Yeast One- and Two-Hybrid High-Throughput Screenings Using Arrayed Libraries

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Abstract

Since their original description more than 25 years ago, the yeast one- and two-hybrid systems (Y1H/Y2H) have been used by many laboratories to detect DNA–protein (Y1H) and protein–protein interactions (Y2H). These systems use yeast cells (*Saccharomyces cerevisiae*) as a eukaryotic "test tube" and are amenable for most labs in the world. The development of highly efficient cloning methods has fostered the generation of large collections of open reading frames (ORFs) for several organisms that have been used for yeast screenings. Here, we describe a simple mating based method for high-throughput screenings of arrayed ORF libraries with DNA (Y1H) or protein (Y2H) baits not requiring robotics. One person can easily carry out this protocol in approximately 10 h of labor spread over 5 days. It can also be scaled down to test one-to-one (few) interactions, scaled up (i.e., robotization) and is compatible with several library formats (i.e., 96, 384-well microtiter plates).

1 Introduction

Molecular interactions are required to ensure proper function of every cell and thus growth and development in living organisms. In particular, interactions between nucleic acids and proteins are an integral part of the regulatory mechanisms controlling gene expression. Transcriptional regulation is an important mechanism underlying gene expression that is mediated by short DNA sequences (*cis*-elements) located in gene promoters that are bound by transcription factor (TF) proteins. Combinatorial interactions between different *cis*-elements and their corresponding TF proteins at a given promoter will render a specific transcriptional output. To reveal the complexity of this regulation as well as to study other interactomes not directly involved in the regulation of gene expression, the yeast one-hybrid (Y1H; Fig. 1a) and two-hybrid (Y2H; Fig. 1b) systems have been successfully and extensively used for detecting

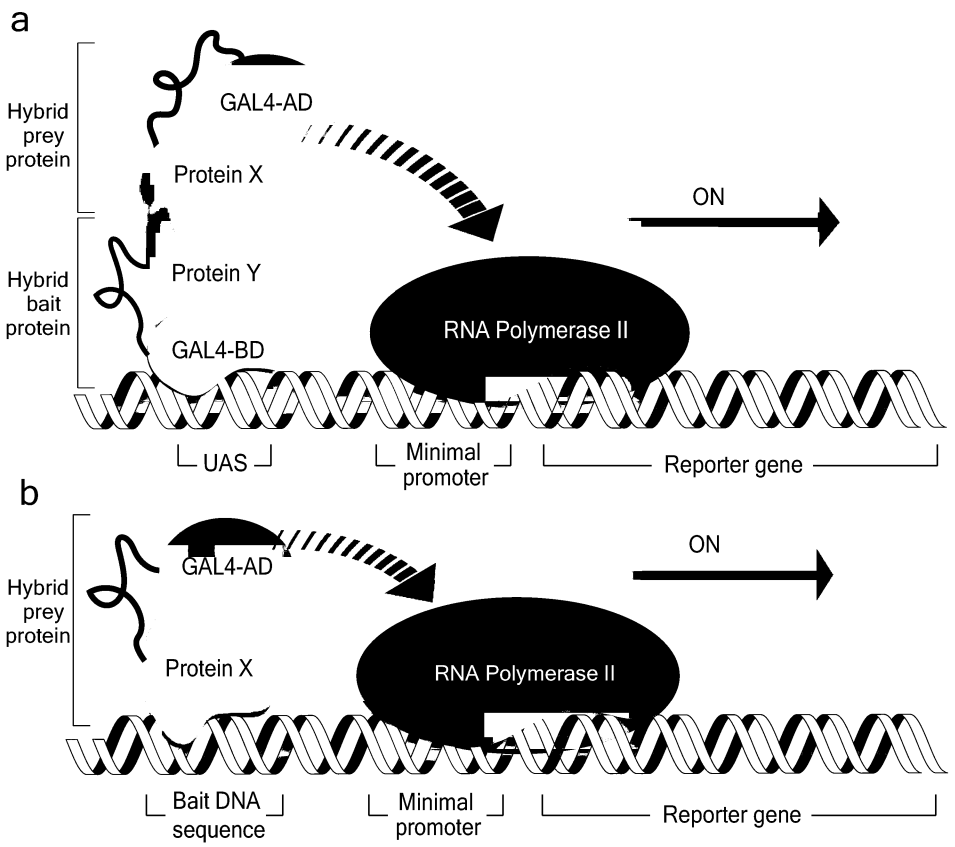

Fig. 1 Overview of the yeast one (Y1H) and two hybrid (Y2H) systems. (**a**) In the Y2H system, a coding sequence for a protein of interest (protein Y) is cloned in frame with the GAL4 binding domain (GAL4-BD) to produce a hybrid protein (Bait). Then, the coding sequence of another protein of interest (protein X) is cloned in frame with the GAL4 activation domain (GAL4-AD) to produce a hybrid protein (Prey). Both constructs are introduced in the same yeast cells where the reporter gene is under the control of a GAL4 binding DNA sequence (upstream activating sequence; UAS) that will be bound by the bait. Transcription of the reporter gene will be enhanced if prey and bait proteins interact. The proteins of interest may be known (i.e.: arrayed ORF libraries or in one-to-one interactions) or unknown (i.e.: cDNA libraries). (**b**) In the Y1H system, the bait construct is obtained by cloning a DNA sequence of interest (Bait DNA sequence) upstream of a reporter gene carrying a minimal promoter. Both bait DNA and prey constructs are introduced in the same yeast cells where transcription of the reporter gene will be activated only if the prey binds the DNA bait

