing for expression of hybrid proteins containing either T25 or T18 adenylate cyclase fragments. The vectors

pKT25 (Kan^r, *cyaA*T25 fragment) and pUT18 (Amp^r, *cyaA*T18) are available in several variants (see table 1).

Genes encoding the proteins of interest (X and Y) are amplified by PCR and cloned by traditional or recombination cloning (e.g. using the Gateway system, Hartley et al. 2000). The latter method is preferred for preparation of clone sets as it can be performed with higher cloning efficiency than traditional cloning. With Gateway cloning, the attL-flanked ORFs can be recombined into the attR-flanked BACTH-DEST plasmids (pST25 and pUT18C) using the LR reaction to generate attB-flanked ORFs within the BACTH vectors. Expression clones may be prepared with the vectors pST25, pSTM25, pUT18, and pUTM18, among others. Outer membrane (OM) bound, peripheral or extracytoplasmic proteins should be transferred to pSTM25 or pUTM18 B2H expression vectors. These modified vectors have an extra transmembrane segment fused downstream to T25 and T18 cyclase domains to detect interactions for OM or peripheral proteins (Ouellette et al., 2014). The Gateway vectors should be propagated in *ccdB* resistant *E. coli* strains at 30°C.

2.2.2. Reagents and media—*E. coli* cultures may be grown with LB media. For LB/X-Gal indicator plates, add X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to 40 µg/ml. IPTG may also be included in these plates (at 0.5 mM) to improve protein expression and hence observation of interactions.

MacConkey agar with 1% maltose may also be used for indicator plates.

Where specified, ampicillin (Amp) and spectinomycin (Spc) should each be used at 100 µg/ml.

One liter of Z buffer is prepared as follows:

16.1 g Na₂HPO₄ • 7H₂O (or 8.5 g anhydrous)

5.5 g NaH₂PO₄ • H₂O

0.75 g KCl

0.246 g MgSO₄ • 7H₂O

Add H₂O to the final volume and adjust to a pH of 7 if needed. Store at 4°C.

Add 2.7 µl β-mercaptoethanol (β-ME) per ml of Z buffer immediately before use in an assay. Retain a portion of Z buffer without β-ME added (see review by Battesti and Bouveret 2012 for further details.)

ONPG (2-Nitrophenyl β-D-galactopyranoside) is used to assay β-galactosidase activity and hence the strength of a protein interaction. Solutions may be prepared as 4 mg/ml in Z-buffer and should be prepared fresh on the day of each assay.

This procedure also requires the following solutions: 0.1% SDS, chloroform for cell lysis and 1M Na₂CO₃ to stop the enzymatic reaction.

2.2.3. Bacterial two hybrid procedure—See Fig. 2 for a summary of this procedure.

Co-transformation of expression constructs into reporter strains: Expression constructs of test proteins encoding the T25-X (or X-T25) and T18-Y (or Y-T18) fusions are co-transformed into competent BTH101 *E. coli*. Competent cells can be prepared on the same day as transformation. Routine heat-shock based transformation protocols are ideal, especially for high-throughput screens. See Chung et al. (1989) for a basic protocol.

1. Transfer 30 μ l of competent cells per reaction to pre-chilled microcentrifuge tubes or 96 well plate(s) on ice.
2. Add 100 ng of both expression constructs (both bait and prey) to the cells.
3. Keep cells on ice for at least 30 min. Heat shock at 42°C for 45 s. After the heat shock, return the cells to ice for 5 min.
4. Add 100 μ l of sterile LB broth to each tube or well and incubate at 37°C for 45 to 60 min.
5. Plate the cells on selective agar plates (e.g., LB/Amp/Spc) and incubate at 30°C for 24 to 36 h.

Screening co-transformants for interactions: Select the co-transformants for further screening on indicator plates at 30°C.

Indicator or selective media plates can be used to detect positive interactions between test proteins. The positive interactions should produce specific phenotypes on respective indicator plates, i.e. blue colonies on LB-X-Gal or red on MacConkey-maltose. This may take up to 4 days. If no interaction (negative result) occurs, all colonies should be colorless on indicator plates. This method can be scaled up for screening libraries.

The same batch of cells can be used for both plating on indicator plates and for quantification using the β -galactosidase assay.

