Array-Based Yeast Two-Hybrid Screens: A Practical Guide

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Abstract

Yeast two-hybrid screens are carried out as random library screens or matrix-based screens. The latter have the advantage of being better controlled and thus typically give clearer results. In this chapter, we provide detailed protocols for matrix-based Y2H screens and give some helpful instructions how to plan a large-scale interaction screen. We also discuss strategies to identify or avoid false negatives and false positives.

Key words: ORFeome, Mating, Pooling, Protein–protein interactions, Yeast two hybrid, Array, Vectors, Yeast strains

Abbreviations

3-AT	3-Amino-1,2,4-triazole
AD	Activation domain
DBD	DNA-binding domain
ORF	Open reading frame
Y2H	Yeast two hybrid

1. Introduction

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 at a genome scale, automated robotic procedures are necessary. The protocols described here were established for yeast proteins, but they can be applied to any other genomes or subsets thereof; for example, viral and bacterial genomes have been screened for interactions in our lab. Different high-throughput cloning methods used to generate two-hybrid

clones, i.e., proteins with AD fusions (preys) and DBD fusions (baits), are presented below. The steps of the process involve the construction of the array and screening of the array by either manual or robotic manipulation, 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 an important issue. Options to reduce the screening effort are discussed. A prerequisite for array-based genome-wide screens is the existence of a cloned *ORFeome* (typically defined as full-length ORF sets) or at least a number of protein-coding clones; we briefly mention strategies how to create such ORFeomes.

1.1. General Screen Requirements

1.1.1. 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 format of the 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 better than others (e.g., splicing proteins, bacterial flagellum proteins, and proteins involved in DNA replication) while others do not appear to work at all (e.g., many metabolic enzymes and membrane proteins). 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 does.

1.2. 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 ORFs, site-specific recombination-based systems are commonly used (e.g., Gateway or Univector cloning (1, 2)) (Fig. 1). Some of these systems require expensive enzymes and vectors, although both may be produced in the lab.

1.2.1. Cloning by Homologous Recombination in Yeast

An alternative to site-directed systems is the cloning by homologous recombination directly in yeast (3). A two-step PCR protocol is used to make DNA with sufficient homology to vector DNA at the terminal ends to allow homologous recombination in the yeast cell (Fig. 1). In the "first-round" PCR reaction, the ORF is amplified with primers that contain ~20 nucleotide tails which are homologs to sequences in the two-hybrid vectors. In the
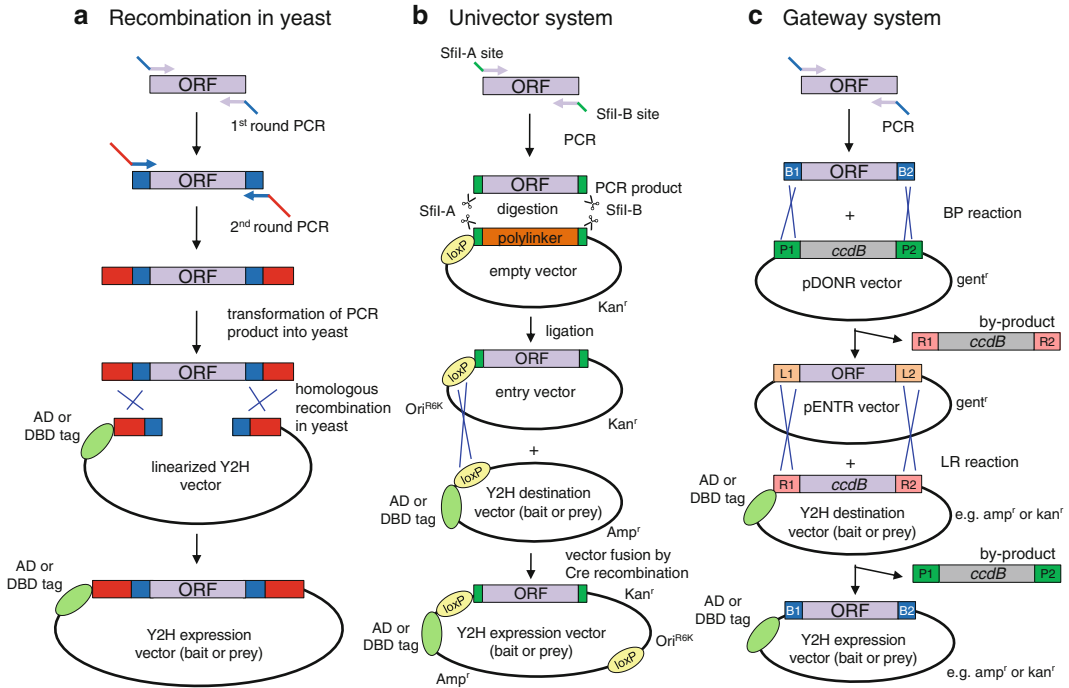
Fig. 1. ORFeome cloning systems. (**a**) Homologous recombination in yeast: ORFs are amplified (first PCR) with gene-specific primers that generate a product with common 5′ and 3′ 20-nucleotide tails. A second PCR generates a product with common 5′ and 3′ 70-nucleotide tails. The common 70-nucleotide ends allow cloning into linearized two-hybrid expression vectors by cotransfection into yeast. The endogenous yeast homologous recombination machinery performs the recombination reaction and results in a circular plasmid. (**b**) Univector plasmid-fusion system: ORFs are amplified with gene-specific primers that generate a product with common 5′ and 3′ rare-cutting restriction sites. The PCR product is cloned into a pUNI entry vector by DNA ligation. Cre–*loxP*-mediated site-specific recombination fuses the pUNI entry clone and yeast two-hybrid expression plasmids (bait/prey) at the *loxP* site. As a result, the gene of interest is placed under the control of the yeast two-hybrid expression vector promoter. (**c**) Gateway cloning: The ORFs are amplified with gene-specific primers that generate a product with common 5′ and 3′ recombination sites (*attB1* and *attB2*). The entry clones are made by recombining the ORFs of interest with the flanking *attB* sites into the *attP* sites of a suitable Gateway entry vector (e.g., pDONR201 or pDONR207) mediated by the Gateway BP Clonase II Enzyme Mix (Invitrogen). Subsequently, the fragment in the entry clone can be transferred to any yeast two-hybrid destination vector that contains the *attR* sites by mixing both plasmids and using the Gateway LR Clonase II Enzyme Mix.

