Yeast Two-Hybrid Assay to Identify Interacting Proteins

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This article describes the general method to perform the classical two-hybrid system. Although it has already been more than 25 years since this technique was developed, it still represents one of the best and most inexpensive, time saving, and straightforward methods to identify and study protein-protein interactions. Indeed, this system can be easily used to identify interacting proteins for a given protein, to check interactions between two known proteins, or to map interacting domains. Most of the interactions revealed using the two-hybrid assay have been proven to be binary direct interactions. Data comparison with other systems, such as mass spectrometry, have demonstrated that this system is at least as reliable. In fact, its use is increasing with time, and at present numerous variants of the yeast two-hybrid assay have been developed, including high-throughput systems that promote the generation of a proteome-scale map of protein-protein interactions in specific system. © 2018 by John Wiley & Sons, Inc.

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INTRODUCTION

The yeast two-hybrid system, a powerful method used for the analysis of protein-protein interactions *in vivo,* was developed by Fields and Song (1989).

The assay is based on the observation that eukaryotic transcription factors are organized into functionally separable domains such as the DNA-binding domain (BD), which mediates the recruitment of the transcription factor on to specific genome DNA sequences, and the activation domain (AD), which recruits the transcriptional machinery promoting transcription (Causier, 2004; Fields & Song, 1989). These domains are physically separable, as it has been established that BD and AD domains do not need to be present within the same protein in order to function. In fact, gene expression occurs also when the two domains are simply in close proximity (Fig. 1; Causier, 2004; Ma & Ptashne, 1988).

In the two-hybrid system, the cDNA encoding a protein of interest is cloned into a vector that allows the expression of the protein, defined as "bait," fused to a transcription factor BD, while the cDNA encoding another given protein is cloned into a vector for the expression of the protein, defined as "prey," fused to a transcription factor AD. If the two expressed proteins interact in the nucleus of the yeast, BD and AD are brought in close

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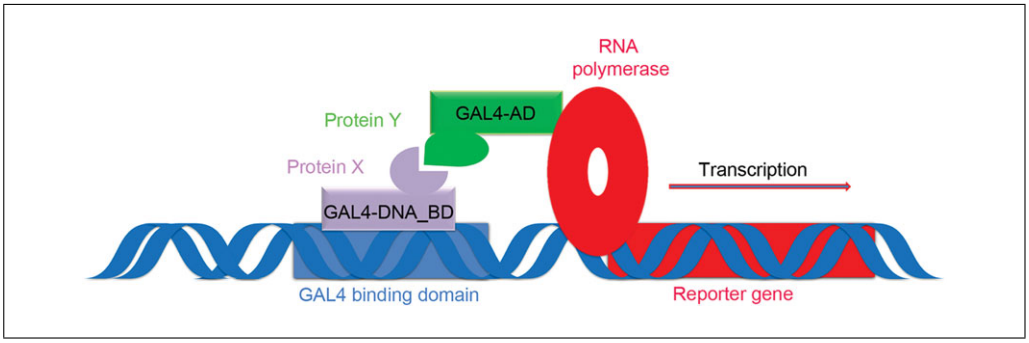

Figure 1 Principle of the two-hybrid assay. The protein X is fused to the DNA binding domain (BD) and the protein Y is fused to an activation domain (AD). If protein X and Y interact, the BD and the AD will combine, reconstituting an active transcription factor that promotes recruitment of the transcription machinery and the transcription of the reporter gene.

proximity and thus become able to activate the transcription of reporter genes under the control of promoters containing sequences recognized and bound by BD (Fig. 1).

Thus, the yeast two-hybrid assay, using the modular nature of eukaryotic transcription factors, allows detection of protein-protein interactions *in vivo*. As this system can be used to screen libraries to identify novel interacting proteins for one or more given proteins, it has, together with its numerous variants, greatly contributed to protein interactome mapping (Parrish, Gulyas, & Finley, 2006).

Strategic Planning

This article describes how to perform the classical yeast two-hybrid assay in order to identify and possibly quantify protein-protein interactions *in vivo*. It is a collection of procedures to perform the yeast two-hybrid assay according to the needs of the operator. In particular, there are four different general protocols (called "basic protocols") that allow the operator to screen for new potential interactions (Basic Protocols 1 and 2), test the specificity of interactions in order to exclude false positives (Basic Protocols 3 and 4), and, possibly, quantify the strength of the interactions (Basic Protocol 5). Then, we describe step by step an alternative method for screening interactions or checking the specificity of these interactions (Alternate Protocol 1). Some of the protocols are integrated with videos in order to increase clarity. Finally, we provide some introductory protocols ("support protocols") that are relevant for the basic protocols and that may be useful for users who are not familiar with bacteria and yeast manipulation. The last section of the article is dedicated to recipes for reagents and solutions for fast reference, and to general comments on the procedures (Commentary section).