Quantification of PPIs with the β -galactosidase assay: The interactions between proteins (X and Y) can be quantified by measuring β -galactosidase activity in liquid cultures using the classical Miller assay (Miller 1972). The β -galactosidase activity is usually expressed in Miller units (one unit corresponds to 1 nmol of ONPG hydrolyzed per min at 28°C) per mg of bacterial dry weight.

This protocol is used to determine the level of β -galactosidase activity in a bacterial two hybrid interaction screen and may be scaled up for high-throughput use in 96 well plate format. The level of galactosidase activity will vary depending on the level of interaction for strongly and weakly interacting proteins. Thus, we can quantify the strength of an interaction between two proteins. The time required for the assay to develop reliable readings/color can vary depending on the strength of the interactions, especially if the interaction is very weak.

Prepare overnight cultures of each co-transformed culture to be assayed. Transfer cultures to a 96 well plate after their overnight growth. Set an incubator or water bath to 30°C.

1. Use 0.1 ml of each overnight culture to determine the absorption at OD₆₀₀ with a plate reader.
2. Collect the cells in the plate by centrifugation at 2,000 to 4,000 rpm in a benchtop centrifuge (at least 1,000 × *g*) for 5 to 10 min.
3. Remove the supernatant and re-suspend each cell pellet in 80 µl of Z-buffer (with β-ME).
4. Add 10 µl of chloroform to each well.
5. Add 10 µl of 0.1% SDS to each well.
6. Vortex each plate, gently, for 30 s.
7. Centrifuge the lysate as in Step 2.
8. Transfer 0.1 ml of each lysate to a clean 96 well plate. Ensure each lysate is clear and that no turbidity is transferred to the new plate.
9. Pre-warm the ONPG solution to 30°C. The plate can also be pre-incubated at 30°C for 5 min to equilibrate the temperature of the suspension.
10. Start the reaction by adding 20 µl of ONPG (4 mg/ml) to each well. Record the start time.
11. Monitor the reaction at 30°C until you see a yellow color (similar to that of LB media) develop or the reaction has run for 90 to 120 min.
12. Stop the reaction by adding 30 µl of a 1M Na₂CO₃ solution. Record the reaction stop time.
13. Determine the OD₄₂₀ Abs of each well using a plate reader.
14. Convert the OD₄₂₀ into Miller units (see Miller 1972) using the following conversion:

$$\text{Miller Units} = 1000 * (\text{OD}_{420} / (\text{OD}_{600} \text{ of culture} * \text{culture volume} * \text{reaction time (min)}))$$

3. The yeast two hybrid system

3.1. Overview of the yeast two hybrid system

The Y2H assay is an *in vivo* genetic method developed by Fields and Song (1989) to screen for binary protein-protein interactions. In this method, the yeast transcription factor Gal4 is split into a DNA-binding domain (DBD) and an activation domain (AD). Protein pairs are fused to the DBD and AD and the resulting DBD-X and AD-Y fusions are generally referred to as bait and prey. Only the simultaneous co-expression of both the bait and an interacting prey will reconstitute the full transcription factor and permit transcription (Fig. 3).

The subjects of a two hybrid screen may be random libraries, such as fragments of genomic or cDNA, or defined clone sets, usually in array format. Arrays are preferred as they are easily scalable (i.e., a full 96 well plate of expression constructs can be screened against one protein of interest on a single plate) and do not require sequencing to verify the identity of each interactor. Using arrays may also help to decrease the incidence of false positive results.

3.2. Yeast two hybrid protocol

3.2.1. Yeast strains and vectors—The following protocol uses the yeast strains AH109 (for baits) and Y187 (for preys) for high-throughput yeast two hybrid screens. These strains are frequently used and grow consistently, especially when provided with supplemental adenine in media (up to 100 mg/L).

Genotypes: *AH109: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4⁺, gal80⁻, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ* (James et al. 1996).

Y187: MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4⁺, met⁻, gal80⁻, URA3::GAL1UAS-GAL1TATA-lacZ (Harper et al. 1993).