second-round PCR, ~50-nucleotide tails (homologous to the destination vector-cloning site) are attached to the first-round PCR product (Fig. 1). The PCR product is then transfected into the yeast cells together with the linearized vector and the recombination event between them takes place inside the yeast cell. The advantage of this strategy is its much reduced cost. The disadvantage is that plasmids have to be recovered from yeast which can be time consuming and inefficient.

1.2.2. Univector Plasmid-Fusion System

Similar to the Gateway system, the Univector Plasmid-Fusion System (UPS) requires an entry vector containing the ORF. The UPS uses *Cre–loxP*-based site-specific recombination to catalyze plasmid fusion between the entry "univector" and destination vectors

containing, e.g., specific promoters, fusion proteins, and selection markers. Cre is a site-specific recombinase, which catalyzes the recombination between two 34 bp *loxP* sequences (Fig. 1).The pUNI plasmid is the entry vector of this system, the vector into which the gene of interest is inserted. The pHOST plasmid is the recipient vector containing the appropriate transcriptional regulatory sequences that eventually control the expression of the gene of interest in the designated host cells. A recombinant expression construct is made through Cre–*loxP*-mediated site-specific recombination that fuses pUNI and pHOST into a dimeric fusion plasmid. A crucial feature of the pUNI plasmid is its conditional origin of replication derived from the plasmid R6Kγ that allows its propagation only in bacterial hosts expressing the *pir* gene (encoding the essential replication protein p). Thus, only dimeric pUNI–pHOST vectors are selected and propagated (1) (Fig. 1).

1.2.3. Gateway® Cloning

Gateway® (Invitrogen) cloning provides another fast and efficient way of cloning ORFs (2). It is based on the site-specific recombination properties of bacteriophage lambda (4); 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 your gene of interest into a specific entry vector. This entry clone is a plasmid containing your 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. 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.

Site-specific recombination systems like the Gateway® or UPS system have got some crucial advantages in comparison to classical ligation cloning: the recombination reaction is highly efficient and fast to perform. The entry vector library can not only be transferred into yeast two-hybrid destination vectors, but also in any other compatible vector system that carries the recombination sites. For instance, the Gateway® technology provides plenty of commercially available destination vectors that can be used for further downstream experiments like protein purification or in vivo expression analysis. Furthermore, bait and prey plasmids can be created simultaneously within the same recombination reaction as long as they contain different bacterial selection markers.

1.3. ORFeome Cloning

The starting point of an array-based Y2H screen is the construction of an ORFeome array. An ORFeome represents all ORFs of a genome or a subset thereof – in our case: the selected gene set individually cloned into entry vectors of a recombination-based cloning system. More and more ORFeomes are available and can be directly used for generating the Y2H bait and prey constructs. Alternatively,

they can be cloned into the entry vector by multiple strategies, such as classical ligation or recombination. Both entry vector construction and the subsequent destination vector cloning can be done for multiple ORFs in parallel. The whole procedure can be parallelized using 96-well plates so that whole ORFeomes can be processed in parallel.