Briefly, Basic Protocol 1 describes the steps for interaction screening of a two-hybrid cDNA library with a specific bait plasmid, yielding yeast cells that potentially contain prey plasmids coding for bait-interacting proteins. It includes the following steps:

- Transformation of yeast cells with the plasmid encoding the bait. Using this procedure, yeast cells that acquire bait plasmid will be selected using the appropriate selective medium. It is important to check, at the end of this procedure, that the fused protein expressed by the bait plasmid does not activate transcription of reporter genes.
- Transformation of yeast cells containing the bait plasmid with a cDNA library. This procedure allows the yeast cells containing the bait plasmid to acquire prey plasmids from a cDNA library. The final aim is to test the interaction between the protein coded by the bait plasmid and the different proteins coded by the different prey plasmids of the cDNA library. At the end of this procedure, only the yeast cells that

have acquired both the bait and the prey plasmids and in which transcription of two reporter nutritional genes has been activated, suggesting the presence of potential new interactions, will be selected. In this context, it is important to have a high transformation efficiency in order to screen an appropriate number of clones that will guarantee adequate cDNA representation in the interaction screening. Importantly, yeast clones isolated at the end of this protocol should be tested for the activation of the third reporter gene in the system, the LacZ gene, which encodes the β-galactosidase enzyme (see Basic Protocol 4).

Basic Protocol 2 allows the isolation of plasmid DNA coding for putative interacting proteins from yeast colonies obtained at the end of Basic Protocol 1, which will be tested for positivity to β-galactosidase (see Basic Protocol 4). Basic Protocol 2 comprises the following steps:

- Extraction of plasmid DNA from yeast cells. As only very little of DNA is obtained with this procedure, usually not visible on an agarose gel and not sufficient for the subsequent steps, the extracted plasmid DNA has to be amplified in bacteria.
- Transformation of bacterial cells with the plasmid DNA extracted from yeast cells. Importantly, after transformation, bacterial cells that have acquired the prey plasmid are selected using the appropriate antibiotic in order to avoid bacteria that instead acquired the bait plasmid.
- Amplification and purification of the prey plasmid from bacteria. With this protocol, bacteria containing the prey plasmid are grown and the plasmid DNA purified and readied for subsequent analysis.

Basic Protocol 3 is needed in order to establish whether activation of transcription of reporter genes in yeast clones, isolated with Basic Protocol 1, was actually due to the interaction between bait and prey. It can be used also to test the interaction between two known proteins or to map their interaction domains by using mutated forms of one or both proteins. Often defined as the "specificity assay," this protocol consists of the co-transformation of yeast cells with bait and prey plasmids to test activation of nutritional reporter genes by growing yeast on selective media. Also, subsequently, activation of the LacZ reporter gene can be tested by performing β-galactosidase assays (Basic Protocols 4 and 5). Briefly, in Basic Protocol 3, yeast cells are transformed with both bait and prey plasmids at the same time and their growth on specific selective medium indicates activation of the nutritional reporter genes. In this step, it is important to make certain that activation of transcription of reporter genes happens only in the presence of both bait and prey proteins, thus indicating interaction between them. Therefore, for each plasmid coding for a putative prey isolated from the library screening, an additional co-transformation with the empty bait vector is needed. If reporter gene transcription activation is detected not only when both bait and prey proteins are present but also when only the prey protein is expressed in yeasts, this indicates that activation of transcription is not dependent on the interaction between bait and prey, and thus the clone was a false positive and that prey should be discarded. The prey plasmids that instead are proven to code for bait-interacting proteins should be sequenced to identify the prey-interacting proteins.