3.2.2. Reagents and media—The selection mechanism used for diploid cultures in yeast two hybrid screens will depend upon the strains and vectors in use. Many methods employ nutritional selection, i.e. if the two yeast strains are auxotrophs for leucine and tryptophan, the yeast two hybrid vectors must contain complementing genes, e.g. LEU2 and TRP1, and diploid cultures must be isolated on media lacking both amino acids. For yeast two hybrid screens using HIS3 as a reporter gene, selective yeast media may be supplemented with 3-AT (3-amino-1,2,4-triazole) at concentrations between 1 mM and 50 mM. 3-AT acts as a competitive inhibitor of the HIS3 enzyme (imidazoleglycerol-phosphate dehydratase) which will therefore reduce background growth, allowing for easier distinction of positive colonies from background.

Selective solid yeast medium, in single well plates (e.g. Nunc Omnitrays) (including –L, –T, –LT, –LTH media)

10X TE buffer (0.1 M Tris-HCl, 10 mM EDTA), pH 7.5

YEPDA medium is prepared as:

Yeast extract	1%
Peptone	2%
Dextrose	2%
Adenine hemisulfate salt	0.01%

Sterilize by autoclaving. For solid medium, include 1.6% agar and pour 40–50 mL of sterile medium into each single-well Omnitray plate. Allow to solidify.

2.2.3. Yeast two hybrid procedure—See Fig. 4 for a summary of this procedure.

Materials: These following methods have been designed to be performed using 96-well plates (or 96-colony plates). The protocol may be used with manual colony transfer or in high-throughput with the aid of a laboratory automation system, such as a Beckman Coulter Biomek 2000, FX or FXP, along with a high density plate replicator attachment (e.g. 96/384 HDR Tool Body, V&P Scientific) and compatible pins (“thin” pins of 0.381 to 0.457 mm or “thick” pins of 1.143 to 1.58 mm in diameter). Plates may also be replicated manually using a 96-pin or 384-pin plate replicator. Solid agar media should be prepared in single-well 86 × 128 mm Omnitray microtiter plates (Thermo Fisher/Nunc).

Plate replicator pins must be sterilized between each pinning or they may introduce cross-contamination. Rinse the tips of each pin in 20% bleach (60 s), sterile dH₂O (20 s), ethanol (95% v/v; 60 s), and sterile dH₂O (20 s).

Screening Protein Interactions Using Genomic or cDNA Libraries: Two-hybrid methods traditionally define screened fusion proteins as baits and preys. This distinction is ensuring that the two vectors contain different genes for selection. Otherwise, nearly any protein may be used as a bait or prey.

Some ORF collections and libraries provide sequences (including, in some cases, nearly complete genomes) in two hybrid vectors. These vectors may require subcloning or a recombination-based cloning procedure (e.g. Gateway cloning) to produce a full set of expression vectors for two hybrid screens. ORFeomes are available for viral genomes (e.g., herpesviruses; Uetz et al. 2006), several bacterial genomes (e.g., *E. coli*; Rajagopala et al. 2010), and some eukaryote species (e.g. *C. elegans* (Lamesch et al. 2004), human (Rual et al. 2004), and *Arabidopsis thaliana* (Gong et al. 2004).

If a bait protein is screened against a *random* genomic (or cDNA) library of prey proteins in a library screen identification of the interacting prey proteins requires isolation and sequencing of positive clones. For further technical details, see Mehla et al. (2015).

Preparations and array construction: Arrays have certain advantages for Y2H screens, especially to validate the positive interactions in a library screen (Mehla et al., 2015 A Tool for Mapping Protein–Protein Interactions). Because certain baits may self-activate (i.e. produce positives without an interactions), usually the preys are arrayed in a defined order before the actual Y2H screen or retest. Prey proteins usually do not generally produce self-activation without a bait protein.

For genome-wide screens, automation is strongly recommended, given that manual work is not only straining, and error prone, but also time consuming and tedious. However, automation can be an expensive option. Collaborating with a lab already in possession of automation equipment may be an alternative.

The ORFs from entry vectors are assembled into Gateway-compatible prey or bait vectors by Gateway cloning. The Gateway-compatible bait and prey vectors are shown in table 1.

The prey and bait vectors are transformed into mating-competent, haploid yeast strains Y187 (mating type 'α') and AH109 (mating type 'a'), respectively.

Yeast transformation: This method is optimized for high-throughput transformation of bait or prey plasmid clones into respective yeast strains and can be scaled up and down as required.