1.4. Prey Array

The Y2H array is made from an ordered set of AD-containing strains (preys), rather than DBD-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 UPS and the Gateway® system are available. In our lab, we preferentially use the Gateway®-adapted pGADT7g (a derivative of pGADT7 from Clontech) and pDEST22 (Invitrogen) vectors (Fig. 2). An alternative is the direct cloning of prey constructs by homologous recombination in yeast (see above). These prey constructs are transformed into haploid yeast cells (e.g., the Y187 strain, *see protocol 3.1*). Finally, individual yeast colonies, each carrying one specific prey construct, are arrayed on agar plates in a 96 format. By a second pinning step, the preys are copied as quadruplicates or duplicates to yield the final prey array that can be used for the screening procedure.

1.5. Baits

Baits are also constructed by recombination-based transfer of the ORFs into specific bait vectors or, alternatively, directly by homologous recombination in yeast. Bait vectors used in our lab are pGBKT7 (Clontech) modified for Gateway® cloning (pGB-KT7g) and pDEST32 (Invitrogen) (Fig. 2). The bait constructs are also transformed into haploid yeast cells; we use the AH109 strain (*protocol 3.1*). After self-activation testing, the baits can be tested for interaction screening against the Y2H prey array.

Bait and prey plasmids must be transformed into haploid yeast strains of opposite mating type to combine bait and prey plasmids by mating. To our knowledge, it does not make a difference whether baits or preys are transformed into either *a* or alpha cells, respectively.

1.6. Self-Activation Test of Baits (One-Hybrid Assay)

Prior to the two-hybrid analyses, the bait yeast strains should be examined for self-activation. Self-activation is defined as a 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. If the HIS3 reporter gene is used, the self-activation background can be suppressed by adding 3-AT, a competitive inhibitor of HIS3.
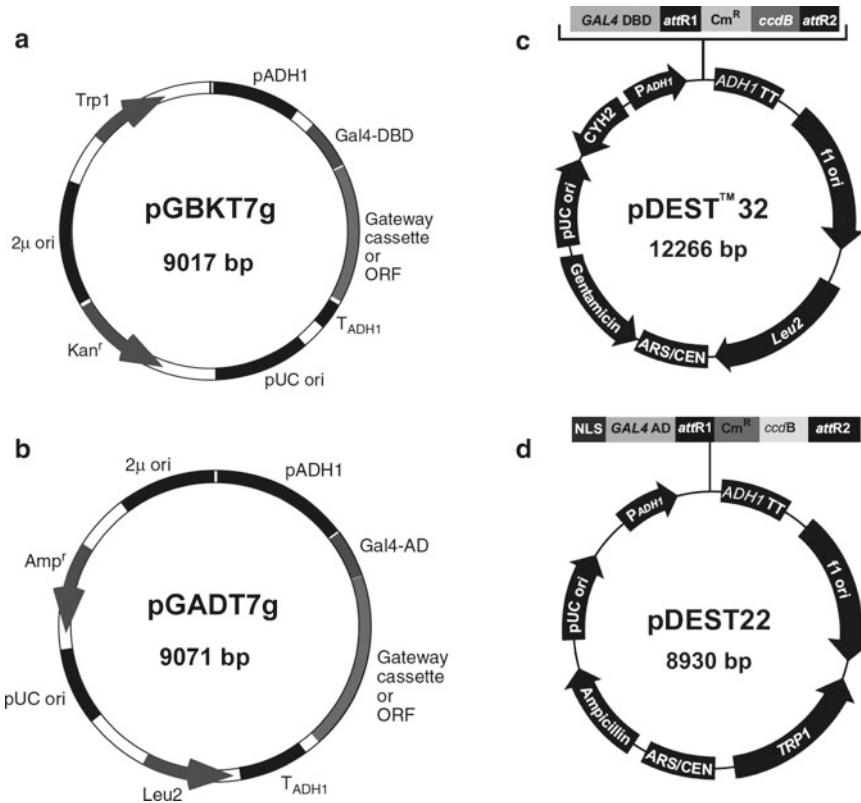
Fig. 2. Commonly used yeast two-hybrid expression vectors. The vectors shown here are all Gateway® system-compatible expression vectors and carry the Gateway cassette which is flanked by the recombination sites *attR1* and *attR2*. The cassette contains *ccdB* whose product inhibits *E. coli* gyrase. Thus, after recombination, positive *E. coli* clones can be directly selected on the respective antibiotic selection medium, whereas negative recombination products are automatically deselected. (**a**, **b**) Bait vector pGBKT7g and prey vector pGADT7g. (**c**, **d**) Bait vector pDEST32 and prey vector pDEST22 (Invitrogen). Here, the Gateway® cassette is shown in closer detail.

