Chapter 1

Analysis of Protein–Protein Interactions Using High-Throughput Yeast Two-Hybrid Screens

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Abstract

The yeast two-hybrid (Y2H) system is a powerful tool to identify binary protein–protein interactions. Here, we describe array-based two-hybrid methods that use defined libraries of open reading frames (ORFs) and pooled prey library screenings that use random genomic or cDNA libraries. The array-based Y2H system is well-suited for interactome studies of existing ORFeomes or subsets thereof, preferentially in a recombination-based cloning system. Array-based Y2H screens efficiently reduce false positives by using built-in controls, retesting, and evaluation of background activation. Hands-on time and the amount of used resources grow exponentially with the number of tested proteins; this is a disadvantage for large genome sizes. For large genomes, random library screen may be more efficient in terms of time and resources, but not as comprehensive as array screens, and it requires significant sequencing capacity. Furthermore, multiple variants of the Y2H vector systems detect markedly different subsets of interactions in the same interactome. Hence, only multiple variations of the Y2H systems ensure comprehensive coverage of an interactome.

Key words: Yeast two-hybrid system, Protein–protein interactions, Two-hybrid array

1. Introduction

Specific interactions between proteins form the basis of most biological processes. Comprehensive analysis of protein–protein interactions on a genome scale is a challenging task of proteomics and has been best explored in budding yeast, human, and other model organisms. Protein interactome analysis on a genome scale was first achieved by using yeast two-hybrid (Y2H) screens (1) and next by large-scale mass spectrometric analysis of affinity-purified protein complexes (2, 3). The Y2H system is a genetic method that detects binary protein–protein interactions in vivo. Classical two-hybrid screens used random libraries (genomic or cDNA) to identify

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novel interactions for a protein of interest. However, more recently, an array-based variation of this original principle has been increasingly used (Fig. 1). This approach can be applied not only to a few proteins, but also to whole genomes. Advantages of arrays are their built-in controls and their systematic nature. However, random library screens are more efficient for large genomes, but not as comprehensive as array screens and may yield more false negatives. Here, we describe the protocols for the array-based and random library two-hybrid screens.

1.1. The Principle of the Yeast Two-Hybrid System

The Y2H system is a genetic method extensively used to detect binary protein–protein interactions in vivo (in yeast cells). The system was developed by Stanley Fields (4) based on the observation that protein domains can be separated and recombined and can retain their properties. In particular, transcription factors can frequently be split into the DNA-binding domain (DBD) and activation domains (ADs). In the two-hybrid system, a DNA-binding domain (e.g., from the yeast Gal4 protein) is fused to a protein "B" (for bait) for which one wants to find interacting partners (Fig. 1). A transcriptional activation domain is then fused to some or all the predicted open reading frames (ORFs or "preys") of an organism. Bait and prey fusion proteins are then coexpressed in the same yeast cell. Usually, both protein fusions are expressed from plasmids that can be manipulated easily and then transformed into yeast cells. If the bait and prey proteins interact, a transcription factor is reconstituted which in turn activates one or more reporter genes. The expression of the reporter gene(s) allows the cell to grow only under certain conditions. For example, the HIS3 reporter encodes imidazoleglycerolphosphate (IGP) dehydratase, a critical enzyme in histidine biosynthesis. In the Y2H screening strain (lacking an endogenous copy of HIS3), expression of a HIS3 reporter gene is driven by a promoter that contains a Gal4p-binding site so that the bait protein fusion can bind to it. However, since the bait fusion should not contain a transcriptional activation domain, it remains inactive. If a protein ORF with an attached activation domain binds to the bait, this activation domain can recruit the basal transcription machinery, and expression of the reporter gene ensues. These cells can now grow in the absence of histidine because they can synthesize their own.

1.2. Applications

Originally, the two-hybrid system was invented to demonstrate the association of two proteins (4). Later, it was demonstrated that completely new protein interactions can be identified with this system, even when there are no candidates for an interaction with a given bait. Over time, it has become clear that the ability to conveniently perform unbiased library screens is the most powerful application of the system. With whole-genome arrays, such unbiased screens can be expanded to complete nonredundant sets of proteins. Arrays, like traditional two-hybrid screens, can also be

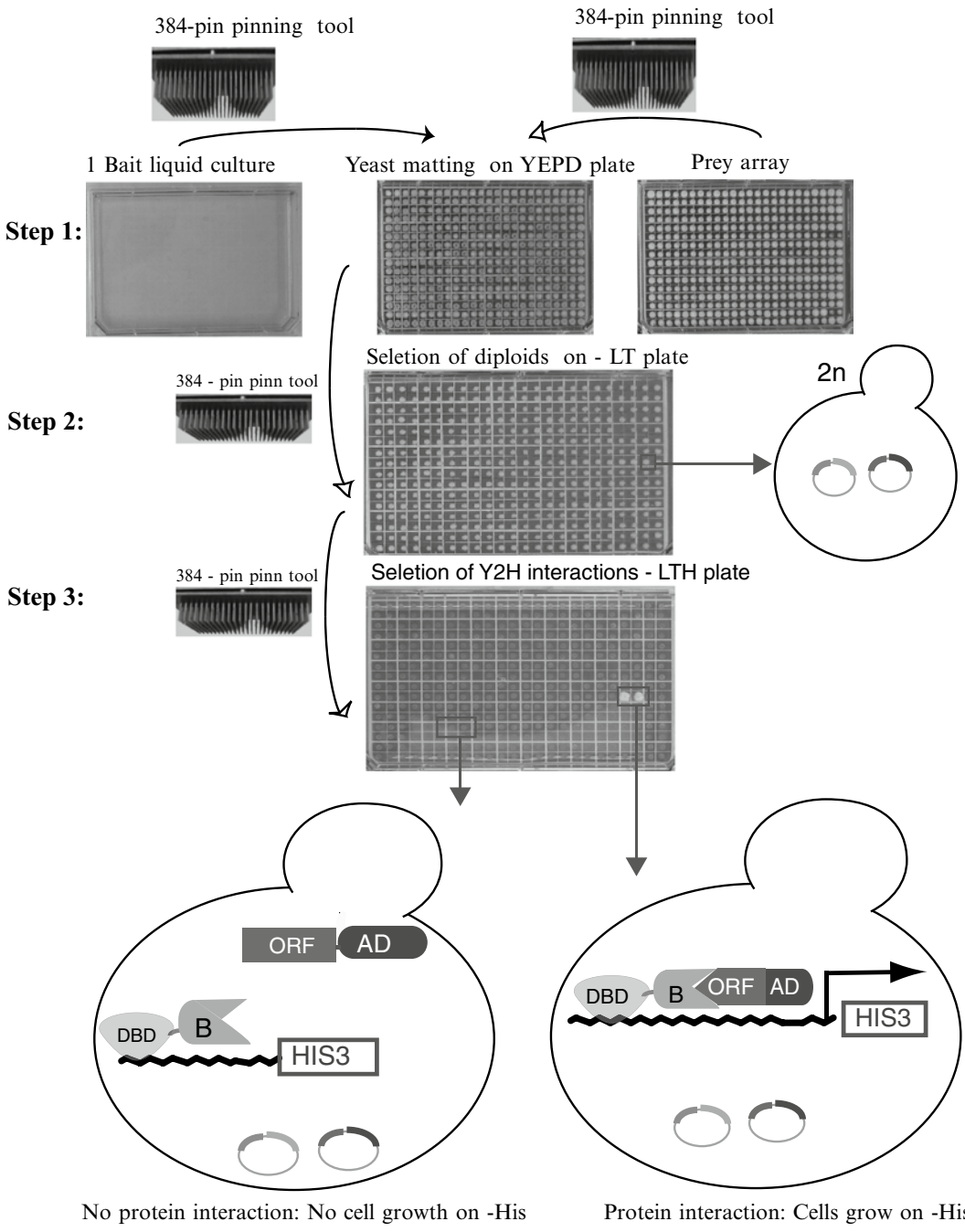

Fig. 1. Scheme of an array-based two-hybrid screen. *Step 1*: Yeast mating combines the bait and prey plasmids. First, the bait (DNA-binding domain (DBD) fusion) liquid culture is pinned onto YEPDA agar plates using a 384-pin pinning tool, and then the prey array (activation domain (AD) fusion) is pinned on top of the baits using the sterile pinning tool. Then, the mating plates are incubated at 30°C for 16 h. *Step 2*: The yeast-mating plates are pinned onto –Leu –Trp medium plates using a sterile 384-pin pinning tool. On –LT plates, only diploid cells grow. Selection on –LT media ensures that both the prey and bait plasmids are combined in the diploid yeast cells. *Step 3*: The diploid cells are pinned onto –Leu –Trp –His medium plates for protein interaction detection. Only if bait and prey proteins interact, an active transcription factor is reconstituted and transcription of a reporter gene is activated (*lower right panel*). The *rectangles* on the selective plate mark negative and positive interactions between the bait and the prey at these specific positions of the array (test is done in duplicates). After Uetz et al. (1).

adapted to a variety of related questions, such as the identification of mutants that prevent or allow interactions (5), screening for drugs that affect protein interactions (6, 7), identification of RNA-binding proteins (8), or semiquantitative determination of binding affinities (9). The system can also be exploited to map binding domains (10), to study protein folding (11), or to map interactions within a protein complex (proteasome (12), flagellum (13)). Finally, recent large-scale projects have been successful in systematically mapping interactions within whole proteomes or subsets thereof (yeast (1, 14); worm (15); fly (16); human (17, 18)). These studies have shown for the first time that most proteins in a cell are actually connected to each other (19).