- 1 Inoculate 10–20 ml of YEPDA with the haploid yeast strain and grow overnight in a shaking incubator (approx. 200 rpm) at 30°C.
- 2 Dilute the cells in 100 ml (or as required) of fresh YEPDA to an OD₆₀₀ of about 0.2 to 0.3. Grow cells for approximately 3 to 4 hours at 30°C with shaking. OD₆₀₀ should be between 0.5 and 0.8 (or, at least one doubling) such that the cells are in log phase of growth.
- 3 Centrifuge the culture in 50 ml sterile tubes and wash them with 25 to 50 ml sterile dH₂O
- 5 Then resuspend the cells in 1 ml of freshly-made 0.1M LiAc/TE and incubate on ice for 15 min followed resuspending again in 1 ml of 0.1M LiAc/TE in 1.5 ml microcentrifuge tube on ice for another 15–20 minutes.

Prepare fresh 0.1M LiAc/TE (1 ml of 1.0M LiAc + 1 ml 10X TE + 8 ml of dH₂O).
- 6 Prepare a transformation mix in a sterile 50 ml centrifuge tube (for 100 transformation reactions) as: 10 ml of 40% PEG (10 ml of 44% PEG + 1 ml of 0.1M LiAc/TE), 250 µl of 10 mg/ml carrier DNA in a 50 ml tube and vortex vigorously followed by 1 to 2 ml of freshly prepared competent yeast cells (previous step). Vortex again for 60 sec.
- 7 Add 100 µl of transformation mix into a 96-well plate using a multichannel pipette or robotic liquid handler. The volume in each well should not exceed 120 µl per well.

For fewer samples, 1.5 ml centrifuge tubes may be used.
- 8 Then add 100 ng of plasmid DNA to each well and mix well with pipette tip. Include one negative (carrier DNA but no plasmid DNA) and positive controls (containing empty vector).
- 9 Seal the 96-well plate with adhesive aluminium foil and Parafilm to secure edges and protect against contamination.
- 10 Vortex the plate gently for 3 to 4 minutes and incubate at 30°C for 45 minutes with gentle shaking (approx. 60 rpm).

Make sure there is no cross-well contamination.
- 12 Then, incubate the plate at 42°C in a water bath for 30 min. Ensure that water is not entering the wells.

For transformation in microcentrifuge tubes, the incubation time at 42°C incubation, may be reduced to 15 min.

- 13 Then, centrifuge the 96 well plate for 8–10 minutes (~700×g, RT.). Discard the supernatant either by pipette or by inverting the plate carefully.
- 14 Add 50 to 100 µl sterile dH₂O to each well and spread it on selective media plates. Make sure the plates are dry
- 15 Incubate plates at 30°C for at least 2 to 3 days.

Strains, such as Y187, may require 3 to 4 days to yield noticeable transformant growth. The specific transformants (each carrying one specific prey vector), are arrayed on selective media plates in a 96- or 384-format in duplicates or quadruplicates. For freeze stocks, the arrays may be cultured in rich (YEPDA) or selective liquid media, resuspended in 20% glycerol, and stored at –80°C for long-term storage. For routine use, the arrays can be stored on solid selective medium for up to 3 months at 4°C and fresh arrays can be propagated using storage plates prior to Y2H screen.

For long term storage, the master prey array should be kept on selective plates (e.g -Leu plates). The master copy of the array should only be used to make fresh copies on YEPDA agar plates for mating. Copying the array onto fresh selective plates every 2 weeks should protect against plasmid loss and contamination.

Bait auto activation test: The bait auto activation test is a prerequisite prior to Y2H screen and is performed to measure the background potential of baits to activate transcription of the reporter gene (here: HIS3) in the absence of prey protein (or presence of empty prey vector). This will help to optimize actual Y2H screen conditions, saving time and resources.