Self-activation of all the baits should be examined simultaneously on plates containing different concentrations of 3-AT (see *protocol 3.2*). For instance, a titration series with 3-AT concentrations of 0, 1, 2, 4, 8, 16, …, 128 mM can be used. The lowest concentration (minimal inhibitory concentration) of 3-AT that suppresses growth in this test is used for the interaction screen because it avoids background growth, whereas true interactions are still detectable.

1.7. Screening Procedure

The Y2H prey array can be screened for protein interactions by a mating procedure that can be carried out manually or using robotics (see *protocol 3.3*). Since the screening procedure used here is based on yeast mating, the bait and prey strains can be mated by manual mixing or by a robotic device that essentially replica plates preys on an array of baits. For large numbers of strains, automation is obviously desirable. Typically, these mating steps are carried out

either in a 96 or 384 format so that colonies can be picked up from equivalent 96- or 384-well microtiter plates and then copied onto solid agar (see step 1 of *protocol 3.3* for details).

1.8. Time Considerations in One-on-One Yeast Two-Hybrid Screens

In the simplest case, a set of baits is tested individually against a set of preys. For ten baits and ten preys, this results in $10 \times 10 = 100$ individual tests (e.g., when all components of a protein complex are tested against each other). For a viral genome of 100 genes, already 10,000 tests are required. Thus, the number of tests grows exponentially with the number of baits and preys.

As a consequence, automation is required for larger projects. For example, in our laboratory, a single Biomek 2000 robot was sufficient for testing about 50 baits against a bacterial genome of 1,000 ORFs per week or all 100 proteins of a viral genome against each other. Note that each interaction also should be tested at least twice as duplicates, just to make sure that the result is reproducible. This doubles the number of tests to be done. In fact, for smaller projects, we recommend to do each test four times, e.g., by spotting quadruplicates of each prey.

In larger projects, all tests can be done once, but then each positive protein pair needs to be retested later, ideally in a coordinated effort to verify all positives. This time, quadruplicates can be used.

In theory, the colony density of the array can be increased as well, e.g., from 384 to 768 or even to 1,536 colonies per plate. However, this approach requires a higher precision of the robot, smaller colony sizes, and thus can reduce the number of detected interactions, e.g., due to a smaller number of transferred cells. While we have used 768-spot arrays on microtiter-sized plates, 1,536 spots turned out to be too error prone with our equipment. In our experience, the higher the number of test positions is, the more noisy becomes the signal since the single colonies start to compete for nutrients and thus clearly slow down growth.

1.9. Pooling Strategies

In an independent chapter, we described a couple of pooling strategies that can be used to speed up high-throughput screening. For very large genome-wide screens, pooling is recommended. The most critical point of pooling is that equal cell numbers of different clones in the pool cannot be adjusted perfectly well (causing over- and underrepresentation) and thus pooling is prone to produce false negatives. Single replication steps must be watched more carefully to yield high mating efficiency and preparation of pool plates takes additional time compared to one-on-one matrix screens. But once the experimental setup is well-established, pooling strategies can yield the same sensitivity as one-on-one screens by lowering cost and time (see *protocol 3.4*).

1.10. Retests

A major consideration when using the Y2H system is the number of false positives. The major source of false positives are nonreproducible signals which arise through little-understood mechanisms. In array screens and probably in random library screen, more than 90% of all interactions can be nonreproducible background (5). Thus, simple retesting by repeated mating can identify most false positives. We routinely use quadruplicate retesting. It is done by 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. Retests can also be used to identify interacting preys of positively tested pools (see *protocol 3.5*).

1.11. Evaluation of Raw Screen Data

Filtering of raw results significantly improves the data quality of the protein interaction set. For filtering, at least three parameters should be considered. *First*, protein interactions that cannot be reproduced in the retest experiment should be discarded. *Second*, for each prey, the number of different interacting baits is calculated. Preys interacting with a large number of baits are assumed to be nonspecific and thus may have no biological relevance. However, the concrete cutoff number depends also on the nature of baits that are screened: if a large family of related proteins is screened, it is not surprising that many of them find the same prey. As a rough guideline, the number of baits interacting with a certain prey should not be larger than 5% of the bait number. The *third* parameter is the background activation activity of the tested bait. The activation strength of interaction pairs must be significantly higher than with all other (background) pairs. In principle, at least with the HIS3 reporter, no activation (i.e., no colony growth) should be observed in noninteracting pairs.