In combination with structural genomics, gene expression data, and metabolic profiling, the enormous amount of data in these interaction networks should allow us eventually to model complex biological phenomena in molecular detail. An ultimate goal of this work is to understand the interplay of DNA, RNA, and proteins, together with small molecules, in a dynamic and realistic way.

1.3. Array-Based Screens

In an array, a number of defined prey proteins are tested for interactions with a bait protein (Fig. 1). Usually, the bait protein is expressed in one yeast strain and the prey is expressed in another yeast strain of different mating type. The two strains are then mated so that the two proteins are expressed in the resulting diploid cell (Fig. 1). The assays are done side-by-side under identical conditions, so they can be well-controlled, i.e., compared. As the identity of the preys is usually known, no sequencing is required after positives have been identified. However, the prey clones need to be obtained or made upfront. This can be done for a few genes or for a whole genome, e.g., an ORFeome (i.e., all ORFs of a genome).

In an array, each element has a known identity, and therefore it is immediately clear which two proteins are interacting when positives are selected. In addition, it is often immediately clear if an interaction is stronger than another one (but see below). Most importantly, since all these assays are done in an ordered array, background signals can be easily distinguished from true signals (Fig. 1, step 3). Until recently, it was much easier to construct a random library and screen it rather than to construct many individual clones and screen them individually. However, now whole genomes become increasingly available as ordered clone sets in a variety of vectors. Modern cloning systems also allow direct transfer of entry clones into many specialized vectors (20). For most model organisms, such genome-scale clone collections are already available (e.g., 21) or will be soon. One of the first applications of such clone collections is often a protein interaction screen.

In fact, in some cases, only an array screen may do the job. For example, if you have a bait protein that activates transcription on its own, a carefully controlled array may be the only way to

distinguish between signal and background (see Fig. 1, step 3). Similarly, weak interactors may be detectable only when compared with a uniformly weak or no background.

1.4. Pooled Array Screening

A completely different screening strategy, the pooling strategy, has the potential to accelerate screening significantly, but might also have the disadvantage of increasing the number of false negatives. This may have been a reason why pooled screens in *Campylobacter jejuni* resulted in more false negatives than in one-by-one screens of *Treponema pallidum* (13, 22).

In the pooled array screening, preys of known identity (systematically cloned or sequenced cDNA library clones) are combined and tested as pools against bait strains. The identification of the interacting protein pair commonly requires either sequencing or retesting of all members of the respective pool. Zhong et al. established a method which allows for pooling up to 96 preys (23). It was estimated that this pooling scheme reduces the number of interaction tests required to 1/8–1/24 in the case of the yeast proteome. Two recent large-scale interaction mapping approaches for human proteins employed such a pooling strategy: Rual et al. tested baits against pools of 188 preys and identified individual interactions by sequencing (17); Stelzl et al. tested pools of 8 baits against a systematic library of individual preys and identified interactions by a second interaction mating (18). Recently, a "smart-pool-array" system was proposed, which allows the deconvolution of the interacting pairs through the definition of overlapping bait pools (24), and thus usually does not depends on sequencing or a second pair-wise mating procedure.

1.5. Random Library Screening (Genomic or cDNA)

Random library screens do not require systematic cloning of all prey constructs; however, the prey library must be created. Therefore, the complete DNA sequence of the genome of interest is no prerequisite. Random prey libraries can be made using genomic DNA- or cDNA-based libraries. For genomic libraries, the genomic DNA of interest is randomly cut, size-selected, and the resulting fragments ligated into one or more two-hybrid prey vector(s). Many two-hybrid screening projects used random genomic DNA libraries (10, 25). A cDNA library is made through reverse transcription of mRNA collected from specific cell types or whole organisms. To simplify the task even more, many cDNA libraries are commercially available. For example, Clontech has a collection of human and tissue-specific cDNA libraries. However, the bait clones that need to be screened with a random library need to be made independently as for an array screen.

In a random library screen, a library of prey proteins is tested for interactions with a bait protein (Fig. 2). Similar to array screens, the bait protein is expressed in one yeast strain and the preys are expressed in another yeast strain of different mating type. The two

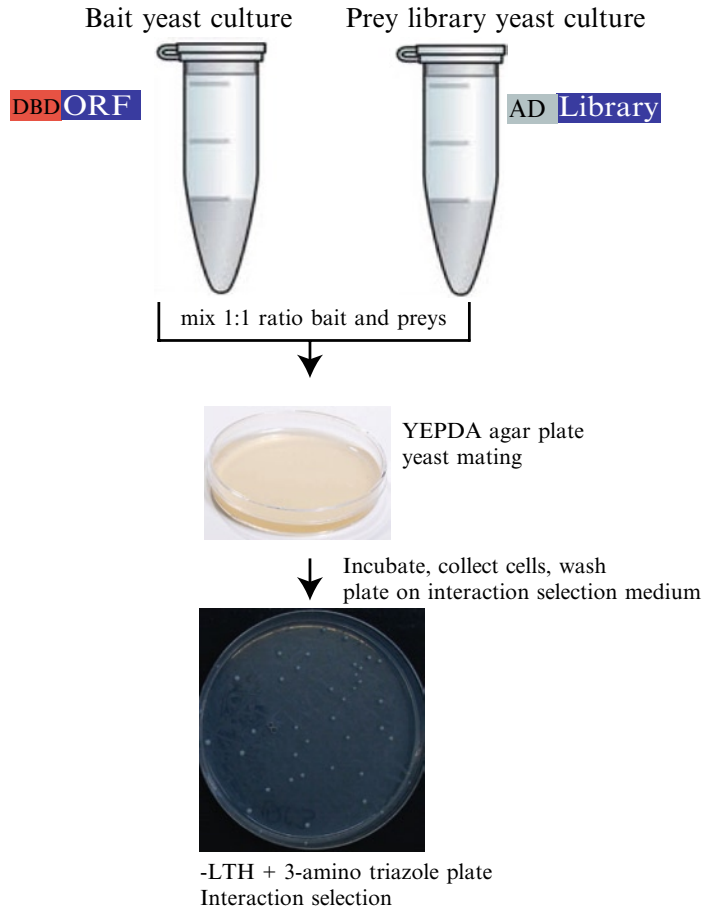

Fig. 2. Scheme of a random library two-hybrid screen: A haploid yeast strain expressing a single protein as a DBD fusion is mixed with the yeast haploid strains expressing cDNA or random genomic library (prey library). The bait and prey (1:1 ratio) culture is plated on YEPDA agar plate. The mating plates are incubated at 30°C for 6 h or overnight at room temperature. During this process, both the prey and bait plasmids are combined in the diploid yeast cells. The cells from the mating plates are collected and transferred onto –Leu –Trp –His medium plates (supplemented with different concentrations of 3-amino-triazole) for protein interaction detection, and plates are incubated at 30°C for 4–6 days. The identity of interacting prey is identified by yeast colony PCR of positive yeast colonies, followed by DNA sequencing of the PCR product.

strains are then mated so that the two proteins are expressed in the resulting diploid cell (Fig. 2). The diploids are plated on interaction selective medium, where only yeast cells having bait and its interacting prey grow. The prey is identified by isolating the prey plasmids, PCR amplification of the insert, and sequencing.