DNA–protein and protein–protein interactions, respectively. These systems use yeast cells (*Saccharomyces cerevisiae*) as a convenient eukaryotic in vivo "test tube" that require little specific optimization for each interaction compared to other approaches, and are more likely than in vitro methods to provide an appropriate environment for interactions that depend on posttranscriptional modifications. The basic principle relies on the observation that many TFs have a modular structure, with a DNA-binding domain (DBD) and a transactivation domain (AD) that can be physically separated

while retaining their functions [1, 2]. In a classic Y2H system ([3]; Fig. 1a), a given protein (protein of interest X) is fused to the DBD of the yeast TF GAL4 (GAL4-BD) while a second one (protein of interest Y) is fused to the AD of the GAL4 TF (GAL4-AD). The resulting hybrid proteins are called bait (GAL4-BD-protein of interest X) and prey (GAL4-AD-protein of interest Y), respectively. If the bait and prey proteins are able to interact when expressed in the same yeast cells, the GAL4-BD and GAL4-AD will be brought into close proximity, enabling reconstitution of a fully functional GAL4 TF. When using appropriate yeast strains, the reconstituted GAL4 TF will bind (by its BD domain) GAL4-specific sequences present in the promoters of reporter genes and activate (by its AD domain) their expression (Fig. 1a). The Y1H system (Fig. 1b) is conceptually similar to the yeast two-hybrid system [4–8]. In this case, the bait construct contains a reporter gene under the control of a DNA sequence of interest. The interaction of a prey protein with the DNA bait will activate the expression of the reporter gene (Fig. 1b). In both Y2H and Y1H systems, the positive effect of the GAL4-AD on the transcription of the reporter gene is dominant over the transcriptional properties that may have proteins of interest fused to this domain (a repressor domain, absence of regulatory domains, etc.), which enable the identification of a wider range of interactions [1, 2].

Since the original description of the Y1H and Y2H systems, several variations and refinements have allowed to enlarge the range of interactors that can be tested as well as to increase the throughput [9–14]. Basically, the most common considerations in the design of the experimental setup are as follows (for recent reviews *see* [15, 16]): yeast strains, reporter genes, method to introduce constructs in yeasts (transformation or mating), plasmid types (episomal, high or low copy or integrated) and cloning method (restriction enzymes or recombination). Apart from these considerations, the type of DNA sequence (size and multimerization) selected as bait for Y1H can be critical in determining the outcome of the experiment (*see* **Note 1**). Also, in Y2H assays, protein baits containing strong transactivation domains may impede detection of further increases in activation levels of the reporter gene upon interaction with a prey (*see* **Note 2**).

In plants, collections of open reading frames (ORFs), several of them dedicated to TFs, have been generated and used in Y1H and Y2H screenings [17–27]. Here we describe a simple method for high-throughput screenings of arrayed ORF libraries with DNA (Y1H) or protein (Y2H) baits not requiring robotics. This methodology has been used to screen an Arabidopsis library of TFs (ca. 1,200 Arabidopsis TFs arrayed in fifteen 96-well microtiter plates; available at the Nottingham Arabidopsis Stock Centre) with DNA or protein baits [22, 28–32]. Particularly successful

have been the Y1H assays by using DNA promoter sequences identified by phylogenomic analyses, since this approach appears to filter genetic redundancy [22, 29, 30]. The liquid assay described here is compatible with Y1H and Y2H screenings of arrayed ORF libraries and requires approximately 10 h of labor spread over 5 days. Briefly, after introducing bait constructs into sexually compatible haploid yeast strains (α mating type if preys are in "a" mating type), mating is used to combine bait and prey constructs in the same yeast cells (diploids). After mating, liquid cultures are scored for diploids according to their ability to grow on selective solid media (positive interactions). Diploid colony size can be taken into account to compare and normalize the strength of positive interactions, a very useful feature if only a fraction of the positives can be chosen for further characterization. Another useful aspect of the arrayed libraries is that non-mating clones can be flagged as not screened. Additionally, more complex matrix interaction schemes involving several baits can be performed, and diploid cells can be stored at 4 °C and respotted at any time on different types of screening plates (i.e., containing hormones or other chemicals) to reevaluate positive and negative interactions from the initial screening. Other yeast strains carrying bait constructs could be adopted to be compatible with screening ORF libraries prepared in different yeast strains to the one used here. In this regard, our methodology can be adapted to other library formats (i.e., 384-well microtiter plates) and scaled up (i.e., robotization, increasing man power). Finally, this protocol can be easily scaled down to titrate bait activation of the reporter gene, to test one-to-one (few) interactions and to confirm positive interactions.

2 Materials

2.1 Media and Reagents

Prepare all media using deionized water. Add adenine hemisulfate (30 mg/L; *see* **Note 3**) to minimal and YPAD media. If preparing solid media, add 20 g/L agar. Unless otherwise indicated, sterilize by autoclaving media for 20 min at 120 °C. We store media at 4 °C in darkness or subdued light.

1. Minimal media (*see* **Note 4**): Dissolve 26.7 g/L of dropout base medium (DOB; it contains 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 20 g glucose) with the appropriate amount of a complete supplement mixture (CSM; MP pharmaceuticals) of amino acids lacking the one/s used for auxotrophic selection: 0.69 g/L CSM-leucine (CSM-L); 0.74 g/L CSM-tryptophan (CSM-W); 0.64 g/L CSM-leucine-tryptophan (CSM-L-W); 0.62 g/L CSM-leucine-tryptophan-histidine (CSM-L-W-H).

The pH does not need to be adjusted but it should be in the 5–5.5 range. Autoclave for only 10 min at 120 °C (*see* **Note 5**).

2. YPAD: Dissolve 20 g/L peptone, 10 g/L yeast extract, adjust pH to 5.8 with HCl, top up to 950 ml with water and autoclave. When the media cools down to 50–60 °C, add 50 ml of a 40% w/v glucose solution (2% final concentration).
3. Glucose 40%: Dissolve 40 g glucose in 100 ml water and autoclave.
4. 3-amino-1,2,4-triazole (3-AT; *see* **Note 6**): Dissolve the appropriate amount of 3-AT in water to obtain a 2 M solution (i.e., 4.2 g in 25 ml water). Sterilize by filtration (0.45 or 0.22 μm pore size) and store this stock solution at −20 °C. When required, the appropriate amount of the 3-AT stock solution (depending on the desired final concentration) should be added to autoclaved minimal media (DOB-L-W-H) once it has cooled down to 50–60 °C.
5. Yeast transformation solution (PATE): Prepare and autoclave stock solutions of 50% w/v polyethylene glycol 4000 (PEG; *see* **Note 7**), 1 M lithium acetate (LiAc) and 10×Tris–EDTA pH: 8 (10×TE: 100 mM Tris–HCl, 10 mM disodium EDTA, pH 8.0 with HCl). To prepare the PATE solution, mix the stock solutions to obtain 40% w/v PEG 4000, 0.1 M LiAc and 1×TE.
6. Salmon sperm DNA (SsDNA): To prepare a 10 mg/ml stock salmon sperm DNA (carrier DNA) dissolve 10 mg of type III salmon sperm DNA (sodium salt) in 1 ml of water (it is recommendable to leave the mix dissolving overnight). Pass the solution vigorously through a gauge 21 needle (21G) 20 times to shear DNA. Sonicate in an ice–water bath until the viscosity of the solution decreases (~3–4 min), boil it for 10 min in a water bath and cool it down quickly by transferring it to ice. Store at −20 °C in small aliquots (i.e., 100 μl).
7. Glycerol 50%: Mix equal volumes of glycerol and water and autoclave.
8. Absolute ethanol (*see* **Note 8**).
9. Yeast plasmid miniprep kit (Zymoprep yeast plasmid miniprep II; Zymoresearch) or equivalent protocol.
10. Oligonucleotides: LO1040 (5′-CACGAGGCCCTTTCGTC TTC-3′), LO1041 (5′-TTCTTCGAAGAAATCACATTAC-3), LO1218 (5′-TATAACGCGTTTGGAATCACT-3′), LO1219 (5′-AGCCGACAACCTTGATTGGAGAC-3′) and LO1537 (5′-TCATCGGAAGAGAGTAGTAA-3).
11. Yeast strains: Tables 1 and 2.
12. Plasmids: Table 3.