Basic Protocol 4 describes the filter β-galactosidase assay. This assay tests expression, and thus activation, of transcription of another reporter gene, β-galactosidase, and is important for reducing the number of false positives due to the use of nutritional markers subjected to selective pressure as gene reporters in the screening. While the β-galactosidase filter assay is useful only for this, the liquid β-galactosidase assay (Basic Protocol 5) may also be used to quantify the strength of interaction between bait and the newly identified preys. In fact, this assay evaluates the level of bait-prey interaction by determining the

amount of β-galactosidase enzyme activity present in yeast cells that express both bait and prey proteins. Basic Protocols 4 and 5 contain the instructions to perform:

- The qualitative β-galactosidase filter assay using X-gal as a substrate (Basic Protocol 4). This assay is normally performed immediately after Basic Protocol 1 on the yeast clones isolated from the interaction screening or on the yeast colonies obtained after Basic Protocol 3.
- The liquid β-galactosidase assay (Basic Protocol 5), an assay used to quantify the strength of the interaction for selected transformants. This assay is based on the hydrolysis of ONPG (o-nitrophenyl β-D-galactopyranoside) and it is used mainly on selected yeast colonies obtained after the specificity tests (Basic Protocol 3), and thus on the colonies expressing bait-interacting proteins.

An alternative to Basic Protocols 1 and 3 is provided as Alternate Protocol 1, which describes yeast mating. Yeast mating overcomes the possible problems of low transformation efficiency in Basic Protocol 1 or 3, and thus can be used as an alternative method to identify novel protein-protein interactions by screening a cDNA library, or to test the interaction between two known proteins. The yeast mating procedure requires haploid yeast strains of opposite mating type, for instance AH109 and Y187 strains, to be transformed with the prey and bait plasmids, respectively. The selected transformants are then mixed and incubated together to mate and subsequently plated onto selective plates.

Finally, support protocols describe how to prepare a yeast strain working plate (Support Protocol 1) and how to make competent bacteria (Support Protocol 2).

Vectors and Yeast Strains

In the two-hybrid assay, the cDNA sequence encoding the protein of interest is subcloned into a vector that allows expression of a given protein fused to a BD. The vector used in this article is pGBKT7 (Clontech), which contains a DNA sequence coding for the GAL4 transcription factor BD followed by a Multiple Cloning Site (MCS) to insert the cDNA of interest (Fig. 2A). In the pGBKT7 vector, the constitutive ADH1 promoter induces high levels of expression of the fusion protein in yeast, while transcription is terminated by the ADH1 transcription termination signals. pUC ori (pUC replication origin) and 2μ ori (Yeast 2μ replication origin) promote the replication of the vector in *E. coli* and *S. cerevisiae*, respectively. pGBKT7 carries the kanamycin resistance gene (Kan^r) for selection in *E. coli* and the TRP1 nutritional marker (gene that encodes phosphoribosylanthranilate isomerase, an enzyme important for the biosynthesis of tryptophan) for selection in yeast.

A cDNA sequence encoding another protein is cloned into a vector for the expression of the proteins fused to an AD. The vector that we used was the pGADGH vector (Clontech), which is used to express the protein fused to the GAL4 AD (Fig. 2B) and to the SV40 nuclear localization signal (NLS) for nuclear targeting of the fusion protein. The expression of the fusion protein is ensured by the constitutive ADH1 promoter and the ADH1 transcription termination signal. Col E1 (colicin E1) ori and 2μ ori allow replication in *E. coli* and *S. cerevisiae*, respectively. The pGADGH vector carries the ampicillin resistance gene (Amp^r) for selection in *E. coli* and the LEU2 marker (gene that encodes isopropylmalate dehydrogenase, an enzyme important for the biosynthesis of leucine) for nutritional selection in yeast.

Importantly, the choice of the vector coding for BD has to match the engineered yeast strain. Indeed, if the BD used is from a given transcription factor, the engineered strain has to carry the proper UAS (Upstream Activation Sequence) upstream of reporter genes in order for that specific transcription factor BD to recognize and bind the UAS. In contrast,