1.6. Multiple Variants of Y2H System

Y2H screens often produce vastly nonoverlapping interaction data when the screens are conducted using different vectors, strains,

Table 1
What you need for a yeast two-hybrid screen (examples)

		Gal4-Fusion		Selection			
Vector	**Promoter**	**DBD**	**AD**	**Yeast**	**Bacterial**	**Ori**	**Source**
Bait and prey vectors							
pDEST22	fl-ADH1	–	N-term	Trp1	Amp.	CEN	Invitrogen
pDEST32	fl-ADH1	N-term	–	Leu2	Gent.	CEN	Invitrogen
pGBKT7g	t-ADH1	N-term	–	Trp1	Kan.	2μ	(35)
pGBGT7g	t-ADH1	N-term	–	Trp1	Gent.	2μ	(29)
pGADT7g	fl-ADH1	–	N-term	Leu2	Amp.	2μ	(35)
pGBKCg	t-ADH1	C-term	–	Trp1	Kan.	2μ	(27)
pGADCg	fl-ADH1	–	C-term	Leu2	Amp.	2μ	(27)
Yeast strains							
Bait yeast strain	AH109						
Prey yeast strain	Y187						
Media and instruments							
Yeast media	YEPDA, selective liquid media, and agar plates						
Pin tool	Optional, but necessary, when large numbers are tested						

Fl full-length, *t* truncated, *N/C-term* N/C-terminal (fusion), *Amp* Ampicillin, *Kan* Kanamycin, *Gen* Gentamicin

and reporter genes or conducted in different laboratories. Rajagopala et al. investigated the underlying reasons for such inconsistencies and compared the effect of different vectors on their Y2H interactions. Low-copy Y2H vectors (yeast CEN ori) produce a higher fraction of interactions that are conserved and biologically relevant when compared to the high-copy vectors (yeast 2μ ori), but the latter appear to be more sensitive and thus detect more interactions (26).

Over the years, several Y2H vectors have been engineered and used for the interaction screening (Table 1). Traditionally, Y2H screens have been performed using N-terminal fusion proteins of DNA-binding and activation domains. Stellberger et al. constructed two new vectors that allow us to make both C-terminal fusion proteins of DNA-binding and activation domains and showed that permutations of C- and N-terminal Y2H vectors detect different subsets of interactions (27).

A recent study by Chen et al. benchmarked a number of two-hybrid vectors using a human positive reference set and a random reference set (92 protein pairs each) from Braun et al. (28). Chen et al. (29) cloned the positive reference set and the random reference set into five pairs of bait–prey vectors (pGBGT7g–pGADCg, pGBGT7g–pGADT7g, pDEST32–pDEST22, pGBKCg–pGADT7g, and pGBKCg–pGADCg). In addition to

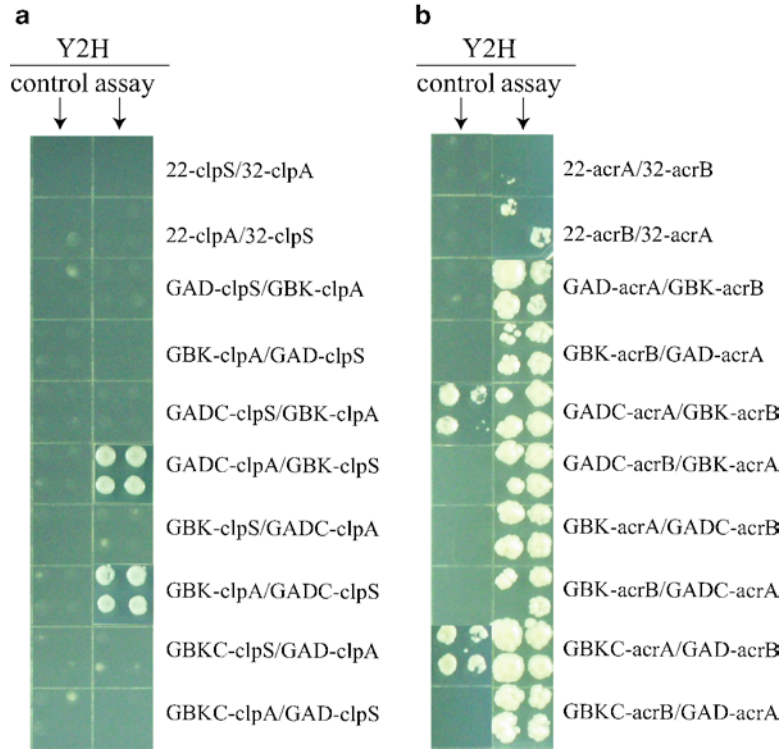

Fig. 3. An example to show the variations of the different two-hybrid vector systems. (**a**) The known protein interaction between *E. coli* AcrA and AcrB proteins tested in five two-hybrid vector pairs. Because each protein is tested as both bait and prey fusion, the interaction is tested in ten different configurations. The control column is tested with the bait and the empty prey vector which serves as a negative control. Only two out of ten tested combinations detected the interaction. (**b**) The known interaction between *E. coli* ClpS and ClpA proteins is tested in ten different configurations (as in (**a**)). Seven out of ten tested combinations show a positive interaction. Hence, protein interactions should be tested with multiple Y2H systems to reduce the number of false negatives. Note that vector names are abbreviated, but correspond to those listed in Table 1 (e.g., "22" = pDEST22 etc.).

each vector pair, they tested each protein both as activation (prey) and DBD fusion (bait), including C-terminal fusions in pGBKCg and pGADCg. This way, they tested each protein pair in ten different configurations (Figs. 3 and 4). The remarkable outcome of this study was that different Y2H variants detect markedly different subsets of interactions in the same interactome. All ten different configurations of bait–prey fusions were required to detect 73 of 92 interactions (79.3%), whereas individual vector pairs detected only 23.3 out of 92 interactions (25.3%) on average (29). Having multiple variants of Y2H that detect different subsets of interactions is of great value to generate more comprehensive protein interaction dataset; thus, future interactome projects must incorporate multiple Y2H vector systems with proper controls and adequate stringency.