Table 1
***Saccharomyces cerevisiae* strain genotypes**

Strain (mating type)	Genotype	Reporters	Auxotrophy/ Transformation markers	Reference
Y187 (α)	*MAT* α, *ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ, *met-, gal80*Δ, *MEL1, URA3::GAL1UAS-GAL1TATA-lacZ*	MEL1, LacZ	trp1, leu2, his3, ade2, met2	[33]
pJ694 (α)	*MAT* α, *trp1-901 leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ*	His3, Ade2, LacZ	trp1, leu2, ura3, met2	[34]
YM4271 (a)	*MAT* a , *ura3-52, his3-200, ade2-101, ade5, lys2-801, leu2-3112, trp1-901, tyr1-501, gal4* Δ, *gal80* Δ, *ade5::hisG*		trp1, leu2, his3, ura3, lys2	[35]

Table 2
Strain growth on different minimal media

Strain	–His	–Leu	–Trp	–Met	–Ade	–Ura
Y187	–	–	–	–	–	+
pJ694	–	–	–	–	–	–
YM4271	–	–	–	+	–	–

Growth in minimal media with a complete supplement of amino acids/nucleotides minus the indicated one (His: Histidine; Leu: Leucine; Trp: Tryptophan; Met: Methionine; Ade: Adenine; Ura: Uracil)

Table 3
Plasmids used for Y1H and Y2H assays

Plasmid	Bacterial selection	Yeast selection	Cloning	Reference
pTUY1H (DNA bait for Y1H)	Ampicillin	Leucine (L)	*XmaI-XbaI*	[22]
pDEST32 (protein bait for Y2H)	Gentamicin	Leucine (L)	Gateway	Invitrogen
pDEST22 (protein prey for Y1H and Y2H)	Ampicillin	Tryptophan (W)	Gateway	Invitrogen

2.2 Disposables and Small Equipment (See Fig. 2)

1. 120 mm square petri plastic plates.
2. 96-well plates (standard sterile clear plates with lid and flat bottom; ~300 µl max. volume/well).
3. Surgical tape.
4. Parafilm tape.
5. Multichannel pipettes (electronic or manual 12- or 8-channel pipettes to dispense volumes in the range of 100–250 µl.
6. 96-well replicator (*see* **Note 9** and Fig. 2 bottom).
7. Erlenmeyer flasks (500 ml or 1 L).
8. Standard shaker or microtiter plate shaker (*see* **Note 10**).
9. Laminar flow cabinet.
10. Optional: 90 mm diameter petri plates, cryogenic vials.

Fig. 2 Photographs of disposables and small equipment required for screening arrayed ORF libraries. *Top*: 500 ml Erlenmeyer flask (*1*); 90 mm diameter petri plate (*2*); electronic 8-channel pipette (*3*); cryogenic vial (*4*); surgical tape (*5*); 96-well replicator (*6*); reagent reservoir (*7*); 96-well microtiter plate with lid (*8*); Parafilm tape (*9*); 120 mm square petri plate (*10*). *Bottom*: Close-up picture of the custom-made replicator

3 Methods

Manipulation of yeasts should always be done under sterile conditions (i.e., laminar flow cabinet).

3.1 Preparation of DNA and Protein Baits for Y1H and Y2H Screenings

1. Bait for Y1H: Prepare a construct with your **bait DNA** sequence in the pTUY1H plasmid (Table 3; *see* **Notes 1** and **11**; [22]). For sequencing or PCR amplification of DNA baits in the pTUY1H plasmid, the oligonucleotides LO1040 (forward) or/and LO1041 (reverse) can be used. Introduce this construct (DNA-bait) into *S. cerevisiae* Y187 (α mating type; Tables 1 and 2; [33]) and select transformants in DOB-L plates (*see* Subheading 3.2).
2. Bait for Y2H: Prepare a construction with your **bait protein** coding sequence in the pDEST32 gateway plasmid (Invitrogen; Table 3; *see* **Notes 2** and **12**). For sequencing or PCR amplification of ORF baits in the pDEST32 plasmid, the oligonucleotides LO1537 (forward) or/and LO1219 (reverse) can be used. Introduce this construct (bait) into *S. cerevisiae* pJ694 (α mating type; Tables 1 and 2; [34]) and select transformants in DOB-L plates (*see* Subheading 3.2).
3. Preys for Y1H and Y2H: As a prey negative control and to titrate your baits, prepare another construct with the coding sequence of an unlikely protein interactor (i.e., the GFP coding sequence) in the pDEST22 plasmid (Invitrogen; Table 3; *see* **Notes 2** and **12**). For sequencing or PCR amplification of ORF preys in the pDEST22 plasmid, the oligonucleotides LO1218 (forward) or/and LO1219 (reverse) can be used. Separately, introduce this construct (AD-GFP) and the empty pDEST22 plasmid (AD-empty) into *S. cerevisiae* YM4271 ("a"mating type; Tables 1 and 2; [35]) and select transformants in DOB-W plates (*see* Subheading 3.2). If available, generate a prey strain with the pDEST22 plasmid containing an ORF known to interact with your bait, as a positive control.