1. Plate bait clones on YEPDA media or selective media in a single-well Omnitray or Petri plate in a standard 96 or 384-spot format depending on the number of clones.
2. The bait strains are mated with a prey strain carrying the prey empty vector (i.e., Y187 strain with pGADCg empty vector) on a YEPDA plate at 30°C.
3. Select diploid cells on appropriate selective media (i.e., -LT, see above) at 30°C.
4. Transfer the diploid colonies to selective media (i.e., -LTH, see above) with and without 3-AT for selecting the *HIS3* reporter gene activity. 3-AT concentrations of 0, 1, 3, 5, 10, and 25.mM are recommended for an initial screen. Almost all background should be gone within this range. However, in special circumstances concentrations up to 100 mM may be required.
5. Incubate the selective plates at 30°C for about 1 week. Record the lowest 3-AT concentration suppressing background growth for each bait and should be added to selective plates in the actual Y2H screens.

Screening for protein interactions: After the bait self-activation test, the actual Y2H screens are performed as described below.

1. Use the sterile pin replicator to transfer the yeast prey array from selective plates to single-well microtiter plates containing solid YEPDA medium and grow the array overnight in a 30°C incubator. Alternatively, the fresh prey array can be made in YEPDA broth in a 96 well plate.
2. Inoculate 15–20 ml of YEPDA broth medium in a 50-ml sterile tube with a bait strain, overnight in a shaking incubator (at ~200 rpm) at 30°C.

If the bait strains are frozen, they should be activated prior to experiment by pinning them on rich media and followed by pinning on selective solid medium plates. Overnight grown baits and preys culture may increase mating efficiency.
3. Pin the overnight bait cells onto solid YEPDA media in an Omnitray plate by dipping the sterile pins in bait liquid culture. Replicate as required and allow the spots to dry for 15 – 20 min.

YEPDA plates should be free of any moisture before pinning cells.
4. Transfer overnight prey array cells onto bait spots. Touch the prey cells with sterilized pins and transfer them onto the plated bait spots. Incubate for 36–48 hours at 30°C to allow mating.

Addition of adenine in the culture media may increase the mating efficiency of some baits.
5. Select diploid cells by transferring the colonies from mating plates to double-dropout selective medium plates (i.e., –LT) using the sterilized pinning tool. Grow the cells at 30°C for 2–3 days or until the colonies are ~1 mm in diameter.

Only the diploid cells containing both the prey and bait vectors will grow on -LT plates. Low mating efficiency will reduce the quality and coverage of Y2H screen results.
6. Transfer the colonies from -LT plates to screening media plates (i.e., –LTH, with or without 3-AT) using the sterilized pinning tool. The required concentration of 3-AT will depend on the bait auto-activation test (see steps 16 through 20). Incubate the plates at 30°C for 4 to 7 days monitoring every day.
7. Score the positive interactions by counting colonies that are significantly large in size compared to background.

The screening plates should be monitored daily for positive colonies. Typical positive interactions should appear within 3 to 5 days.

Retesting of positive interactions: The main concern associated with the Y2H screen is the possibility of a large number of false positives. The use of different vectors and retesting of all the positive interactions through repeated mating can help to reduce the number of false positives. Only the reproducible interactions in retest Y2H screens will be counted as positive interactions. Moreover, screening the same baits and preys using multiple vectors provides additional evidence for positive interactions. Alternative methods are also available

for confirmation of Y2H interactions (for protocols see Serebriiskii et al. 2005; Rajagopala et al 2007).

4. Comparison of yeast and bacterial two hybrid methods

4.1. Comparison of method usage

The Y2H screens can capture only direct pairwise interactions, including weak interactions. Y2H screens are a heterologous system for bacterial proteins which comes with some limitations. For example, non-physiological level of protein expression, absence of necessary cofactors, ligands or chaperones for functional activity and issues with the translocation of bacterial proteins to the yeast nucleus. This leads to low sensitivity and coverage and up to 70% of PPIs are not detected even in comprehensive screens. The specificity of the Y2H screens is enhanced with the advancement of multi vector and next generation Y2H screens for mapping membrane interactomes (Stellberger et al. 2010, PMID: 22610515).

The B2H methods are developed to be performed in their natural host and thus can detect both direct and indirect protein interactions. The adenylyl cyclase based B2H is the most commonly used B2H technique and is suitable for detecting interactions between cytoplasmic, membrane proteins and periplasmic proteins. Most of the B2H studies are performed at small scale. Traditionally, the yeast two hybrid (Y2H) system has been the preferred choice over B2H, for detecting protein–protein interactions on genome wide scale. However, there is no objective reason why the B2H should not be applicable on a large-scale.