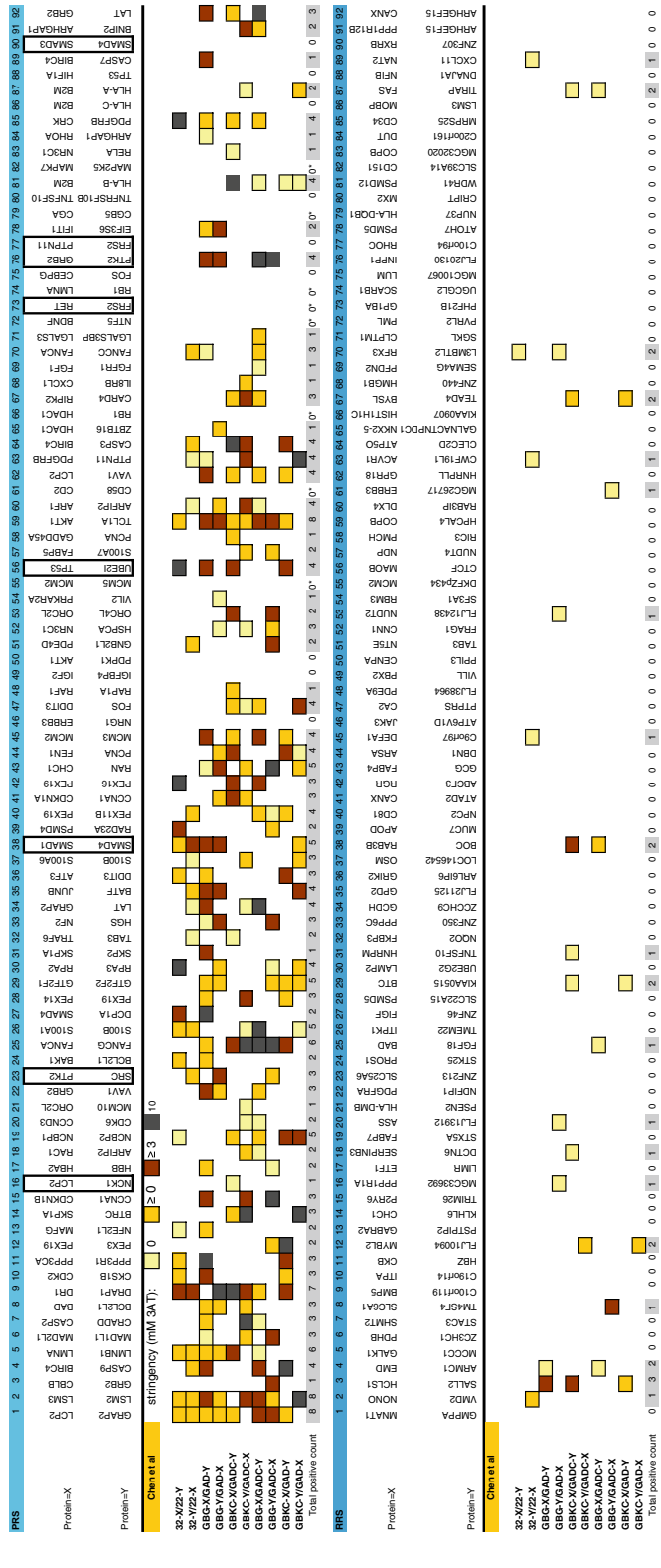

Fig. 4. Verification of known protein interactions by different two-hybrid vector systems: PRS is the Positive Reference Set of 92 known interactions among human proteins. The RRS panel shows the Random Reference Set of (most likely) noninteracting protein pairs from Braun et al. (28). X and Y indicate bait and prey proteins, respectively. Each of the 92 interaction pairs of PRS and RRS is tested in ten different configurations. The total positive count is the sum of vector pairs that detect the interaction (*gray boxes*). All ten different configurations of bait–prey fusions detect 73 out of 92 interactions (79.3%), whereas individual vector systems on average detected only 23.3 out of 92 interactions (25.3%). *Shades of gray* indicate levels of 3-AT (ranging from 0 [*light*] to 10 mM [*dark*]). Vector names are abbreviated but correspond to those listed in Table 1. After Chen et al. (29).

1.7. Evaluation of Raw Y2H Screening Data

Filtering of raw results significantly improves the data quality of the protein interaction set. In array screens, at least three parameters should be considered. *First*, protein interactions that are not reproduced in a retest experiment should be discarded. *Second*, for each prey, the number of different interacting baits (prey count) is counted; preys interacting with a large number of baits are nonspecific ("sticky" preys) and, thus, may have no biological relevance. The cutoff number depends also on the nature of baits and the number of baits screened: if a large family of related proteins is screened, it is not surprising that many of them find the same prey. As a general guideline, the number of baits interacting with a certain prey should not be larger than 5% of the bait number in genome-wide screenings. The *third* parameter is the background self-activation strength of the tested bait. The activation strength of interacting pairs must be significantly higher than with all other (background) pairs. Ideally, no activation (i.e., no colony growth) should be observed in noninteracting pairs. In the random library screening, the interacting protein is identified by sequencing. The interacting prey should be picked up at least two or more times with a bait.

In addition to the above parameters, more sophisticated statistical evaluations of the raw results have been suggested. For instance, filtering the raw interaction dataset by Logistic Regression (which uses positive and negative training sets of interactions) can help to identify the most reliable data (30, 31).

1.8. Quality of Large-Scale Two-Hybrid Interaction Data

Two-hybrid screens are not perfect. It is quite unlikely that you will detect all physiologically relevant interactors of your bait protein. *False negatives* may arise from steric hindrance of the two fusion proteins so that physical interaction or subsequent transcriptional activation is prevented. Other explanations for false negatives include instability of proteins or failure of nuclear localization, absence of a prey protein from a library, and inappropriate posttranslational modification of a bait or a prey, prohibiting an interaction. Earlier studies estimate that the false negative rate in array-based two-hybrid screens is on the order of 75%; i.e., up to 75% of all "true" interactions may be missed (13). This large number can be reduced by several strategies. For example, we investigated the interactome of bacterial flagella by using ORFs from both *T. pallidum* and *Campylobacter jejuni* which had estimated false negative rates of 76 and 77%, respectively. However, a combination of both datasets recovered 33% of all known flagellar interactions and, thus, had a false negative rate of 67%. When protein domains and fragments are used, this number can be further reduced. A recent study by Chen et al. has shown that different two-hybrid systems detect markedly different subsets of interactions in the same interactome (29). Using multiple variants of Y2H vectors (Figs. 4 and 5) for the same interactome can result in much

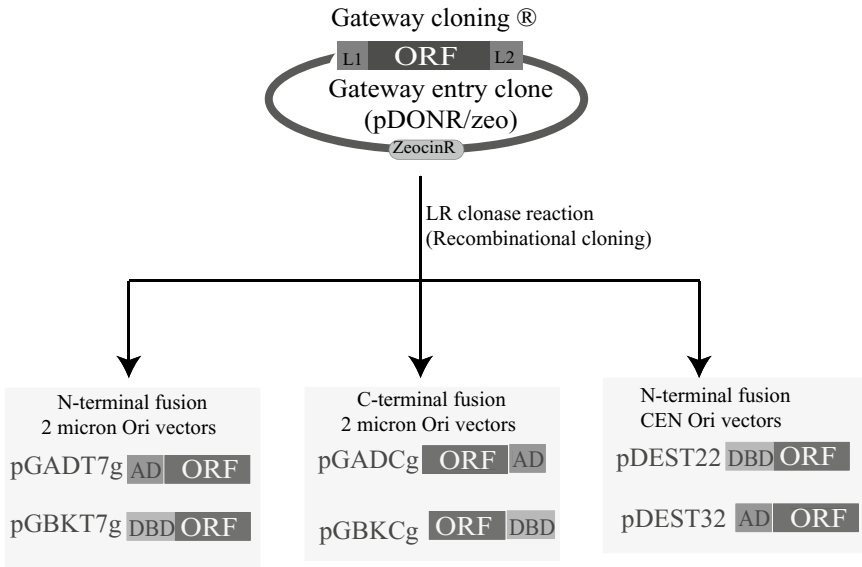

Fig. 5. Gateway cloning strategy for creating baits and preys. The Gateway-based Y2H expression clones are made by combining the ORFs of interest from a Gateway entry vector (such as pDONR/zeo or pDONR201) and the Y2H expression vectors (such as pDEST22 and pDEST32, Table 1) in the so-called Gateway LR reaction (20).

fewer false negatives, possibly on the order of 25–20%, compared to an average of 75% with single-vector systems (Fig. 4).

False positives: As with most assay systems, the two-hybrid system has the potential to produce false positives. "False positives" may be of technical or biological nature. A "technical" *false positive* is an apparent two-hybrid interaction that is not based on the assembly of two-hybrid proteins (i.e., the reporter gene(s) gets activated without a protein–protein interaction between bait and prey). Frequently, such false positives are associated with bait proteins that act as transcriptional activators. Some bait or prey proteins may affect general colony viability and, hence, enhance the ability of a cell to grow under selective conditions and activate the reporter gene. Mutations or other random events of unknown nature may be invoked as potential explanations as well. A number of procedures have been developed to identify or avoid false positives, including the utilization of multiple reporters, independent methods of specificity testing, or simply repeating assays to make sure that a result is reproducible (32–34) (described below).

A biological false positive involves a bona fide two-hybrid interaction with no physiological relevance. This includes the partners that can physically interact but that are never in close proximity to one another in the cell because of distinct subcellular localization or expression at different times during the life cycle. Examples may include paralogs that are expressed in different tissues or at different developmental stages. The problem is that the "false positive" nature can rarely be proven, as there may be unknown conditions

under which these proteins do interact with a biological purpose. Overall, few technical false positives can be explained mechanistically.

While it often remains difficult to prove the biological significance of an interaction, many studies have attempted to validate them by independent methods. Finding an interaction by several methods certainly increases the probability that it is biologically significant. Recently, Uetz et al. (35) validated Y2H interactions of Kaposi Sarcoma Herpes virus (KSHV) by CoIP and found that about 50% of them can be confirmed. Similarly, when subsets of the large-scale human Y2H interactomes were evaluated, 78 and 65% of them could be verified by independent methods (17, 18).

1.9. Combination of AP/MS and Y2H Data and Protein Complex Topology

It is important to note that most methods detect either direct binary interactions (Y2H and related technologies) or indirect interactions (e.g., mass spectrometry) without knowing which proteins are interacting. Protein complexes are often interpreted as if the proteins that copurifiy are interacting in a particular manner consistent with either a spoke or a matrix model (36). The Y2H and other fragment complementation systems detect direct binary interactions. Combination of both methods gives a better picture of protein complex topology and an experimentally derived confidence score for each interaction. However, even the combination of both methods is usually not sufficient to establish accurate topology as some interactions may be too weak to be detected individually.

1.10. General Requirements for a Screen and Alternatives

Although the protocols in this chapter are based on the DNA-binding and activation domains of the yeast Gal4 protein, other DBDs and activation domains can be used.