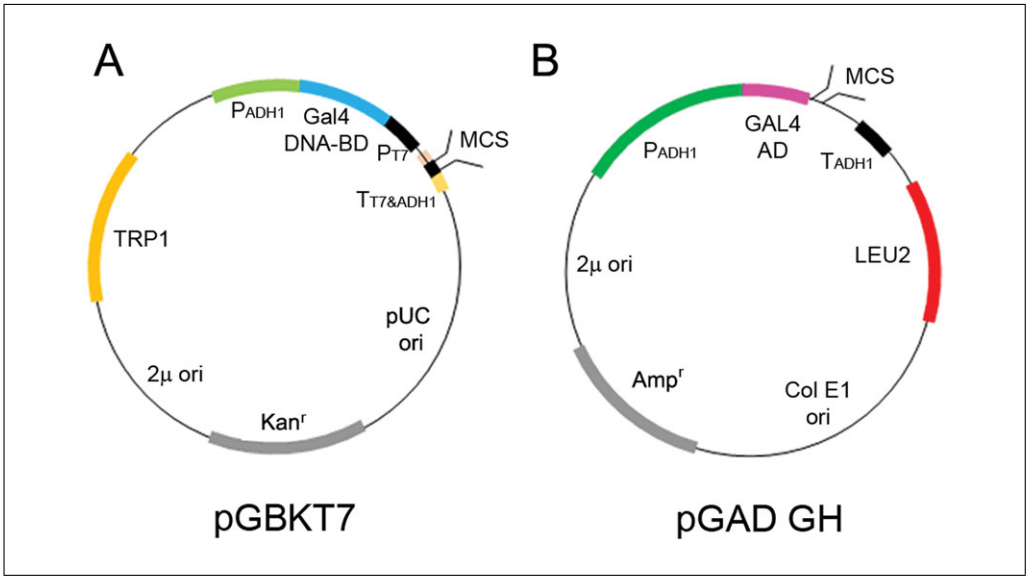

Figure 2 Maps of the vectors used for two-hybrid assay. (**A**) In the pGBKT7 vector (Clontech), the cDNA sequence encoding the bait is fused to the cDNA encoding the GAL4 DNA binding domain (DNA-BD). The vector contains the constitutive ADH1 promoter (P_{ADH1}) and the T7 and ADH1 transcription termination signals ($T_{T7\ \&\ ADH1}$). pUC and 2 μ ori promote replication in *E. coli* and *S. cerevisiae,* respectively. The vector carries the kanamycin resistance gene (Kan^r) for selection in *E. coli*, and the TRP1 nutritional marker for selection in yeast. pGBKT7 also contains the T7 promoter and a MCS. (**B**) In the pGAD GH vector (Clontech), the cDNA encoding the prey is cloned in the MCS and fused to the cDNA encoding the GAL4 activation domain (AD). The vector contains the SV40 nuclear localization signal for nuclear targeting of the fusion protein. Expression of the cDNA is guaranteed by the constitutive ADH1 promoter (P_{ADH1}) and the ADH1 transcription termination signal (T_{ADH1}). Col E1 ori and 2 μ ori allow the replication in *E. coli* and *S. cerevisiae*, respectively. The ampicillin resistance gene (Amp^r) is present for selection in *E. coli*, as well as the LEU2 marker for the nutritional selection in yeast.

vectors coding for AD can be interchangeable because, in order to start transcription, it is only important for the AD to be present near the site of transcription initiation, and this happens if the two fusion proteins interact.

For the two-hybrid assay, the yeast strain used in this article is AH109. This strain carries deletions of the *gal4* and *gal80* genes to avoid interference by endogenous GAL4 and GAL80 proteins during the assay, and it is auxotrophic for leucine, tryptophan, histidine, and adenine. In this system, LEU2 and TRP1, present in bait and prey vectors, are used as selectable markers that allow selection of the transformed yeast cells (Fig. 2). AH109 yeast cells are able to grow in the absence of histidine and adenine when the GAL promoters are activated by the binding of BD and the close proximity of an AD, possibly due to the interaction of bait and prey. This is due to the presence of the nutritional reporter genes ADE2 (coding for phosphoribosylaminoimidazole carboxylase, an enzyme important for the synthesis of adenine) and HIS3 (encoding imidazoleglycerol-phosphate dehydratase, a key enzyme for histidine biosynthesis) in the AH109 strain that allow control of the stringency of selection and reduce the incidence of false positives, with ADE2 providing strong nutritional selection (James, Halladay, & Craig, 1996). In addition to ADE2 and HIS3, the AH109 strain contains two more reporter genes (LacZ and MEL1). MEL1 and LacZ, which encode α-galactosidase and β-galactosidase enzymes, respectively, can be used in a colorimetric assay to further reduce the number of false positives. In fact, as they are neutral markers, they are not subjected to selective pressure. Furthermore, they can be used to roughly estimate the strength of the interaction.