In addition to these 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 qualify the most reliable data (6, 7).

2. Materials

2.1. Yeast Media

2.1.1. YEPD

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. *YEPD 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 media to ~60°C and 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 (see Note 1).

2.1.2. Selective Media

Dropout mix (-His, -Leu, -Trp): 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, 1 g uracil. Mix all components and store under dry conditions at room temperature.

1. *Medium concentrate (5×):* 8.5 g yeast nitrogen base, 25 g ammonium sulfate, 100 g glucose, 7 g dropout mix. Make up to 1 L with water and sterile filter (e.g., Millipore sterile filter). Store at 4°C (see Notes 2 and 3).

2. *Amino acid stock solutions* (see Note 4):
   Histidine (His): Dissolve 4 g of histidine in 1 L water and sterile filter.
   Leucine (Leu): Dissolve 7.2 g of leucine in 1 L water and sterile filter.
   Tryptophan (Trp): Dissolve 4.8 g of tryptophan in 1 L water and sterile filter.

3. *3-Amino-triazole (3-AT) stock solution*: 0.5 M. Sterile filter (see Note 4).

For one liter of minimal medium, autoclave 16 g of agar in 800 ml of water, cool the medium to ~60°C, then add 200 ml 5× medium concentrate, and mix. Pour ca. 40 ml into each sterile Omnitray plate under sterile hood and let them solidify (see Note 1). Depending on the required selective plates, you have to add the missing amino acids or 3-AT. Liquid minimal media can be prepared without adding agar. Corresponding amino acids are added from the amino acid stock solutions as follows (see Note 5):
   Selection of baits (-Trp plates): 8.3 ml leucine and 8.3 ml histidine.
   Selection of preys (-Leu plates): 8.3 ml tryptophan and 8.3 ml histidine.
   Selection of diploids (-Leu-Trp plates): 8.3 ml histidine.
   Readout medium (-Leu-Trp-His plates): Add 3-AT from 0.5 M stock solution as needed for screening self-activating baits.

2.2. Yeast Transformation

1. *Carrier DNA* (salmon sperm DNA): Dissolve 7.75 mg/ml salmon sperm DNA (e.g., Sigma D1626) in water and store at –20°C following a 15 min 121°C autoclave cycle.

2. *96 PEG solution* (100 ml): Mix 45.6 g PEG, 6.1 ml of 2 M LiOAc (lithium acetate), 1.14 ml of 1 M Tris-HCl, pH 7.5, and 232 µl 0.5 M EDTA; make up to 100 ml with sterile water and autoclave. Store at room temperature.

3. *CT110*: Mix 20.73 ml 96 PEG, 0.58 ml boiled salmon sperm DNA (boil frozen salmon sperm DNA at 95°C for 5 min), and 2.62 ml DMSO. Add DMSO last and mix quickly after adding by shaking vigorously and vortexing for 30 s (see Note 6).

2.3. Screen Procedure, Retests, and Bait Self-Activation Test

1. 96-well microtiter plates, round bottom.

2. 1-well plates (Nunc OmniTray plates, Nunc).

3. Bleach solution (20%): Dilute a 12% sodium hypochlorite solution 1:5 with water (see Note 7).

4. 95% ethanol solution, industrial.

5. Autoclaved water.

6. Replication tool or robot (e.g., Biomek, Beckman Coulter), 96- and 384-pinning tool.

7. 1% (w/v) adenine solution (1% in 0.1 M NaOH), sterile filter.

8. YEPD and selective media as liquids and agar plates as described (see Subheading 2.1).

2.4. Vectors (Examples)

1. Bait plasmid(s): pGBKT7 (Clontech), pOBD2 (8), pDEST32 (Invitrogen), pGBKT7g (9), pGBKCg (10), pAS1, pAS2-1 (Clontech).

2. Prey plasmid(s): pGADT7 (Clontech), pDEST22 (Invitrogen), pGADT7g (9), pGADCg (10).

Any other vectors can be used as long as they are compatible with each other and the yeast strains.

2.5. Yeast Strains (Examples)

1. AH109: Genotype (MAT a, trp 1-901, leu2-3,112, ura3-52, his3-200, Δgal4, Δgal80, LYS2: GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3: MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ) (11, 12).

2. Y187: Genotype (MAT α, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, Δgal4, met, Δgal80, URA3: GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ) (13).

3. Methods

The following protocols are described for the *HIS3* reporter and the pGBKT7g and pGADT7g vector system. The protocols are applicable for the pDEST32/pDEST22 system and others as well. However, the different yeast and *E. coli* selection markers have to be considered during the selection steps and the selection media have to be exchanged.