In the LexA two-hybrid system, the DBD is provided by the entire prokaryotic LexA protein, which normally functions as a repressor in *Escherichia coli* when it binds to LexA operators. In the Y2H system, the LexA protein does not act as a repressor. An activation domain often used in the LexA two-hybrid system is the heterologous 88-residue acidic peptide B42 that strongly activates transcription in yeast. An interaction between the target protein (fused to the DBD) and a library-encoded protein (fused to an AD) creates a novel transcriptional activator with binding affinity for LexA operators.

In general, every component of the "classic" two-hybrid system can be replaced by different components: For example, the reporter gene does not need to be *HIS3*. Alternatively, *LEU2*, an enzyme involved in leucine biosynthesis, can be used. The reporter does not have to be a biosynthetic enzyme at all; green fluorescent protein (GFP) has been successfully used as a reporter gene (37), beta-galactosidase (lacZ) is common (38), and many others are under investigation. Finally, the two-hybrid system does not need to be based on transcription. Johnsson and Varshavsky (1994) developed

a related system that is based on reconstituting artificially split ubiquitin, a protein that tags other proteins for degradation. As long as the function of a protein can be used as a selective marker, it is theoretically possible to divide it into fragments and drive the reassociation of the two fragments by exogenous "bait and prey" proteins, which are attached to each half. Several other variations have been developed and are described elsewhere (39, 40).

1.11. Genome-Wide Yeast Two-Hybrid Screening

The construction of an entire proteome array of an organism that can be screened in vivo under uniform conditions is a challenge. When proteins are screened on a genome scale, automated robotic procedures are necessary (see below). The procedure can be modified for manual use or for use with alternative screening strategies, such as synthetic lethal screens. With minor modifications, the array can be used to screen for protein interactions with DNA, RNA, or even small-molecule inhibitors of the Y2H interactions.

The protocols described here have been tested with yeast, bacterial, and viral proteins, but they can be applied to any other genome or subset thereof. Different high-throughput cloning methods used to generate two-hybrid clones, i.e., proteins with AD fusions (preys) and DBD fusions (baits), are therefore included below. The process involves the construction of the prey and bait array (Subheading 3.2) and screening of the array by either manual or robotic manipulation (Subheadings 3.5–3.8) screening the random cDNA or genomic library (Subheadings 3.7–3.9), including the selection of positives and scoring of results.

High-throughput screening projects deal with a large number of proteins; therefore, hands-on time and amount of resources become important issues. Options to reduce the screening effort are discussed. A prerequisite for array-based genome-wide screens is the existence of a cloned ORFeome; we briefly mention strategies to create such ORFeomes. Many ORFeome projects are currently being done (see ORFeomes below). We expect readily available complete ORFeomes for all major model organisms in the near future. Large-scale random library screening requires sequencing; having sequencing capacity becomes an important issue.

2. Materials

2.1. Yeast Media

1. *YEPD liquid medium*: 10 g yeast extract, 20 g peptone, 20 g glucose. Make up to 1 L with sterile water and autoclave.
2. *YEPDA liquid medium*: 10 g yeast extract, 20 g peptone, 20 g glucose. Make up to 1 L with sterile water and autoclave. After autoclaving, cool the medium to 60–70°C and then add 4 ml of 1% adenine solution (1% in 0.1 M NaOH).

3. *YEPDA solid medium*: 10 g yeast extract, 20 g peptone, 20 g glucose, 16 g agar. Make up to 1 L with sterile water and autoclave. After autoclaving, cool the medium to 60–70°C, then add 4 ml of 1% adenine solution (1% in 0.1 M NaOH), pour 40 ml into each sterile Omnitray plate (Nunc) under sterile hood, and let them solidify.
4. *Medium concentrate*: 8.5 g yeast nitrogen base, 25 g ammonium sulfate, 100 g glucose, 7 g dropout mix (see below). Make up to 1 L with sterile water and filter sterilize (Millipore).

2.2. Yeast Minimal Media (Selective) Plates

1. For 1 L of selective medium, autoclave 16 g agar in 800 ml water, cool the medium to 60–70°C, and then add 200 ml medium concentrate. Depending on the required selective plates, add the missing amino acids (see below) and/or 3-amino-1,2,4-triazole (3-AT) and pour plates.
2. –Trp plates (media-lacking tryptophan): Add 8.3 ml leucine and 8.3 ml histidine stock solution (see below).
3. –Leu plates (media-lacking leucine): Add 8.3 ml tryptophan and 8.3 ml histidine solution.
4. –Leu –Trp plates (media-lacking tryptophan and leucine): Add 8.3 ml histidine stock solution.
5. –Leu –Trp –His plates (media-lacking tryptophan, leucine, and histidine): Nothing needs to be added.
6. –Leu –Trp –His+ 3 mM 3-AT plates: Add 6 ml of 3-AT (0.5 M) to a final concentration of 3 mM.
7. *Dropout mix* (–His, –Leu, –Trp): Mix 1 g methionine, 1 g arginine, 2.5 g phenylalanine, 3 g lysine, 3 g tyrosine, 4 g isoleucine, 5 g glutamic acid, 5 g aspartic acid, 7.5 g valine, 10 g threonine, 20 g serine, 1 g adenine, and 1 g uracil and store under dry, sterile conditions.
8. *Amino acid stock solutions*: **Histidine** (His): Dissolve 4 g of histidine in 1 L sterile water and filter sterilize. **Leucine** (Leu): Dissolve 7.2 g of leucine in 1 L sterile water and sterile filter. **Tryptophan** (Trp): Dissolve 4.8 g of tryptophan in 1 L sterile water and filter sterilize.

2.3. Yeast Transformation

1. Salmon sperm DNA (carrier DNA): Dissolve 7.75 mg/ml salmon sperm DNA (Sigma) in sterile water, autoclave for 15 min at 121°C, and store at –20°C.
2. Dimethylsufoxide (DMSO, Sigma).
3. Competent host yeast strains, e.g., AH109 (for baits) and Y187 (for preys).
4. Lithium acetate (LiOAc) (0.1 M).

5. Selective plates (depending on the selective markers, described in Subheading 2.2).
6. 96 PEG solution: Mix 45.6 g PEG (Sigma), 6.1 ml of 2 M LiOAc, 1.14 ml of 1 M Tris, pH 7.5, and 232 µl 0.5 M EDTA. Make up to 100 ml with sterile water and autoclave.
7. Plasmid clones or linearized vector DNA and PCR product (for homologous recombination).

2.4. Bait Self-Activation Test

1. YEPDA liquid medium and selective media agar in single-well microtiter plates (Omnitray plates, Nunc).
2. –Trp –Leu ("–LT") plates (see Subheading 2.2).
3. Selective plates without Trp, Leu, and His ("–LTH"), but with different concentrations of 3-AT, e.g., 0 mM, 1 mM, 3 mM, 10 mM, 50 mM, and 100 mM (–LTH/3-AT plates).
4. Bait strains and the prey strain carrying the empty prey plasmid, e.g., Y187 strain with pDEST22 plasmid (Invitrogen).

2.5. Two-Hybrid Screening Protocol

1. 20% (v/v) bleach (1% sodium hypochlorite).
2. 95% (v/v) ethanol.
3. Single-well microtiter plate (e.g., OmniTray; Nalge Nunc) containing solid YEPD + adenine medium (see Subheading 2.1), –Leu –Trp, –His –Leu –Trp, and –His –Leu –Trp+ different concentrations of 3-AT.
4. 384-Pin replicator for manual screening or robot (e.g., Beckman Biomek FX).
5. Bait liquid culture (DBD fusion-expression yeast strain).
6. Yeast prey array on solid YEPDA plates.

2.6. Retest of Protein Interactions

1. 96-well microtiter plates (U- or V-shaped).
2. YEPDA medium and YEPDA agar in Omnitrays (Nunc).
3. Selective agar plates (–LT, –LTH with 3-AT).
4. Prey yeast strain carrying empty prey plasmid, e.g., pDEST22 in Y187 strain.
5. Bait and prey strains to be retested.

2.7. Beta-Galactosidase Filter Lift Assay

1. Selective plate (–LT) with diploid yeast colonies (from Subheading 3.6). The diploid cells carry the bait and prey combinations to be tested for activation of the beta-galactosidase reporter.
2. Omnitray plate.
3. Nitrocellulose membrane and Whatman paper.
4. Z-buffer: 60 mM Na_2HPO_4 (anhyd.), 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$.
5. X-GAL solution: 40 mg/ml in dimethylformamide (DMF).