3.2 Yeast Transformation (Modified from [36])

1. Streak a YPAD plate with the appropriate yeast strain from a frozen stock and incubate for 48 h at 28 °C (incubation over a weekend is also fine).
2. Inoculate 5 ml of liquid YPAD with a fresh colony and grow for 24 h at 28 °C with shaking.
3. Fill an eppendorf tube with the grown liquid culture (usually having OD_{600} ~ 1.5–2) and centrifuge at 3500 rcf for 2 min. Discard supernatant and repeat this step until a pellet of ~50 μl of yeast cells is obtained (~3 ml of culture). After the last centrifugation, pour off supernatant by inverting the tube and remove excess liquid by gently stroking the eppendorf twice in

an inverted position. From now on, mixing should be done with the researcher's fingertips by flicking the bottom of the eppendorf tube. Resuspend yeast cells in the remaining liquid (~100 µl total volume), add freshly denatured (10 min at 95 °C and chilled in ice for 2 min) carrier DNA (usually 10–20 µl of a 10 mg/ml salmon sperm DNA stock; *see* **Note 13**) and mix; add between 0.5–1 µg of plasmid DNA (≤20 µl) and mix; add 500 µl PATE solution and mix. Incubate the tube overnight (*see* **Note 14**) at room temperature in darkness (i.e., inside a drawer).

4. Centrifuge yeast cells at 3500 rcf for 2 min, remove completely the supernatant with the help of a pipette, and resuspend the cells in 1 ml sterile water. Repeat this step twice and resuspend the cells in 150 µl sterile water after the second repetition (if required, pipetting up and down can be used to help with cell resuspension).
5. Plate yeast cells onto appropriate auxotrophic minimal media for positive selection of cells carrying the introduced plasmid. Colonies will appear after 48 h incubation at 28 °C (incubation over a weekend is also fine).

3.3 Titrating Bait Autoactivation of the HIS3 Reporter Gene Before the Screening (See Note 15 *and Fig. 3a)*

This titration protocol can also be used to test one-to-one (few) interactions.

1. Pick several colonies (3–5) from transformation plates and streak them onto new plates of the appropriate minimal media. After 48 h incubation at 28 °C, use fresh colonies to inoculate 10 ml tubes containing 2 ml YPAD each (tube 1: bait strain; tube 2: AD-GFP prey strain; tube 3: AD-empty prey strain). Grow at 28 °C with shaking for 24 h (overnight is usually enough).
2. Mating: Mix 500 µl of the bait culture with 500 µl of the prey cultures (tube 1 + tube 2 and tube 1 + tube 3) in sterile eppendorf tubes and incubate 24–48 h at 28 °C without shaking (24 h is usually enough to obtain diploids).
3. Enrichment for diploid cells: Use 200 µl of the mated cultures to inoculate 2 ml of liquid DOB-L-W media in 10 ml tubes. Grow at 28 °C with shaking for 48 h (24 h is usually enough).
4. Plating diploid cells (*see* **Note 16**): Prepare two serial dilutions from the diploid enriched cultures (10^{-1} and 10^{-2}). Plate 5 µl drops of each dilution and undiluted culture onto the following agar media: DOB-L-W (for quantification of diploid cells), DOB-L-W-H ± 3-AT (for quantification of bait activation of the reporter gene). Incubate plates at 28 °C and score yeast growth over the next 7 days after plating.

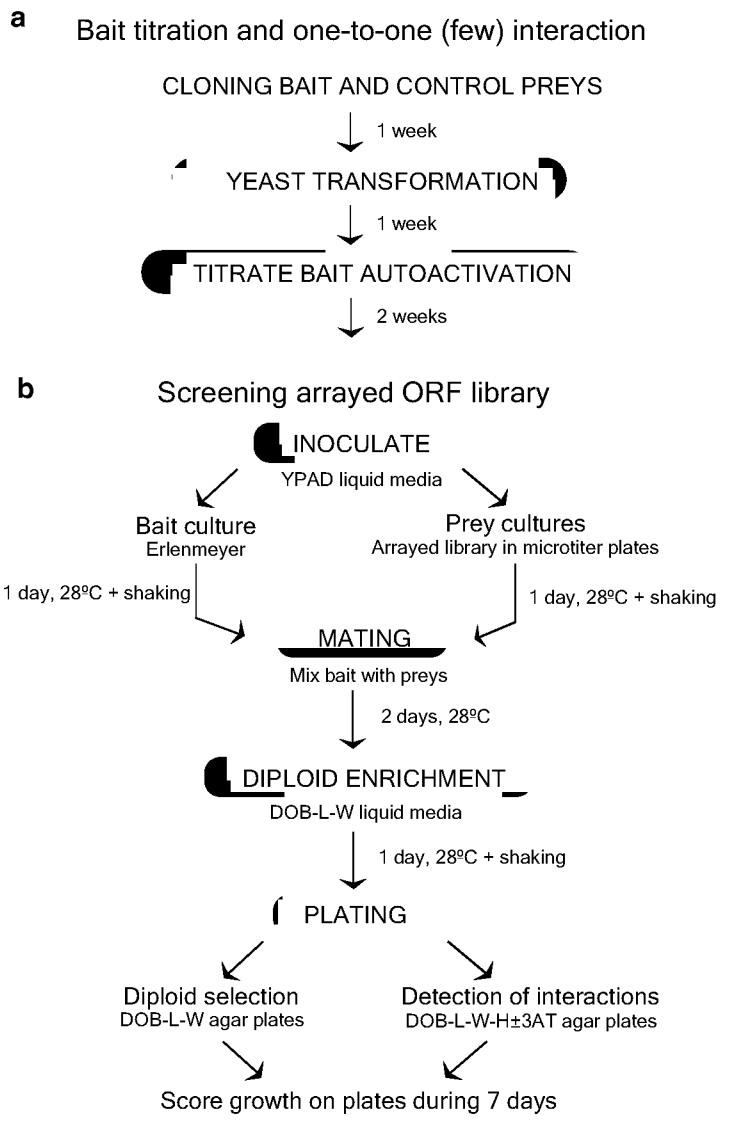

Fig. 3 Outline for bait titration and screening methods. (**a**) After cloning bait (DNA or protein ORFs) and preys (control and proteins of interest ORFs) into appropriate plasmids, they are introduced into sexually compatible yeast strains (yeast transformation), which are mated to obtain diploids that are tested for activation of the reporter gene. Conditions that block reporter activation in yeast cells containing bait and control preys should be chosen for the screening (*see* Subheading 3.3). The bait titration procedure can also be used for testing one or few selected interactions. (**b**) Bait and ORF library clones growing on plates with their corresponding auxotrophic media (DOB-L for baits and DOB-W for preys), are used to inoculate Erlenmeyer flasks and 96-well microtiter plates, respectively, containing YPAD. After 24 h incubation, bait and preys are then mixed and incubated for 48 h to allow mating. Mated cells are used to inoculate 96-well microtiter plates with DOB-L-W for diploid enrichment. After incubation for 24 h, diploid cells are replica-spotted onto diploid (DOB-L-W) and screening (DOB-L-W-H ± 3-AT) plates. The 3-AT concentration required to block reporter activation in the absence of a true interaction should have been previously determined as indicated in (**a**). Positive interactions (growth on screening plates) should appear between 1 and 7 days of incubation (*see* Subheading 3.4). Typically, the total time from inoculation of bait and preys until final screening results are obtained is about 12 days