3.1. Yeast Transformation for Bait and Prey Construction

This protocol is suitable for 100 yeast transformations, and may be scaled up or down as needed. 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 under GAL4 control.

1. Inoculate 50 ml YEPD liquid medium with ~200 μl liquid stock of yeast strains (e.g., AH109, Y187, or any other appropriate yeast strain; we use Y187 strains for preys and AH109 for baits) in a 250-ml flask and grow overnight with shaking at 30°C (minimum 15 h, max. 24 h).

2. Spin down cells in 50-ml conical tube (3,500×*g*, 5 min at room temperature), discard supernatant, dissolve the pellet by adding 2 ml LiOAc (0.1 M), and transfer resuspended yeast to two 1.5-ml microfuge tubes. Spin and pellet yeast and resuspend in a total volume of 1.8 ml LiOAc (0.1 M).

3. Prepare CT110 solution.

4. Add all the competent yeast cells prepared above and mix vigorously by hand or by vortexing for 1 min. Immediately pipette 245 μl into each of 96 wells of a 96-well plate.

5. Add 50–100 ng of plasmid or 5 μl of PCR products (in case of cotransformation and homologous recombination in yeast) and positive control (empty vector) and negative control (only CT110). Seal the 96-well plate with plastic or aluminum tape and vortex for 4 min.

6. Incubate at 42°C for 30 min.

7. Spin the 96-well plate for 10 min at 2,000×*g*; discard the supernatant and aspirate with eight-channel wand or by tapping on cotton napkin for a couple of times. Add 150 μl of sterile water to all 96 wells, resuspend, and plate cells on selective agar plates (e.g., standard Petri dishes) with -Leu for pGADT7g or -Trp for pGBKT7g.

8. Incubate the plates at 30°C for 3 days. After 2 days, the colonies start to appear; pick colonies after 3 days.

9. Rearray baits and preys in 96-well plates. Grow them up again for 1–2 days in -Leu or -Trp liquid minimal medium at 30°C.

10. The bait and prey plate can now be used to make a couple of copies on selective agar medium, to back up the arrays as glycerol (25%) stocks for –80°C long-term storage, and to use the baits directly for the self-activation test (see below) (see Note 8).

3.2. Bait Self-Activation Test

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 during the interaction screen and can be achieved by mating individual bait strain with a single prey strain that carries the empty prey plasmid. Ninety-six individual bait activation tests can be carried out on one plate simultaneously.

1. Load a 96-well plate (round button) with ~200 μl YEPD liquid medium.

2. Inoculate plate with baits by replicating the 96-format bait plate from solid medium into the destination plate by using a sterile 96-pinning tool (see step 1 of Subheading 3.3 for sterilization details).

3. Inoculate the yeast strain Y187 which carries the empty prey vector in 30–50 ml YEPD liquid medium.

4. Grow yeast for ~18 h at 30°C (it is not necessary to shake the 96-well plate, whereas shaking of the prey strain in a flask is recommended).

5. Pellet yeast in the 96-well plate by centrifugation for 10 min at 2,000×g; discard the supernatant and aspirate with eight-channel wand or by tapping on cotton napkin for a couple of times.

6. Use 96-replication tool to pin baits from 96-well source plate onto a YEPD single-well agar plate as quadruplicates.

7. Pour the yeast strain with the empty prey vector into a single-well plate.

8. Use 384-replication tool to pin yeast onto the YEPD single-well agar plate that harbors the baits already.

9. Mating occurs at 30°C for 1–2 days.

10. Replicate from mating plate on -Leu-Trp agar single-well plates to select diploids.

11. Incubate for 2–3 days at 30°C.

12. Pin diploids on -Leu-Trp-His agar medium in single-well plates with different concentrations of 3-AT (e.g., 0, 1, 2, 4, 8, …, 128 mM).

13. Select yeast for 7 days at 30°C.

14. Determine minimal inhibitory concentration of 3-AT which is needed for each bait to suppress self-activation growth for use in the interaction screen.

3.3. Yeast Two-Hybrid Screen

1. Preparations
   (a) *Sterilization steps*: Sterilize the pinning tool by dipping the pins into 20% bleach for 20 s, sterile water for 1 s, 95% ethanol for 20 s, and sterile water again for 1 s. Repeat this sterilization after each transfer (see Note 9).
   (b) *Prepare prey array for screening*: Use the sterile replicator to transfer the yeast prey array (e.g., 384 format) from selective plates to single-well plates containing solid YEPD medium and grow the array overnight in a 30°C incubator (maximum 24 h). Ideally, the template prey array should be kept on selective plates (see Note 10).