3. Methods

3.1. Strategic Planning

Before starting an array-based screen, the size and character of the array must be designed and the ultimate aims of the experiment need to be considered. Factors that may be varied include the form of protein array (e.g., full-length protein or single domain, choice of epitope tags, etc.). Similarly, the arrayed proteins may be related (e.g., a family or pathway of related proteins, orthologs of a protein from different species, the entire protein complement of a model organism). In our experience, certain protein families work extremely well (e.g., splicing proteins) while others do not appear to work at all (e.g., many metabolic enzymes). We recommend to carry out a small-scale pilot study incorporating positive and negative controls before committing to a full-scale project.

Although high-throughput screening projects can be performed manually, automation is strongly recommended. Highly repetitive tasks are not only boring and straining, but also error-prone when done manually. If you do not have local access to robotics, you may have to collaborate with a laboratory that has.

3.2. Generation of a Protein Array Suitable for High-Throughput Screening

Once the set of proteins to be included in the array is defined, the coding genes need to be PCR-amplified and cloned into Y2H bait and prey vectors. In order to facilitate the cloning of a large number to proteins, site-specific recombination-based systems are commonly used (e.g., Gateway (20), see Fig. 5). Gateway cloning requires expensive enzymes and vectors, although both may be produced in the lab.

3.2.1. Gateway Cloning

Gateway (Invitrogen) cloning provides a fast and efficient way of cloning the ORFs (20). It is based on the site-specific recombination properties of bacteriophage lambda (41); recombination is mediated between the so-called attachment sites (att) of DNA molecules: between attB and attP sites or between attL and attR sites. The first step to Gateway cloning is inserting the gene of interest into a specific entry vector. One way of obtaining the initial entry clones is by recombining a PCR product of the ORF flanked by attB sites with the attP sites of a pDONR vector (Invitrogen). The resulting entry clone plasmid contains the gene of interest flanked by attL recombination sites. These attL sites can be recombined with attR sites on a destination vector, resulting in a plasmid for functional protein expression in a specific host. For example, a Gateway entry clone (in a pDONR vector) can be subsequently transferred to multiple Y2H expression vectors (such as pGADT7g, pGBKT7g, pGADCg, pGBKCg, pDEST22, and pDEST32, Fig. 5).

3.2.2. The ORFeome

The starting point of a systematic array-based Y2H screening is the construction of an ORFeome. An ORFeome represents all ORFs

of a genome – in our case, the selected gene set individually cloned into entry vectors. More and more ORFeomes are available and can be directly used for generating the Y2H bait and prey constructs. These ORFeomes range from small viral genomes, e.g., KSHV and VZV (35), to several bacterial genomes, such as *E. coli* (21), *Bacillus anthracis*, or *Yersinia pestis*. These bacterial ORFeomes are available from BEI Resources (http://www.beiresources.org/). Clone sets of multicellular eukaryotes, e.g., *Caenorhabditis elegans* (42), human (43), or plant (44), have also been described. However, not all genes of interest are already available in entry vectors. Both entry vector construction and the subsequent destination vector cloning can be done for multiple ORFs in parallel. The whole procedure can be automated using 96-well plates so that whole ORFeomes can be processed in parallel.

3.3. The Prey Array

The Y2H array is set up from an ordered set of AD-containing strains (preys) rather than BD-containing strains (baits) because the former do not generally result in self-activation of transcription. The prey constructs are assembled by transfer of the ORFs from entry vectors into specific prey vectors by recombination. Several prey vectors for the Gateway system are available. In our lab, we primarily use the Gateway-compatible pGADT7g vector, a derivative of pGADT7 (Clontech), or pDEST22 (Invitrogen) (Fig. 5). These prey constructs are transformed into haploid yeast cells (Subheading 3.4), e.g., the Y187 strain (mating-type alpha) (Table 2). Finally, individual yeast colonies, each carrying one specific prey construct, are arrayed on agar plates in a 96- or 384-format in duplicates or quadruplicates.

3.3.1. Bait Construction

Baits are also constructed by recombination-based transfer of the ORFs into specific bait vectors. Bait vectors used in our lab are the Gateway-adapted pGBKT7g, pGBKCg (Clontech), and pDEST32

Table 2
Yeast strains and their genotypes

Yeast strains	Genotypes
Y187	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ,URA3::GAL1UAS-GAL1-TATA-lacZ (after Harper et al. (47))
AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1 TATA-lacZ (after James et al. (48))

(Invitrogen) (Fig. 5). The bait constructs are also transformed into haploid yeast cells (described in Subheading 3.4), e.g., the AH109 strain (mating-type a) (Table 2). After self-activation testing, the baits can be tested for interactions against the Y2H prey array or random prey library (see Note 1).

3.4. High-Throughput Yeast Transformation

This method is recommended for the high-throughput transformation of the bait or prey plasmid clones into respective yeast strains, and is based on the method of Cagney et al. (45). This protocol is suitable for 1,000 transformations, it can be scaled up and down as required, and most of the steps can be automated. Selection of the transformed yeast cells requires leucine- or tryptophan-free media (–Leu or –Trp depending on the selective marker on the plasmid). Moreover, at least one of the haploid strains must contain a two-hybrid reporter gene (here, HIS3 under GAL4 control).

1. Prepare competent yeast cells: Inoculate 250 ml YEPD liquid medium with freshly grown yeast strains on YEPD agar medium in a 2-L flask and grow in a shaker (shaking at 200 rpm) at 30°C. Remove the yeast culture from the shaker when the cell density reaches OD 1.0–1.3. This usually takes 12–16 h.
2. Spin out the cells at 2,000 × *g* for 5 min at room temperature; pour off the supernatant.
3. Dissolve the cell pellet in 30 ml of LiOAc (0.1 M); make sure that pellet is completely dissolved and there are no cell clumps.
4. Spin the cells in a 50-ml Falcon tube at 2,000 × *g* for 5 min at room temperature, pour off the supernatant, and dissolve the cell pellet in a total volume of 10 ml LiOAc (0.1 M).
5. Prepare the *yeast transformation mix* without yeast cells by mixing the following components in a 200-ml sterile bottle:

Component	For 1,000 reactions (ml)
96-PEG	100
Salmon sperm DNA	3.2
DMSO	3.4

6. Add the competent yeast cells prepared above (steps 1–4) to the yeast transformation mix; shake the bottle vigorously by hand or vortex for 1 min.
7. Pipette 100 µl of the yeast transformation mix into a 96-well transformation plate (we generally use Costar 3596 plates) by using a robotic liquid handler (e.g., Biomek FX) or a multistep pipette.
8. Now add 25–50 ng of plasmid; keep one negative control (i.e., only yeast transformation mix).

9. Seal the 96-well plates with plastic or aluminum tape and vortex for 2–3 min. Care should be taken to seal the plates properly; vigorous vortexing might cause cross-contamination.
10. Incubate the plates at 42°C for 30 min.
11. Spin the 96-well plate for 5 min at 2,000 × *g*; discard the supernatant and aspirate by tapping on a cotton napkin for a couple of times.
12. Add 150 µl of selective liquid media to each well, depending on the selective marker on the plasmid construct (for example, trytophan- or leucine-free liquid media). Seal the plates with AirPore tape (Qiagen) to protect from evaporation.
13. Incubate at 30°C for 36–48 h.
14. Pellet the cells by spinning at 2,000 × *g* for 5 min, discard the supernatant, and add 10 µl sterile H_2O to each well.
15. Transfer the cells to selective agar plate to select yeast with transformed plasmid (single-well Omnitrays, 128 × 86 mm, from Nunc are well-suited for robotic automation). Typically, we use a 96-pin tool (see reagent setup for the sterilization of the pin tool). As an alternative to the pin tool, one can use a multichannel pipette to transfer the cells. Allow the yeast spots to dry on the plates.
16. Incubate at 30°C for 2 days. Colonies start appearing after 24 h.

3.5. Self-Activation Test

Prior to the two-hybrid analyses, the bait yeast strains should be examined for self-activation. Self-activation is defined as detectable bait-dependent reporter gene activation in the absence of any prey interaction partner. Weak- to intermediate-strength self-activator baits can be used in two-hybrid array screens because the corresponding bait–prey interactions confer stronger signals than the self-activation background. In case of the *HIS3* reporter gene, the self-activation background can be suppressed by adding different concentrations of 3-AT, a competitive inhibitor of *HIS3*. Self-activation of all the baits is examined on plates containing different concentrations of 3-AT. The lowest concentration of 3-AT that suppresses growth in this test is used for the interaction screen (see below) because it avoids background growth while still detecting true interactions.

The aim of this test is to measure the background reporter activity (here, *HIS3*) of bait proteins in the absence of an interacting prey protein. This measurement is used for choosing the selection conditions used for Y2H screening described in Subheading 3.6.