These four reporter genes allow monitoring of transcription activation, possibly due to interaction between bait and prey, thanks to the fact that they are under the control

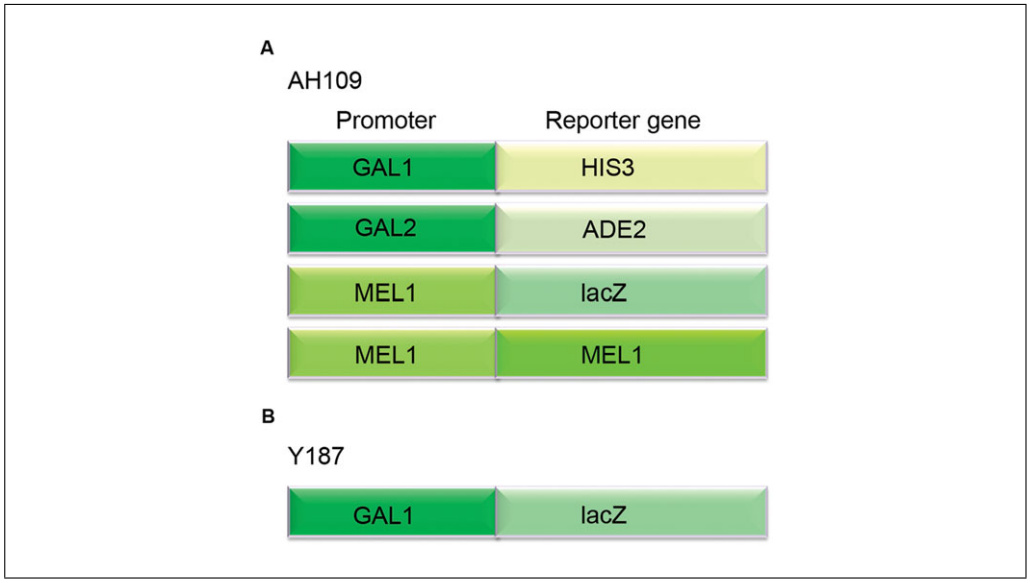

Figure 3 Reporter genes in AH109 e Y187 strains. (**A**) In AH109 yeast strain, the HIS3 and ADE2 reporter genes are under the control of different GAL4-UAS (GAL1, GAL2), while MEL1 and LacZ reporter genes are under the control of MEL1 UAS. (**B**) Strain Y187 contains the lacZ reporter gene under the control of the GAL1 UAS.

of three distinct GAL4-responsive promoters (Fig. 3A). These distinct promoters reduce false positives by eliminating proteins (i) that interact upstream of the reporter construct's binding site, (ii) that interact directly with the sequences flanking the GAL4 binding site, and (iii) that interact with transcription factors bound to specific TATA boxes.

Y187 (MATα genotype) cells are used as mating partners of AH109 (MATa genotype). Mating is a rapid and efficient method used to introduce two plasmids into the same host cells. The Y187 strain contains the LacZ reporter gene under control of the GAL1 UAS and exhibits a higher level of induced β-galactosidase activity compared to AH109 (Fig. 3B).

For quantitative β-galactosidase assays, the yeast strain we used is L40. This strain is auxotrophic for leucine, tryptophan, and histidine, and contains two reporter genes, HIS3 and LacZ. In this strain, β-galactosidase is produced at higher levels, making it more suitable for quantitative assays.

BASIC PROTOCOL 1

IDENTIFICATION OF CLONES THAT COULD INDICATE POSSIBLE NOVEL PROTEIN-PROTEIN INTERACTIONS THROUGH THE SCREENING OF A cDNA LIBRARY

This protocol describes the screening of a cDNA library for the identification of proteins that interact with a protein of interest (bait). The library consists of a collection of expression plasmids in which the coding DNA sequence for AD is fused to individual cDNAs. There are many commercially available cDNA libraries for the two-hybrid screening (e.g., from Clontech - Takara) or, if needed, the library can be constructed by inserting the cDNA prepared from a specific cell type or tissue into the appropriate two-hybrid vector (e.g., Clontech - Takara). In a sequential transformation, the yeast strain AH109 is transformed with the BD/bait plasmid and plated on SD/–W agar plates ("synthetically defined" agar plates lacking tryptophan) that allow the selection of cells that have been transformed (Fig. 4A). The selected transformants are then grown (Fig. 4B), transformed with the AD/cDNA library plasmid (encoding prey), and plated on SD/–WL agar plates (plates lacking tryptophan and leucine) for the selection of yeast cells that have taken up both the bait and the prey plasmid (Fig. 4C). To select clones in