(c) *Prepare bait liquid culture* (DBD fusion-expressing yeast strain): Inoculate 20–30 ml of liquid YEPD medium in a 50-ml conical flask with a bait strain from plates with selective medium and grow overnight in 30°C shaker for 18–22 h (see Note 10).

2. Mating Procedure

   (a) Add a corresponding volume adenine from a 1% adenine stock solution to a final concentration of 0.004% into the bait liquid culture (see Note 11).

   (b) Pour the overnight liquid bait culture into a sterile Omnitray plate. Dip the sterilized pins of the pin replicator (thick pins should be used to pin baits) into the bait liquid culture and place directly onto a fresh single-well (Omnitray) plate containing solid YEPD media. Repeat with the required number of plates (see Note 12).

   (c) Pick up the prey array yeast colonies with sterilized pins (thin pins (≤1-mm diameter] should be used) and transfer them directly onto the baits pinned onto the YEPD plate so that each of the 384 bait spots per plate receives different prey yeast cells (i.e., a different AD fusion protein) (see Note 13).

   (d) Incubate for 1–2 days at 30°C to allow mating (see Note 14).

3. Selection of Diploids

   For the selection of diploids, transfer the colonies from YEPD mating plates to single-well 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 (see Note 15).

4. Interaction Selection

   Transfer the colonies from -Leu-Trp plates to a single-well 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 + the specific concentration of 3-AT which was determined in the self-activation assay (*see* Subheading 3.2). Incubate at 30°C for 6–10 days.

   Score the interactions by looking for growing colonies that are significantly above background by size and that are present as duplicate (or quadruplicate) colonies (see Note 16). 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.

3.4. Mini-Pooling Screens

This protocol describes pooling of a prey array that contains 960 different clones resulting in one pool plate with 10-prey mini pools. The protocol can be adjusted to bait pools as well or to create larger pools than 10. For this, the corresponding volumes have to be adjusted.

1. Load ten 96-well plates with ~200 µl liquid YEPD medium per well.

2. Inoculate these plates with preys from the prey array grown on -Leu selective agar medium with a sterilized 96-pinning tool.

3. Grow yeast for 18–22 h at 30°C.

4. Resuspend yeast in all wells by pipetting with a 12-channel pipette (see Note 17).

5. Transfer from each source plate 20 µl yeast suspension in the destination pooling plate. Pool, therefore, all preys from source positions A1, A2, …, H12.

6. Once the transfer is done, mix the wells of the pooling plate by pipetting (see Note 17).

7. Spin the pooling plate for 10 min at $2,000 \times g$.

8. Discard the supernatant and aspirate with eight-channel wand or by tapping on cotton napkin a couple of times.

9. Pin yeast with a sterile 96-pinning tool from the prey pool plate onto YEPD solid medium as, e.g., quadruplicates.

Mating of a single bait and selection steps follow as described in the previous protocol (Subheading 3.3). The prey pooling plate can be used directly to be mated against ten different baits by using thick pins (≥1 mm). Then, the pools are depleted. The pooling plate can be stored as –80°C glycerol culture and grown up again as master plate for further screens. Although the preparation time is quiet intensive (because of the pipetting steps), we recommend always to prepare fresh master plates. So, equal cell numbers of single clones in the pooling plates can be assured.

Preys can be pooled in different ways, e.g., pooling columns, rows, or similar positions from different source plates. In the simplest case of pooling screens, the interacting prey of a positively tested pool can be identified by retesting by one-on-one retest assays (see the next protocol).

The situation becomes more complicated if preys must be rearrayed for "smart" pooling as described in a separate chapter. For large libraries and to ensure equal cell numbers, preys can be picked by a robot which has a picking and rearraying function (e.g., Q-bot, Genetix).

3.5. Retests

Testing for reproducibility of interactions greatly increases the reliability of the interaction data. This protocol is used for specifically retesting interaction pairs detected in a one-on-one or pooling screen.

1. Rearray bait and prey strains or positively tested prey pool of each interaction pair to be tested in 96-well microtiter plates. Use an individual 96-well plate for the baits, as well as for the preys. For each retested interaction, fill one well of the bait plate and one corresponding well of the prey plate with ~200 µl YEPD.

2. For each retested interaction, inoculate the bait strain into a well of the 96-well bait plate and the prey strain at the corresponding position of the 96-well prey plate.

   For example, bait "X" is transferred at positions B1, B2, and B3 of the bait plate. The preys to be tested are arrayed into B1 (prey "Y"), B2 (prey "Z"), and the prey strain that carries the empty prey vector into B3 of the prey plate. The B3 test position is the control that helps to verify the background/self-activation.