Two hybrid methods are popular fixtures in the biomedical literature, as a chronological search of PubMed reveals (Fig. 5). Here, we show the total number of publications per year containing the phrase “two hybrid” anywhere in their title or abstract. At its peak in the early 2000’s, two hybrid methods were highlighted in more than 900 publications each year, with yeast two hybrid approaches providing the majority of those methods. Bacterial two hybrid methods have been comparatively less popular but have been modified to be used in concert with other methods (Serebriiskii et al. 2005) or to more easily confirm expression of the desired fusion proteins (Battesti and Bouveret 2008). Some of the difference in popularity between yeast and bacterial two hybrid methods may simply be due to the time since their introduction: Karimova et al. did not publish details of their bacterial two hybrid method until 1998, nearly a decade after Fields and Song described Y2H (see Fig. 6 for counts of citations per year to this paper alone). It should also be noted that two hybrid methods of any type may not always serve as a project’s primary data source: 370 publications from 2015 in PubMed included bacterial two hybrid methods though just 40 in the same year mentioned the method in their title or abstract. Some publications may use two hybrid approaches to generate further experimental evidence and may therefore avoid discussing the method in detail. Though perhaps increasingly seen as a basis for new approaches rather than a core methodology, two hybrid methods remain prominent among protein-protein interaction screens.

Likely owing to their popularity and ease of implementation, two hybrid methods have not only remained popular approaches but have contributed more than a third of all interactions in the IntAct molecular interaction database (Fig. 7). These counts include all interactions in the database – even if the interaction was studied by more than one group or with more than one method – and therefore serve as a summary of publicly available interaction data and the methods responsible. For some species, two hybrid methods are especially popular: more than half of the protein-protein interactions in IntAct among bacterial proteins were identified using two hybrid approaches.

4.3. Experimental comparison of bacterial and yeast two hybrid

The main concerns of two hybrid methods are the rate of false positives and the bait self-activation especially for Y2H screens. Despite its extensive use, two hybrid methods have rarely been explored simultaneously or in parallel. To better understand the experimental outcomes of B2H and Y2H methods, an extensive study should be done to compare different types of vector, types of proteins and parameters affected by the host. The types of proteins may be further classified in terms of their functions and location. More extensive studies are required to comprehend the outcomes and suitability of two hybrid methods. We tried to get an idea of the outcomes of B2H and Y2H screens by screening the same set of interacting proteins by both B2H and Y2H (Fig. 8). A protein “X” was tested against a set of 12 prey partners by both methods. Few positive interactions are captured in our B2H screens, however not even a single interaction was detected by Y2H method. There can be several reasons for this result. The most plausible explanation is that the fusion proteins in both systems are sufficiently different to allow an interaction in one system but not the other. Rajagopala et al. (2009) as well as Chen et al. (2010) have shown that small differences even within the Y2H can cause substantial outcomes in the set of detected PPIs. Given that only a fraction of all interactions are detected in each screen, this explains even non-overlapping resulting PPIs in different screens. Most of the proteins selected in our example are membrane proteins which is another confounding reason that not a single interaction was captured in Y2H screen. Also, the function of the interacting proteins might have some implications. For instance, directly or indirectly, the selected proteins are predicted to be involved in the LPS assembly or outer membrane asymmetry in bacteria and thus could lead to lower mating efficiency and even may cause toxicity in the yeast cells. The interactions detected by Y2H screens took place in the nucleus and thus the membrane or outer membrane proteins which are having hydrophobic transmembrane region/domains could not be able to reach the nucleus and ultimately no interaction (Auerbach and Stagljar 2005). In B2H screens, the interactions are not restricted to the nucleus and only the protein complementation is required to detect the positive interactions. That could give the B2H screens a certain advantage for screening inner membrane (or even peripheral membrane) proteins unless a specialized membrane yeast two hybrid assay is used. That is why the interactions shown here are captured only by B2H screens.