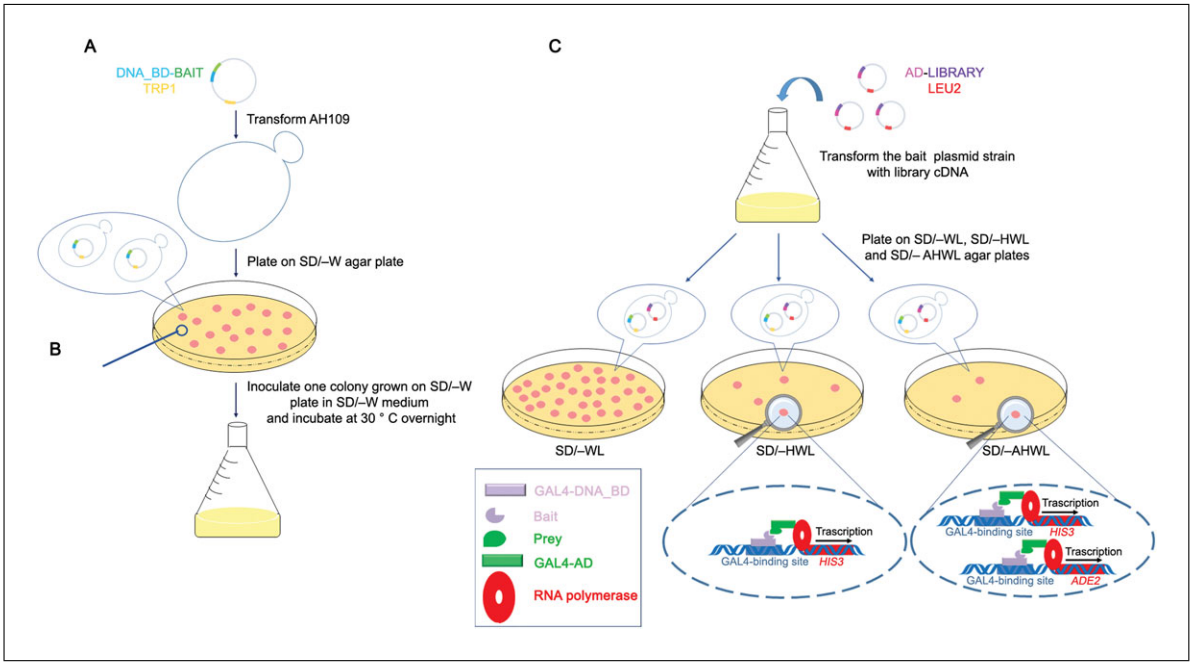

Figure 4 Screening of a cDNA library. (**A**) The yeast AH109 strain is transformed with the BD/bait plasmid and plated on SD/–W agar plate for the selection of the transformed cells. (**B**) The selected transformants are then grown up in SD/–W medium, (**C**) transformed with the AD/cDNA library plasmid (prey), and plated on SD/–WL agar plates for the selection of cells that have taken up both the bait and the prey plasmid; they are grown on SD/–HWL and SD/–AHWL agar plates for the selection of weak and strong interactions, respectively.

which possibly an interaction between the bait and the prey has occurred, yeast cells are grown on SD/–HWL (plates lacking histidine, tryptophan, and leucine) and SD/–AHWL agar plates (plates lacking adenine, histidine, tryptophan, and leucine), respectively, in order to check for activation of transcription of the HIS3 and ADE2 reporter genes (Fig. 4C).

Materials

AH109 yeast strain grown on agar plate (Support Protocol 1)
YPDA liquid medium (see recipe)
10× TE buffer (see recipe)
Sterile double-distilled water
1 M lithium acetate (LiAc; see recipe)
50% (w/v) polyethylene glycol (PEG; see recipe)
Carrier DNA (see recipe)
Bait-plasmid DNA
Dimethyl sulfoxide (DMSO; e.g., Sigma, cat. no. D4540) sterilized using a 0.22-µm pore filter
SD/–W 10-cm-diameter agar plates (see recipe)
SD/–L 10-cm-diameter agar plates (see recipe)
SD/–H 10-cm-diameter agar plates (see recipe)
SD/–A 10-cm-diameter agar plates (see recipe)
SD/–W liquid medium (-W medium, see recipe)
cDNA library (Clontech - Takara)
SD/–HWL 15-cm-diameter agar plates (see recipe)
SD/–AHWL 15-cm-diameter agar plates (see recipe)
SD/–WL 10-cm-diameter agar plates (see recipe)