1. Bait strains are arrayed onto a single-well Omnitray agar plate; either the standard 96-spot format or the 384-spot format is used (see Note 2).

2. The arrayed bait strains are mated with a prey strain carrying the empty prey plasmid, e.g., Y187 strain with pDEST22 (Invitrogen). Mating is conducted according to the standard screening protocol as described in Subheading 3.6. Note that here an array of baits is tested, whereas in a "real" screen (Subheading 3.6) an array of preys is tested.
3. After selecting for diploid yeast cells (on –LT agar), the cells are transferred to media selecting for the HIS3 reporter gene activity as described in Subheading 3.6. The –LTH transfer may be done to multiple plates with increasing concentrations of 3-AT. Suggested 3-AT concentrations are 0, 1, 3, 10, 25, 50, and 100 mM.
4. These –LTH+ 3-AT plates are incubated for 1 week at 30°C. The self-activation level of each bait is assessed: the lowest 3-AT concentration that completely prevents colony growth is noted. As this concentration of 3-AT suppresses reporter activation in the absence of an interacting prey, this 3-AT concentration is added to –LTH plates in the actual interaction screens as described in Subheading 3.6.

3.6. Screening for Protein Interactions Using a Yeast Protein Array

The Y2H prey array can be screened for protein interactions by a mating procedure that can be carried out manually or using robotics. A yeast strain expressing a single candidate protein as a DBD fusion is mated to all the colonies in the prey array (Fig. 1, step 1: shown for one prey plate). After mating, the colonies are transferred to a diploid-specific medium and then to the two-hybrid interaction selective medium. To manually screen with more than one bait, replicate copies of the array are used.

In many cases, a handheld 384-pin replicating tool can be used for routine transfer of colonies for screening. For large projects, however, a robotic workstation (e.g., Biomek 2000 or Biomek FX, Beckman Coulter) may be used to speed up the screening procedures and to maximize reproducibility. A 384-pin steel replicating tool (e.g., High-Density Replication Tool; V&P Scientific) can be used to transfer the colonies form one plate to another. Between the transfer steps, the pinning tool must be sterilized (see below).

Note that not all plasticware is compatible with robotic devices, although most modern robots can be reprogrammed to accept different consumables. In the procedure described here, the prey array is gridded on 86 × 128-mm single-well microtiter plates (e.g., OmniTray, Nalge Nunc International) in a 384-colony format (see Fig. 1).

1. *Sterilization*: Sterilize a 384-pin replicator by dipping the pins into 20% bleach for 20 s, sterile water for 1 s and 95% ethanol for 20 s, and sterile water again for 1 s. Repeat this sterilization after each transfer. Note: Immersion of the pins into these

solutions must be sufficient to ensure complete sterilization. When automatic pinning devices are used, the solutions need to be checked and refilled occasionally (especially ethanol, which evaporates faster than others).

Day 1

2. *Preparing prey array for screening*: Use the sterile replicator to transfer the yeast prey array from selective plates to single-well microtiter plates containing solid YEPD medium and grow the array overnight in a 30°C incubator (see Note 3).
3. *Preparing bait liquid culture (DBD fusion-expressing yeast strain):* Inoculate 20 ml of liquid YEPD medium in a 250-ml conical flask with a bait strain and grow overnight in a 30°C shaker (see Note 4).

Day 2

4. *Mating procedure*: Pour the overnight liquid bait culture into a sterile Omnitray plate. Dip the sterilized pins of the pin replicator (thick pins of ~1.5 mm diameter should be used to pin baits) into the bait liquid culture and place directly onto a fresh single-well microtiter plate containing YEPDA agar media. Repeat with the required number of plates and allow the yeast spots to dry onto the plates for 10–20 min.
5. Pick up the fresh prey array (i.e., AD) yeast colonies with sterilized pins (thin pins of ~1 mm diameter should be used to pin the preys) and transfer them directly onto the baits on the YEPDA plate so that each of the 384 bait spots per plate receives different prey yeast cells (i.e., a different AD fusion protein). Incubate overnight at 30°C to allow mating (Fig. 1, step 1, see Note 5).
6. *Seletion of diploids*: For the selection of diploids, transfer the colonies from YEPDA mating plates to plates containing –Leu –Trp medium using the sterilized pinning tool (thin pins should be used in this step). Grow for 2–3 days at 30°C until the colonies are >1 mm in diameter (Fig. 1, step 2, see Note 6).
7. *Interaction selection:* Transfer the colonies from –Leu –Trp plates to a single-well microtiter plate containing solid –His –Leu –Trp agar using the sterilized pinning tool. If the baits are self-activating, they have to be transferred to –His –Leu –Trp+ a specific concentration of 3-AT (Subheading 3.5). Incubate at 30°C for 6–8 days.
8. Score the interactions by looking for growing colonies that are significantly above background by size and are present as duplicate colonies.
9. The plates should be examined every day. Most two-hybrid positive colonies appear within 3–5 days, but occasionally positive interactions can be observed later. Very small colonies are

usually designated as background; however, there is no absolute measure to distinguish between the background and real positives. When there are many (e.g., >20) large colonies per array of 1,000 positions, we consider these baits as "random" activators. In this case, the screening should be repeated or the interactions should be retested.

10. Scoring can be done manually or using automated image analysis procedures. When using image analysis, care must be taken not to score contaminated colonies as positives.

3.7. Screening for Protein Interactions Using Random cDNA Libraries

A yeast strain expressing a single protein as a DBD fusion is mated to all the cells in the prey library (Fig. 2, step 1: shown for one bait sample). After mating, the resulting diploid cells are transferred to the Y2H selective medium, and the interacting prey is identified by yeast colony PCR of the resulting positive yeast colony, followed by DNA sequencing of the PCR product.

Day 1

1. Prepare prey library for screening: Inoculate 200 ml of selective medium (medium lacking leucine or tryptophan, depending on the selective marker on the prey plasmid) with an aliquot of yeast prey library (e.g., an *E. coli* prey library plasmid prep) to an O.D of 0.05. Grow at 30°C with shaking at 180 rpm for 16 h. Cultures should have an OD600 of 0.9–1.0 when they are harvested for the mating (the amount of prey library culture required depends on the number of baits you want to screen and the complexity of the prey library, see step 4).
2. Inoculate the empty prey vector in 200 ml selective medium (Y2H negative control).
3. Preparing bait liquid culture (DBD fusion-expressing yeast strain): Inoculate 10 ml of selective medium (medium lacking leucine or tryptophan, depending on the selective marker on the bait plasmid) with bait fusion-expressing yeast strain and grow the yeast overnight in a 30°C incubator (see Note 4).

Day 2

4. Mating procedure: Mix bait and prey at a 1:1 ratio, for example, 4 OD bait (4 ml of OD = 1) and 4 OD prey (4 ml of OD = 1) culture in 15-ml Falcon tubes (see Note 7).
5. For each bait, include one negative control, mix bait, and empty prey vector (1:1).
6. Centrifuge for 2 min at 3,000 × *g* at room temperature, and discard supernatant.
7. Resuspend pellet in 500 µl YPDA, plate on YEPDA agar plate (60 mm × 15 mm), and air dry the plates.

8. Incubate the plates at 30°C for 6 h or overnight at room temperature.
9. After incubation, collect the cells by washing the plate with 2 ml of sterile water.
10. Spin down the cells, remove the supernatant, and wash one time with 2 ml of sterile water.
11. Resuspend in 2 ml of selective medium (media lacking tryptophan, leucine, and histidine).
12. Plate 500 μl on the interaction selective agar plates –Leu –Trp –His+ 3 mM 3-AT (media lacking tryptophan, leucine, and histidine+ 3 mM 3-AT). To measure the number of diploids, make an aliquot of 1:100 dilution and plate the cells on –Leu –Trp plates (see Note 6); the screening depth in millions should be >0.1 million up to 1 million diploids in case of *E. coli* library screening, i.e., at least 20 times the number of library size. Store the remaining sample at 4°C for further use.
13. Interaction selection: Incubate the –Leu –Trp –His+ 3 mM 3-AT for 4–6 days at 30°C until the colonies are ~1 mm in diameter. If the baits are self-activating, they have to be transferred to –His –Leu –Trp+ a specific concentration of 3-AT (Subheading 3.5). Incubate at 30°C for 6–8 days.
14. Two-hybrid positives: The plates that show colony growth (but no colonies on control plates, i.e., bait mated to empty prey vector) identify the two-hybrid positive yeast clones.
15. Identity of interacting preys: The positive yeast colonies are picked either manually or using robotics and subjected to yeast colony PCR (Subheading 3.9), followed by DNA sequencing to identify the preys.