Also, the proper folding of the membrane proteins might need the phospholipids bilayers and thus can lead to false negatives or to no interaction in Y2H screens. These limitations could be overcome by screening only the cytoplasmic domain of the membrane proteins or using a low copy number plasmid to avoid any toxicity (if any). Not only the localization of

the proteins causes false negatives, also the specific condition (e.g oxidative, cofactors etc.) or posttranslational modification required for the proper folding/activity of the proteins can lead to higher rate of false negatives. Another reason for higher false negatives in Y2H screens, could be the toxicity of interacting proteins upon overexpression (Brückner et al. 2009).

4.4. Comparison of method strengths and weaknesses

Affinity purification coupled with mass spectrometry (AP-MS) is the most popular biochemical method for detecting PPIs. In AP/MS, the tagged bait proteins are used to pull out interacting prey proteins. The two hybrid methods are widely used and have produced large number of interactions available in different databases (Fig. 7). Two hybrid methods are preferred because of following:

1. **Accessibility:** Two hybrid methods can be carried out in a lab with basic and inexpensive microbiological reagents and tools. They do not require any specific equipment unless being carried out on a large scale when expensive robots may be required.
2. **Type of interactions:** Two hybrid methods detect the in vivo binary interactions between individual proteins. The interacting proteins may not be from the same organism. They can be from two different bacteria or a bacterium and its phage, etc. Entire proteins, protein domains or protein fragments can be used for screening interactions. There is no size limit for proteins being screened.
3. **Sensitivity, scalability and coverage:** The assay is highly sensitive. Even transient and weak interactions can be detected using two hybrid methods. Two hybrid methods are easy to scale up and down.

However, several features of two hybrid assay impose limitations on the type and nature of interactions that can be analyzed.

1. **False positives and self-activation of baits.** Potentially high rate of false positives is one of the major drawbacks of two hybrid assays. However, false positives can be reduced by including stringent experimental conditions (e.g. higher 3-AT concentrations, using different vector combinations) and by filtering the resulting dataset (e.g. biological relevance or plausibility of interactions). Another related problem is self-activating baits. Self-activating baits activate the reporter gene without interacting with the prey protein. The bait self-activation consequences are not typically associated with B2H screening.
2. **Lack of detection of interactions for certain proteins.** Proteins that cannot fold intracellularly (e.g. membrane proteins) or cannot enter the nucleus cannot be studied using transcription-based assays. Protein fusions that are either toxic to or unstable in yeast (Y2H) or *Escherichia coli* (B2H) cannot be screened using two hybrid methods. In general, all interactions dependent on posttranslational events that do not occur in yeast (Y2H) or *Escherichia coli* (B2H) will not be detected. Two hybrid systems have transcription factor domains fused either to

the N or C terminus of proteins which may block interactions dependent on a free N or C terminus.

5. Conclusion

No single two hybrid method is best suitable for every individual or lab. The selection of methods for mapping protein-protein interactions is dependent on the needs, and resources availability. A comprehensive analysis of these methods in terms of nature of proteins, host and methodology, might help in planning and choosing a better approach for mapping protein-protein interactions.

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Principle of the Bacterial Two Hybrid System

Figure 1. Principle of the bacterial two hybrid system

The T18 and T25 domains of adenylate cyclase are shown in different conditions. The intact adenylate cyclase domains produce residual cAMP synthesis. The segregated T18 and T25 domains do not produce cAMP, and hence there is no observed phenotypic change. The interacting proteins X and Y can bring together T18 and T25 domains of adenylate cyclase in close proximity and restore cAMP synthesis, leading to characteristic phenotypes on indicator plates.

Figure 2. Stepwise overview of the bacterial two hybrid procedure

The test fusion proteins X (bait) and Y (prey) are constructed and cotransformed in the *E. coli* BTH101 strain. The interaction between X and Y can restore the cAMP synthesis, leading to the observed phenotypes on indicator plates: for example, red and blue colonies on MacConkey/maltose and LB/X-Gal plates, respectively.

Principle of the Yeast Two Hybrid System

Figure 3. Principle of the yeast two hybrid system

The segregation of Gal4 DNA-binding domain (DBD) and activation domain (AD) prevents transcription of the reporter gene, leading to a lack of colony growth. The interaction between bait and prey proteins brings together the Gal4 DBD and AD and reconstitutes the transcription factor. This leads to transcription of a reporter gene (i.e., *HIS3*) and results in yeast colony growth.