3.4 Screening Yeast Arrayed Libraries (See Note 17 and Fig. 3b)

Always sterilize the replicator by flaming with absolute alcohol in-between handling of different plates. Modifications to screen independent baits simultaneously are indicated in bold.

1. Day 0: Using a 96-well replicator (Fig. 2 bottom), make a replica of the library (15 × 96-well plates) on DOB-W square agar plates (15 square plates). Also, streak one (**two**) DOB-L plate with the (**two**) bait strain. Incubate all plates at 28 °C for 48 h (weekend incubations are also fine).
2. Day 1: Dispense YPAD in a sterile reagent reservoir and, using a multichannel pipette, aliquot 125 µl (**250 µl**) of YPAD into each well of 96-well microtiter plates (15 microtiter plates). By using a replicator, inoculate 96-well microtiter plates with the library prey strains grown on the DOB-W square agar plates. Cover microtiter plates with the lid and seal them with surgical tape. Shake (250 rpm; *see* **Note 10**) and incubate at 28 °C for 24 h. In parallel, inoculate one (**two**) 0.5-1 L Erlenmeyer containing 200 ml of YPAD with a clump of bait cells (5–10 colonies) from the DOB-L plate and incubate for 24 h at 28 °C with shaking (200 rpm).

   Media and disposables for day 1: 12 ml (**24 ml**) YPAD/96-well microtiter plate (180 ml for 15 microtiter plates; **360 ml**), 200 ml (**400 ml**) YPAD/bait and one sterile reagent reservoir.
3. Day 2 (mating): **Using a multichannel pipette, transfer 100 µl of culture from each well of the microtiter plates from day 1, to a second set of 15 × 96-well microtiter plates (pipette up and down two or three times to resuspend any settled cells at the bottom of the wells before transferring any liquid to a new plate).** Dispense the YPAD culture for the bait into a sterile reagent reservoir and, using a multichannel pipette, add 100 µl to each well of the 15 × 96-well microtiter plates from day 1 (**dispense the YPAD culture for the second bait into the wells of the second set of 15 × 96-well microtiter plates**). Incubate 2 days at 28 °C without shaking (weekend incubations are also fine).

   Media and disposables for day 2: One sterile reagent reservoir (**15 × 96-well microtiter plates and two reagent reservoirs**).
4. Day 4 (diploid enrichment): Dispense DOB-L-W into a sterile reagent reservoir and, using a multichannel pipette, add 200 µl to each well of new 15 × 96-well microtiter plates (**30 × 96-well microtiter plates**). Resuspend settled mated cells in the 15 × 96-well microtiter plates (**30 × 96-well microtiter plates**) from day 2, by hitting the bottom of the wells with the pins of the replicator, and use it to inoculate the new 15 × 96-well DOB-L-W microtiter plates (**30 × 96-well microtiter plates**) previously prepared. Shake (2.8 rcf; *see* **Note 10**) and incubate at 28 °C for 24 h (48–72 h are also fine).

Media and disposables for day 4: 19.2 ml DOB-L-W/96-well plate (288 ml for 15 × 96-well microtiter plates; **576 ml for 30 × 96-well microtiter plates**), 15 × 96-well microtiter plates (**30 × 96-well microtiter plates**) and one sterile reagent reservoir.

5. Day 6: Resuspend any settled cells by hitting the bottom of the wells with the pins of the replicator and, for each 96-well DOB-L-W microtiter plate from day 4, inoculate two square agar plates, one with DOB-L-W (diploid plates) and another with DOB-L-W-H ± 3-AT (screening plates; the concentration of 3-AT required to block bait activation of the *HIS3* reporter should have been previously determined in the previous section; Subheading 3.3). Close square agar plates with their corresponding lids and seal them with Parafilm once the droplets left by the replicator dry out. Incubate the plates at 28 °C and score yeast growth for 7 days (*see* **Note 18**).

   Media and disposables for day 6: 15 (**30**) × DOB-L-W agar square plates and 15 (**30**) × DOB-L-W-H ± 3-AT agar square plates. **In case both baits require different 3-AT concentrations to block autoactivation of the *HIS3* reporter gene, two sets of DOB-L-W-H plates, each with the appropriate 3-AT concentration, will have to be prepared.**

3.5 Confirming Positive Interactions and Quantifying Strength

1. Use diploid cells able to grow in screening plates to inoculate 5 ml DOB-W and incubate for 48 h at 28 °C with shaking (standard shaker).
2. These cultures can now be used to isolate the AD-prey plasmid responsible for the interaction and the activation of the reporter gene. A yeast plasmid miniprep kit (we routinely use the Zymoprep yeast plasmid miniprep II; Zymoresearch) or any other appropriate procedure can be used for this purpose.
3. The isolated plasmid should be transformed in *Escherichia coli* and reisolated from the bacteria in order to sequence the prey ORF with the oligonucleotides LO1218 (forward primer annealing near the C-terminal region of the GAL4-AD in the pDEST22 plasmid) and/or LO1219 (reverse primer annealing downstream of the gateway region in the pDEST22 plasmid).
4. This plasmid should be reintroduced into the YM4271 yeast strain (*see* Subheading 3.2 for a transformation protocol) and the mating with the bait strain repeated. Alternatively, the plasmid may be directly introduced into the bait strain by transformation (*see* **Note 19**).
5. The resulting yeast strains should be analyzed as described in the titration section (from **step 3** of Subheading 3.3) in order to confirm the interactions and their strengths (a more detailed and narrow range of 3-AT concentrations can now be used).