3. Incubate the plates O/N at 30°C.

4. Spin the bait and prey plates for 10 min at $2,000 \times g$.

5. Discard the supernatant and aspirate with eight-channel wand or by tapping on cotton napkin a couple of times.

6. Pin baits with a sterile 96-pinning tool on -Trp and preys on -Leu selective agar medium as quadruplicates.

7. Allow baits and preys to grow at 30°C for 2–3 days.

8. Mating: First, transfer baits with a sterile 384-pinning tool on YEPD mating plates. Second, transfer preys onto baits.

The rest of the procedure can be done according to the screening protocol (Subheading 3.3). For interaction retesting, diploids are pinned on -Leu-Trp-His selective media plates with different concentration of 3-AT (see Note 18). The control test position has to be compared to bait self-activation background signals. Reproducible interactions should show up on different concentrations of 3-AT, whereas the activation control test position indicates clearly no colony growth.

4. Notes

1. Prepared agar plates should be stored for 1–2 days with closed lid under a sterile hood before used. Fresh solidified media is often wet and cannot be used directly.

2. Medium concentrate can be stored at 4°C up to 6 months.

3. Some components of the medium concentrate (e.g., amino acids) are not well-soluble in water. The solution has to be stirred before the filtration step for up to 5 h until all components are dissolved. Thereby, heating is not recommended because of the heat sensitivity of amino acids.

4. Stock solutions can be stored up to 6 months at 4°C. Alternatively, the stock solutions can be frozen as aliquots at –20°C for long-time storage.

5. Selection media may differ due to the used Y2H expression vector system and have to be adapted. For instance, in the

pDEST32/pDEST22 system, the selection markers for baits and preys are interchanged (baits are selected on -Leu and preys on -Trp) while selection of pGBKT7g baits is done on -Trp. pGADT7g preys must be selected on -Leu medium.

6. CT110 has to be prepared fresh before yeast transfection and should not be stored.

7. Sodium hypochlorite solution is not very stable and has to be prepared every day. Alternatively, other disinfection solutions with a bleaching effect can be used. We do not recommend to use higher final concentrations than 2.4% since the steel pins of the replication tool might stain.

8. Yeast on agar medium can be stored for ~2 months at 4°C. The plates should be sealed with parafilm to avoid drying out. Baits and preys should be stored on the corresponding selective media since loss of plasmids can occur on nonselective medium.

9. Sterilization steps have to be established to the robotic system and sterilization solutions that are used. For example, in advance, it should be tested what the minimal time for the sterilization is since this speeds up the whole screen. However, it must be ensured that no cross contamination occurs.

10. The needed baits and prey arrays can also be used for the mating procedure when grown on/in selective medium. To our knowledge, this does not influence the mating efficiency much, but we recommend to use here YEPD medium since yeast grows faster and higher cell numbers can be achieved.

11. Adenine achieves a higher mating efficiency. Many yeast strains (e.g., AH109, Y187) are deficient in synthesizing adenine since they can carry an additional adenine selection marker.

12. After transfer from the liquid culture, allow the plates to dry for ~30 min. The positions should be dry when the preys are copied onto the bait spots. Also the plate should be checked if enough bait cells were transferred. Reasonable amounts were transferred when each spot occurs cloudy. This is critical for a good mating efficiency.

13. Thick pins can be used as well. We use thin pins since more replication steps can be done from a single source plate. If only a replication tool with thick pins is available, more prey array plates have to be prepared since only a couple of transfer steps can be done regarding source plate depletion.

14. Mating takes place in <15 h, but a longer period is recommended because some bait strains show poor mating efficiency.

15. This step is an essential control step because only diploid cells containing the *Leu2* and *Trp1* markers on the prey and bait vectors, respectively, grow on this medium. This step also helps recovery of the colonies and increases the efficiency of the interaction selection step.

16. We normally score interactions after 7 days. But 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 (i.e., >30) large colonies per array of 6,000 positions, we consider these baits as "random" activators. In this case, the screen should be repeated to ensure that these positives are reproducible (unless the screen is done already in duplicate or quadruplicate).

17. Alternatively, the cells can be resuspended by vortexing. To save pipet tips the cells can also be resuspended by vortexing. However, if vortexing is used we recommend to seal the plate with adhesive tape since rigorous vortexing may cause cross-contamination.

18. Pinning the retest onto readout medium with various concentrations, 3-AT can be used to semiquantify interactions. This helps, e.g., to distinguish between "strong" and "weak" signals and might also help to separate spurious ones.

Acknowledgments

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