3.8. Protein Interaction Retesting

A major consideration when using the Y2H system is the number of false positives. The major sources for false positives are nonreproducible signals that arise through little-understood mechanisms. In Y2H screens, more than 90% of all interactions can be nonreproducible background (46). Thus, simple retesting by repeated mating can identify most false positives. We routinely use at least duplicate tests, although quadruplicates should be used if possible (see Fig. 1). Retesting is done by manually mating the interaction pair to be tested and by comparing the activation strength of this pair with the activation strength of a control, usually the bait mated with the strain that contains the empty prey vector.

Testing for reproducibility of interactions greatly increases the reliability of the Y2H interaction data. This method is used for specifically retesting interaction pairs detected in an array screen.

1. Re-array bait and prey strains of each interaction pair to be tested into 96-well microtiter plates. Use separate 96-well plates for baits and preys. For each retested interaction, fill one well of the bait plate and one corresponding well of the prey plate with 150 µl YEPD liquid medium.
2. For each retested interaction, inoculate the bait strain into a well of the 96-well bait.
3. Bait and prey strains should be at the corresponding position of the 96-well prey plate, for example, bait at position B2 of the bait plate and prey at position B2 of the prey plate. In addition, inoculate the prey strain with the empty prey vector (e.g., strain Y187 with plasmid pDEST22) into 20 ml YEPD liquid medium.
4. Incubate the plates overnight at 30°C.
5. Mate the baits grown in the bait plate with their corresponding preys in the prey plate. In addition, mate each bait with the prey strain carrying an empty prey vector as a background activation control. The mating is done according to Subheading 3.6 using the bait and prey 96-well plates directly as the source plates (see Note 8).
6. The transfers to selective plates and incubations are done as described in Subheading 3.6. As before, test different baits with different activation strengths on a single plate and pin the diploid cells onto –LTH plates with different concentrations of 3-AT. For choosing the 3-AT range, the activation strengths (Subheading 3.5) serve as a guideline.
7. After incubating for ~1 week at 30°C on –LTH/3-AT plates, the interactions are scored; positive interactions show a clear colony growth at a certain level of 3-AT, whereas no growth should be seen in the control (bait mated with empty vector strain).

3.9. Yeast Colony PCR and SAP

This protocol is designed to amplify the insert of the preys in the two-hybrid positive yeast clones using primers that bind to the upstream and downstream region of the insert. The PCR is optimized for 30 µl reaction; the total volume of the reaction may be increased if necessary. To clean up PCR products before sequencing, the PCR reaction is subjected to exonuclease I which removes leftover primers while the Shrimp Alkaline Phosphatase (SAP) removes the dNTPs.

1. Pick the yeast colony from interaction selective plate into 100 µl of sterile H_2O in 96-well plate (for longer storage, store at –80°C).
2. Take a 96-well PCR plate and pipette 5 U of zymolyase (1 µl) enzyme to each well.

3. Pipette 9 µl of the above yeast (1.) and incubate at 30°C for 60 min.
4. After incubation, add 20 µl PCR master mix with forward and reverse primers specific to prey vector used in the two-hybrid screening.
5. Run PCR cycles as recommended by the enzyme provider manual.
6. After PCR, load 5 µl of PCR reaction into agarose gel to check PCR products.
7. Purify the PCR reaction by SAP (see below).

SAP Master Mix

Components	100 samples (µl)
10× SAP buffer	50
Water	890
SAP (1 U/µl)	50
Exonuclease I (10 U/µl)	10

8. Spin the yeast colony PCR plate at 2,000 × *g* for 3 min (to sediment yeast debris).
9. Pipette 8 µl of PCR sample without touching the bottom yeast pellet into new PCR plate.
10. Add 10 µl of SAP master mix to 8 µl of PCR sample.
11. Incubate in the thermocycler as follows: 37°C for 60 min, 72°C for 15 min, and then put on hold at 4°C.
12. Use the sample for DNA sequencing using primers specific to prey vector.

3.10. Beta-Galactosidase Filter Lift Assay (Alternative Reporter Genes)

Y2H interactions can be reproduced using other reporter genes in addition to the one used in the actual screen depending on the different reporter genes present in the yeast strains used. Examples include beta-galactosidase or *ADE2* (for selection on adenine-deficient medium). Because of the use of different promoters, these reporter genes have different activation requirements, and Y2H interactions reproduced with different reporter genes are assumed to be more reliable. However, the use of multiple reporters may result in the loss of weaker Y2H positives. The beta-galactosidase reporter has the advantage of giving a semiquantitative output of the activation strength. Other reporters might be advantageous and can be transformed into yeast as additional plasmids or by using alternative strains, which contain the reporter as integrated construct. For example, the strain AH109 carries an alpha-galactosidase reporter gene which produces an enzyme that is secreted into the medium. Therefore, these cells do not require cell

lysis for detection. The following method was adapted from the Breeden lab (http://labs.fhcrc.org/breeden/Methods/yeast%20methods/B-Gal.doc).

1. Use the same diploid plate as in Subheading 3.6. As a control, the bait strains are mated with a prey strain containing an empty vector (following mating steps of Subheading 3.6).
2. Cut a nitrocellulose membrane to the dimensions of an Omnitray plate (Nunc). Place the nitrocellulose membrane on top of diploid yeast colonies and leave for 10 s.
3. Use tweezers to lift the filter and slowly submerge in liquid nitrogen for 1 min.
4. Place the membrane on an empty Omnitray plate (Nunc) to thaw.
5. Cut a Whatman paper to the same size as nitrocellulose membrane. Soak the Whatman paper with 2 ml Z-buffer to which 35 µl X-solution had been added.
6. Overlay the nitrocellulose filter with the Whatman paper and remove air bubbles.
7. Incubate at 30°C for 10–60 min.
8. Evaluate: A blue stain indicates the activation of the beta-galactosidase reporter and, therefore, a positive interaction.

4. Notes

1. Bait and prey must be transformed into yeast strains of opposite mating types to combine bait and prey plasmids by mating and to coexpress the fusion proteins in diploids. Bait and prey plasmids can go into either mating type. However, this decision also depends on existing bait or prey libraries to which the new library may be mated later.
2. Multiple baits are first inoculated at the different positions of a 96-well plate as liquid culture, and then cells are transferred (manually or with the use of a robot) to solid agar single-well plates (Omnitray plates). In this step, the 96-well format can also be converted into the 384-well format. This positions each bait in quadruplicates on the 384-well formatted plate. Full media agar (YEPDA agar) can be used; however, for long-term storage of the array, selective agar is suggested to prevent loss of plasmids.
3. In a systematic array-based Y2H screening, duplicate or quadruplicate prey arrays are usually used. In a random genomic

library screening, the entire experiment should be done in two copies to ensure reproducibility. Ideally, the master prey array should be kept on selective plates. The master copy of the array should only be used to make "working" copies on YEPDA agar plates for mating. These templates can be used for 1–2 weeks; after 2 weeks, it is recommended to copy the array onto fresh selective plates. Preys or bait clones tend to lose the plasmid if stored on YEPDA for longer periods, which may reduce the mating and screening efficiency.

4. If the bait strains are frozen, they are streaked or pinned on selective solid medium plates and grown for 1–2 days at 30°C. Baits from this plate are then used to inoculate the liquid YEPD medium. It is important to make a fresh bait culture for Y2H mating, as keeping the bait culture on reach medium (YEPD) for a long time may cause loss of plasmids. Usually, we grow baits overnight for mating.
5. Mating usually takes place in <15 h, but a longer period is recommended because some bait strains show poor mating efficiency. Adding adenine into the bait culture before mating increases the mating efficiency of some baits.
6. This is an essential control step to ensure successful mating because only diploid cells containing the Leu2 and Trp1 markers on the prey and bait vectors, respectively, grow in this medium. This step also helps the recovery of the colonies and increases the efficiency of the next interaction selection step.
7. The amount of OD units of bait and prey depends on the complexity of prey library; in case of *E. coli* which contains about 4,300 ORFs, we use 4 OD units of baits and preys. In case of human cDNA library screening, we recommend to use 12 OD units of baits and preys each.
8. First, the baits are transferred from their 96-well plate to two YEPDA plates (interaction test and control plate) using a 96-well replication tool. Let the plate dry for 10–20 min. Then, transfer the preys from their 96-well plate onto the first YEPDA plate and the empty prey vector control strain onto the second YEPDA plate.

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