3.6 Making Yeast Glycerol Stocks for Long Term Storage

To avoid maintenance of yeast cells by repeatedly refreshing colonies in agar plates stored at 4 °C, permanent stocks can be prepared by following the steps described in this section.

1. Spread 100–200 μl of a grown culture of the yeast strain onto an appropriate minimal media plate. Alternatively, you can streak a generous quantity of cells evenly over the plate. A lawn of cells should be seen after incubation for 48 h at 28 °C.
2. Collect grown cells (usually a quarter of a 90 mm diameter petri plate is enough) from the agar plate by rubbing them with a sterile loop or pipette tip. Resuspend the cells in a cryogenic vial containing 800 μl sterile water (YPAD or the corresponding selection media is also fine) and 1.2 ml 50% glycerol (30% final concentration). Shake or/and vortex for homogeneous cell suspension.
3. Store the vial directly at −80 °C for long-term storage (*see* **Note 20**). Frozen stocks can be refreshed on the appropriate auxotrophic media or YPAD.

4 Notes

1. Try using promoter fragments not much longer than 100 bp. *S. cerevisiae* genome is more compact than those of plants and it is known that for *U*pstream *A*ctivating *S*equences (UAS) located over 300 bp upstream of a reporter gene, transcription initiates proximally to the UAS and competes with that derived from the reporter gene located downstream [37]. In our hands, the sensitivity of the assay is greatly reduced when the bound DNA sequence is in the context of a long promoter fragment (i.e., an 80 bp sequence in a 400-bp promoter fragment). Also, using multimerized sequences tends to give higher backgrounds than using just one copy of the selected DNA sequence (L. Oñate-Sánchez, unpublished results).
2. Protein baits that activate the reporter gene too strongly may not be amenable for classic Y2H assays since they may hinder detection of higher activation levels of the reporter gene due to a positive interaction. A possibility is to use a truncated version of the protein with reduced activation levels, although the possibility that the deleted portion of the protein may be involved in interactions cannot be ruled out. An alternative is to use a different system in which the activation of the reporter gene does not directly rely on the transcriptional properties of the interactors (i.e., [38, 39]).
3. All yeast strains used here carry the *ade2–101* mutation. If grown on normal media not supplemented with adenine (low in adenine), the colonies will develop a pink or red color due

to the accumulation of a pigment derivative of 5-aminoimidazole ribotide in vacuoles [40, 41]. Adding adenine hemisulfate (30 mg/L) to the medium, will enhance their growth.

4. We purchase DOB and CSMs from MP pharmaceuticals since it reduces labor and variability between media batches (*see* http://www.mpbio.com/ for a more detailed formulation of CSMs). Other suppliers are possible but, in one occasion, different results were obtained when comparing DOB and CSMs with media from a different supplier.
5. We have found that autoclaving minimal media longer times produces browning of the media resulting in poor yeast growth.
6. This is a toxic substance and requires using personal protective equipment. 3-AT is a competitive inhibitor of the product of the *HIS3* reporter gene.
7. We always use PEG 4000 from Merck since we did not obtain transformants when we used the equivalent product from a different supplier.
8. We do not use denatured alcohol to flame the replicator since it usually contains quaternary amines that will inhibit yeast growth.
9. We use a custom-made replicator but any other replicator will do the job. A 3-mm thick stainless steel plate (to protect the methacrylate when flaming) is screwed to the bottom of a rectangular methacrylate block (1.8 cm × 13.3 cm × 10.1 cm). Then, from the top and through the whole block, stainless steel screws (6 cm × 3.5 mm) are screwed in a 96-well format. For easy of handling a methacrylate handle is added. This replicator spots ~5–10 μl droplets on the agar plates.
10. To shake the 96-well microtiter plates we routinely use the HiGro™ Shaker (Fig. 4 left; Genemachines; http://www.americaninstrument.com/pdf/4850-SHAKER.pdf) that combines a small shaking orbital (8 mm), gas flow and temperature controls (2.8 rcf or 250 rpm, 2 s air flow every 30 s and 28 °C). However, in our hands, standard shakers (2.8 rcf) are also amenable to use with this protocol (Fig. 4 right).
11. We recommend cloning the DNA sequence of interest in the *Xma I* and *Xba I* sites of the pTUY1H plasmid since it will remove most of the multicloning site sequences, which reduces background and distance from the *HIS3* start codon.
12. In the case of Y2H, different vectors may not produce equivalent results. The pDEST22/pDEST32 vectors (Invitrogen) produce a higher fraction of interactions that are conserved and that are biologically relevant compared to the pGBKT7/pGADT7-related vectors (Clontech; our protocol is also compatible with these plasmids), but the latter appear to be more sensitive and thus detect more interactions [42]. For these

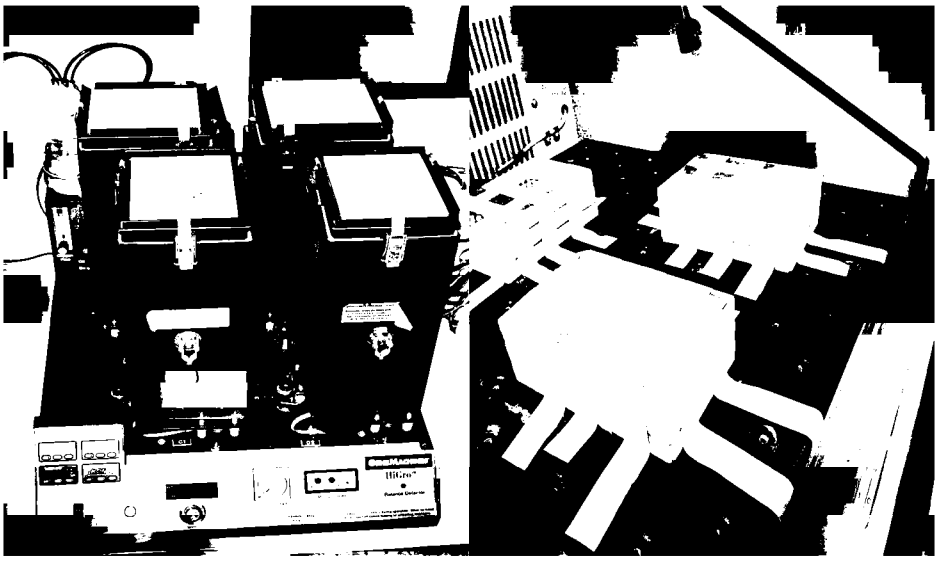

Fig. 4 Photographs of shakers. *Left*: The HiGro shaker (*see* **Note 10**); *Right*: convenient arrangement of microtiter plates when using a conventional shaker

reasons, interactions observed in yeast should be confirmed in the system from where the interactors originate. In silico tools, such as tissue-specific and developmental information on expression patterns, may help determine the temporal and spatial coexistence of any given pair of interactors. This information can be used to prioritize on specific interactions when too many positives are obtained.