Figure 4. Stepwise overview of the yeast two hybrid procedure

The test protein constructs (bait and prey) are transformed into haploid yeast strains of opposite mating type (a and α). The mating of two haploid strains carrying bait and prey plasmids produce the diploid cells. Diploid cells are selected by transferring the mated array to double-dropout (e.g., -LT) medium followed by selection of positive interaction by transferring cells to triple-dropout (e.g., -LTH) medium.

Figure 5. Count of publications in PubMed featuring two hybrid methods

Counts include all publications including the phrases shown in the key at right in their title or abstract. Counts made in July 2016; year to date values for 2016 are shown at far right.

Figure 6. Count of citations to Fields and Song (1989)

Counts include all citations to the Fields and Song paper in *Nature* describing their yeast two hybrid system as of July 2016. Citation counts are from Web of Science; the publication has been cited 4,169 times in total by this metric.

1 square is 10,000 interactions.

Figure 7. Count of interactions in IntAct by identification method

Counts include all interactions in the IntAct database of protein interactions as of August 2016. “Two hybrid interactions” include those identified with any two hybrid, two hybrid array, or two hybrid pooling method and all variations. “Pull down interactions” are those identified with any pull down method, not including method variations. Each square represents 10,000 interactions.

Figure 8. Experimental examples and comparison of results from yeast and bacterial two hybrid screens

A representative high throughput B2H screen is shown here (only a part of 96 well plate). The blue and red color colonies on LB/X-gal and McConkey/Maltose indicator plates are considered as positive interactions (A). The same set of test protein pairs were tested for interactions by both B2H and Y2H methods (B and C). The red color and visible growth of colonies are considered as positives in B2H and Y2H screens, respectively. Few positive

interactions are detected in the B2H screens. However, no single interaction was captured in the Y2H screens as discussed in the text above.

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Table 1

Vectors frequently used in two hybrid screens.

Vector	Pro-moter	Fusion Protein	Fusion		Selection		Ori	Source
			DBD/ T18	AD/ T25	Yeast	Bacterial		
Y2H Bait vectors								
pGBKCg	t-ADH1	Gal4	C	-	Trp1	Kanamycin	2μ	Stellberger
pGBGT7g	t-ADH1	Gal4	N	-	Trp1	Gentamycin	2μ	Rajagopala
Y2H prey vectors								
pGADCg	fl-ADH1	Gal4	-	C	Leu2	Ampicillin	2μ	Stellberger
pGADT7g	fl-ADH1	Gal4	-	N	Leu2	Ampicillin	2μ	Chen <i>et al</i>
B2H vectors								
pKT25	pLac	AC	-	N	-	Kanamycin	p15A	Karimova
pKNT25	pLac	AC	-	C	-	Kanamycin	p15A	Karimova
pUT18C	pLac	AC	C	-	-	Ampicillin	ColE1	Karimova
pUT18	pLac	AC	N	-	-	Ampicillin	ColE1	Karimova
pST25	pLac	AC	-	N	-	Spectinomycin	p15A	Ouel., Bat.
pSNT25	pLac	AC	-	C	-	Spectinomycin	p15A	Ouel., Bat.
pSTM25	pLac	AC	-	N	-	Spectinomycin	p15A	Ouel., Bat.
pUTM18C	pLac	AC	C	-	-	Ampicillin	ColE1	Ouel., Bat.
Gateway-compatible vectors								
pST25-DEST	pLac	AC	-	N	-	Spectinomycin	p15A	Ouel., Bat.
pSNT25-DEST	pLac	AC	-	C	-	Spectinomycin	p15A	Ouel., Bat.
pUT18C-DEST	pLac	AC	C	-	-	Ampicillin	ColE1	Ouel., Bat.
pSTM25-DEST	pLac	AC	-	N	-	Spectinomycin	p15A	Ouel., Bat.
pUTM18C-DEST	pLac	AC	C	-	-	Ampicillin	ColE1	Ouel., Bat.

Notes: Promoter promoter for fusion protein: t = truncated, fl = full-length, ADH1 = alcohol dehydrogenase, AC = adenylate cyclase doain, Fusion proteins N = N-terminal, C = C-terminal, Ori = origin of replication; sources Stellberger et al., Rajagopala et al., Karimova et al., Ouel. = Ouellette et al., Bat. = Battesti et al. (see references).