13. The optimal amount of carrier DNA is 100–200 μg, which increases the number of transformants about twofold. However, when high colony numbers are not required, addition of carrier DNA may be omitted (Dr. Benito, personal communication).
14. Four hours of incubation is enough in many cases (Dr. Benito, personal communication).
15. This titration protocol can also be used to test one-to-one (few) interactions just by including other prey constructs in addition to the controls (i.e., when confirming positive interactions). It also can be used for obtaining a detailed quantification of the strength of the interactions.
16. Plating dilutions of cultures may not be necessary since liquid cultures of diploid cells in minimal media usually reach stationary phase after 48 h of growth, which ensure that equivalent number of cells are being used in all cases. Alternatively, dilutions can be plated just in DOB-L-W media (diploid plates) and undiluted cultures can be used for the rest of the plates

(screening plates). This setup will allow semiquantitative comparisons of growth (interaction strength) between different yeast colonies. Sometimes it is possible to find notable differences in diploid numbers between colonies. Should that be the case, use all colonies to inoculate fresh DOB-L-W liquid media again as in **step 3** of Subheading 3.3, and continue with the protocol. For the screening plates we initially use the following range of 3-AT concentrations (mM): 0, 1, 5, 15, 30, 60, and 100. A different range of concentrations, depending on the information you might have based on previous work with your favorite bait, can be used. It may be necessary to repeat the titration using a tighter range of concentrations to precisely determine the lowest 3-AT concentration that block reporter activation by your bait (after 7 days of incubation) and to be used in the screening.

17. This protocol was designed to manually screen libraries arrayed in a 96-well format, and we have systematically used it with a prey library of ca. 1200 *Arabidopsis thaliana* TF open reading frames (ORFs) cloned in the pDEST22 plasmid [22]. Diploid (DOB-L-W) and screening (DOB-L-W-H ± 3-AT) plates are inoculated with similar number of cells and grown and scored in parallel, allowing eventual non-mating clones to be flagged as not screened. In any case, diploid colony size and density observed after 2 days of incubation of diploid plates can be taken into account to compare and normalize the strength of positive interactions observed in screening plates. We have observed that less diploid cells are recovered when mating Y187 with YM4271 compared to those obtained between pJ694 and YM4271. Using arrayed TF libraries instead of pooled TF collections, reduces labor time since this eliminates the effort required to characterize several positives produced by the same clone. The efficiency of the protocol may be enhanced in some steps by having two people working simultaneously. Typically we screen two baits at the same time to maximize the screening effort. Scaling up the procedure (i.e., robotization) and other library formats (i.e., 384 wells) are also possible.
18. Scoring yeast growth for 7 days after plating cells will provide additional information about the strength of the interaction. Usually, the faster the growth, the stronger the interaction.
19. In some occasions, the genetic background of the yeast cells (haploid versus diploid) may affect the interaction. Although mating (diploid background) detects less interactions compared to transformation (haploid background), the first ones are more reproducible as well as better suited for high-throughput screens [40].
20. Never use liquid nitrogen to freeze cryogenic vials since most yeast cells will not survive such low temperatures.

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References

1. Brent R, Ptashne M (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell 43:729–736
2. Ma J, Ptashne M (1998) Converting a eukaryotic transcriptional inhibitor into an activator. Cell 55:443–446
3. Fields S, Song O (1989) A novel genetic system to detect protein–protein interactions. Nature 340:245–246
4. Wilson TE, Fahrner TJ, Johnston M, Milbrandt J (1991) Identification of the DNA binding site for NGFIB by genetic selection in yeast. Science 252:1296–1300
5. Li JJ, Herskowitz I (1993) Isolation of the ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science 262:1870–1874
6. Wang MM, Reed RR (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364:121–126
7. Dowell SJ, Romanowski P, Diffley JF (1994) Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science 265:1243–1246
8. Inouye C, Remondelli P, Karin M, Elledge S (1994) Isolation of a cDNA encoding a metal response element binding protein using a novel expression cloning procedure: the one hybrid system. DNA Cell Biol 13:731–742
9. Rezwan M, Auerbach D (2012) Yeast "N"-hybrid systems for protein-protein and drug-protein interaction discovery. Methods 57(4):423–429
10. Ferro E, Trabalzini L (2013) The yeast two-hybrid and related methods as powerful tools to study plant cell signalling. Plant Mol Biol 83(4–5):287–301
11. Ji X, Wang L, Nie X, He L, Zang D, Liu Y, Zhang B, Wang Y (2014) A novel method to identify the DNA motifs recognized by a defined transcription factor. Plant Mol Biol 86:367–380
12. Ota K, Feng SY, Ito T (2014) Detecting protein-DNA interactions using a modified yeast one-hybrid system. Methods Mol Biol 1164:39–50
13. Mallick J, Jansen G, Wu C, Whiteway M (2016) SRYTH: a new yeast two-hybrid method. Methods Mol Biol 1356:31–41
14. Snider J, Stagljar I (2016) Membrane Yeast Two-Hybrid (MYTH) mapping of full-length membrane protein interactions. Cold Spring Harb Protoc. doi:10.1101/pdb.top077560
15. Reece-Hoyes JS, Walhout AJ (2012) Yeast one-hybrid assays: a historical and technical perspective. Methods 57(4):441–447
16. Mehla J, Caufield JH, Uetz P (2015) The yeast two-hybrid system: a tool for mapping protein-protein interactions. Cold Spring Harb Protoc 5:425–430
17. Paz-Ares J (2002) REGIA, an EU project on functional genomics of transcription factors from *Arabidopsis thaliana*. Comp Funct Genomics 3:102–108
18. Gong W, Shen YP, Ma LG, Pan Y, Du YL, Wang DH, Yang JY, Hu LD, Liu XF, Dong CX, Ma L, Chen YH, Yang XY, Gao Y, Zhu D, Tan X, Mu JY, Zhang DB, Liu YL, Dinesh-Kumar SP, Li Y, Wang XP, Gu HY, Qu LJ, Bai SN, Lu YT, Li JY, Zhao JD, Zuo J, Huang H, Deng XW, Zhu YX (2004) Genome-wide ORFeome cloning and analysis of Arabidopsis transcription factor genes. Plant Physiol 135:773–782
19. Mitsuda N, Ikeda M, Takada S, Takiguchi Y, Kondou Y, Yoshizumi T, Fujita M, Shinozaki K, Matsui M, Ohme-Takagi M (2010) Efficient yeast one-/two-hybrid screening using a library composed only of transcription factors in *Arabidopsis thaliana*. Plant Cell Physiol 51: 2145–2151
20. Arabidopsis Interactome Mapping Consortium (2011) Evidence for network evolution in an Arabidopsis interactome map. Science 333(6042):601–607

21. Brady SM, Zhang L, Megraw M, Martinez NJ, Jiang E, Yi CS, Liu W, Zeng A, Taylor-Teeples M, Kim D, Ahnert S, Ohler U, Ware D, Walhout AJ, Benfey PN (2011) A stele-enriched gene regulatory network in the Arabidopsis root. Mol Syst Biol 7:459
22. Castrillo G, Turck F, Leveugle M, Lecharny A, Carbonero P, Coupland G, Paz-Ares J, Oñate-Sánchez L (2011) Speeding *cis*-trans regulation discovery by phylogenomic analyses coupled with screenings of an arrayed library of Arabidopsis transcription factors. PLoS One 6:e21524
23. Gaudinier A, Zhang L, Reece-Hoyes JS, Taylor-Teeples M, Pu L, Liu Z, Breton G, Pruneda-Paz JL, Kim D, Kay SA, Walhout AJ, Ware D, Brady SM (2011) Enhanced Y1H assays for Arabidopsis. Nat Methods 8(12): 1053–1055
24. Ou B, Yin KQ, Liu SN, Yang Y, Gu T, Wing Hui JM, Zhang L, Miao J, Kondou Y, Matsui M, Gu HY, Qu LJ (2011) A high-throughput screening system for Arabidopsis transcription factors and its application to Med25-dependent transcriptional regulation. Mol Plant 4:546–555
25. Burdo B, Gray J, Goetting-Minesky MP, Wittler B, Hunt M, Li T, Velliquette D, Thomas J, Gentzel I, dos Santos Brito M, Mejía-Guerra MK, Connolly LN, Qaisi D, Li W, Casas MI, Doseff AI, Grotewold E (2014) The Maize TFome—development of a transcription factor open reading frame collection for functional genomics. Plant J 80:356–366
26. Pruneda-Paz JL, Breton G, Nagel DH, Kang SE, Bonaldi K, Doherty CJ, Ravelo S, Galli M, Ecker JR, Kay SA (2014) A genome-scale resource for the functional characterization of Arabidopsis transcription factors. Cell Rep 8:622–632
27. Taylor-Teeples M, Lin L, de Lucas M, Turco G, Toal TW, Gaudinier A, Young NF, Trabucco GM, Veling MT, Lamothe R, Handakumbura PP, Xiong G, Wang C, Corwin J, Tsoukalas A, Zhang L, Ware D, Pauly M, Kliebenstein DJ, Dehesh K, Tagkopoulos I, Breton G, Pruneda-Paz JL, Ahnert SE, Kay SA, Hazen SP, Brady SM (2015) An Arabidopsis gene regulatory network for secondary cell wall synthesis. Nature 517(7536):571–575
28. Rueda-Romero P, Barrero-Sicilia C, Gómez-Cadenas A, Carbonero P, Oñate-Sánchez L (2012) *Arabidopsis thaliana* DOF6 negatively affects germination in non-after-ripened seeds and interacts with TCP14. J Exp Bot 63: 1937–1949
29. Iglesias-Fernández R, Barrero-Sicilia C, Carrillo-Barral N, Oñate-Sánchez L, Carbonero P (2013) *Arabidopsis thaliana* bZIP44: a transcription factor affecting seed germination and expression of the mannanase encoding gene AtMAN7. Plant J 74:767–780
30. Iglesias-Fernández R, Wozny D, Iriondo-de Hond M, Oñate-Sánchez L, Carbonero P, Barrero-Sicilia C (2014) The AtCathB3 gene, encoding a cathepsin B-like protease, is expressed during germination of *Arabidopsis thaliana* and transcriptionally repressed by the basic leucine zipperP protein GBF1. J Exp Bot 65:2009–2021
31. Marín-de la Rosa N, Sotillo B, Mizckolczi P, Gibbs DJ, Vicente J, Carbonero P, Oñate-Sánchez L, Holdsworth MJ, Bhalerao R, Alabadí D, Blázquez MA (2014) Large-scale identification of gibberellin-related transcription factors defines Group VII ERFs as functional DELLA partners. Plant Physiol 166:1022–1032
32. Ballester P, Navarrete-Gomez M, Carbonero P, Oñate-Sánchez L, Ferrándiz C (2015) Leaf expansion in Arabidopsis is controlled by a TCP-NGA regulatory module likely conserved in distantly related species. Physiol Plant 155:21–32
33. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816
34. James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144:1425–1436
35. Liu J, Wilson TE, Milbrandt J, Johnston M (1993) Identifying DNA-binding sites and analyzing DNA-binding domains using a yeast selection system. Methods 5:125–137
36. Elble R (1992) A simple and efficient procedure for transformation of yeasts. BioTechniques 13(1):18–20
37. Dobi KC, Winston F (2007) Analysis of transcriptional activation at a distance in *Saccharomyces cerevisiae*. Mol Cell Biol 27(15): 5575–5586
38. Aronheim A, Zandi E, Hennemann H, Elledge SJ, Karin M (1997) Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Mol Cell Biol 17:3094–3102
39. Stagljar I, Korostensky C, Johnsson N, te Heesen S (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc Natl Acad Sci U S A 95:5187–5192
40. Smirnov MN, Smirnov VN, Budowsky EI, Inge-Vechtomov SG, Serebrjakov NG (1967)

Red pigment of adenine-deficient yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 27(3):299–304

41. Weisman LS, Bacallao R, Wickner W (1987) Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. J Cell Biol 105(4):1539–1547
42. Rajagopala SV, Hughes KT, Uetz P (2009) Benchmarking yeast two-hybrid systems using the interactions of bacterial motility proteins. Proteomics 9:5296–5302