Sterile inoculating loops
Flasks
Shaking incubator at 30°C for flasks and tubes
Spectrophotometric cuvettes
Spectrophotometer
95°C thermomixer
50-ml conical tube (e.g., Corning Falcon, cat. no. 430829)
Centrifuge (e.g., Eppendorf 5810R)
14-ml tube with round bottom (i.e., Corning Falcon, cat. no. 352059)
Vortex mixer
Water baths at 30°C and 42°C
Sterile glass beads (e.g., Sigma-Aldrich, cat. no. 18406)
Incubator at 30°C for plates

AH109 transformation with the bait plasmid

1. Inoculate a single colony of AH109 yeast from the stock agar plate into a 250-ml flask containing 50 ml of YPDA medium and incubate overnight at 30°C with constant shaking at 250 rpm.

2. Measure the OD_{600} (optical density of the sample at a wavelength of 600 nm) of a 1:10 dilution (100 µl of the culture in 1 ml YPDA final volume) of the overnight culture. Dilute the overnight culture in YPDA medium to obtain a culture with OD_{600} between 0.2 and 0.3 (0.25 ideally) in a final volume of 80 ml into a 500-ml flask. In order to do this, calculate the volume of overnight culture that must be transferred according to the following formula:

$$\frac{V_f \times OD_{600f}}{OD_{600f} \times 10}$$

V_f = Final volume = 80 ml
OD_{600f} = Final OD_{600} = 0.25
OD_{600i} = OD_{600} of the overnight culture diluted 1:10
10 = dilution factor

For example, if the OD_{600} of the overnight culture is 0.6, the amount of overnight culture to add in 80 ml of YPDA final volume in order to obtain a culture with OD_{600} of 0.25 is 3.3 ml: $(\frac{80 \times 0.25}{0.6 \times 10})$

3. Incubate for approximately 3 hr at 30°C with vigorous shaking at 250 rpm until the OD_{600} is 0.5 ± 0.1.

Measure the OD several times during the incubation to prevent overgrowth of yeast cells above 0.6 OD_{600}.

4. Prepare the following:

1× TE (10 ml):

10× TE	1 ml
Sterile double-distilled H_2O	9 ml

1× TE/LiAc (10 ml):

10× TE	1 ml
1 M LiAc	1 ml
Sterile double-distilled H_2O	8 ml

PEG/LiAc (10 ml):

50% (w/v) PEG	8 ml
10× TE	1 ml
1 M LiAc	1 ml

Carrier DNA

Thaw an aliquot of carrier DNA (prepared as described in Reagents and Solutions) stored at −20°C, incubate for 5 min at 95°C in a thermomixer, and put immediately on ice until the use. All these solutions must be prepared immediately before the use.

5. Place the culture (yeast cells grown in 80 ml of YPDA medium) in two 50-ml tubes and centrifuge for 5 min at 1000 × *g*, room temperature.
6. Discard the supernatants and resuspend the cell pellets in 8.0 ml of 1× TE solution (see step 4). Pool the cells in a single 50-ml tube.
7. Centrifuge for 5 min at 1000 × *g*, room temperature.
8. Discard the supernatant and resuspend the cell pellet in 0.5 ml of 1× TE/LiAc solution (see step 4).
9. Add 1 µg of bait-plasmid DNA, 10 µl of carrier DNA, and 100 µl of cells to a 14-ml tube with a round bottom. As negative control, add 10 µl of carrier DNA and 100 µl of cells to a different tube.

   The carrier DNA enhances the uptake of the plasmid during the transformation.

10. Add 750 µl of PEG/LiAc solution to each tube. Vortex for 10 sec to mix.
11. Incubate the tubes for 30 min at 30°C in a water bath, and gently shake them every 5 min.
12. Add 100 µl of DMSO to each tube. Mix gently. Do not vortex.
13. Incubate the tubes for 20 min at 42°C in a water bath and gently shake them every 5 min.
14. Centrifuge for 5 min at 1000 × *g*, room temperature.
15. Aspirate the supernatant with a pipet and discard it in order to remove DMSO.
16. Resuspend the cell pellet in 500 µl of sterile 1× TE solution.
17. Plate 100 µl on a 10-cm SD/–W agar plate in order to select the transformed colonies. To plate, spread the transformation mix over the plate by using sterile glass beads until all the liquid has been absorbed. 100 µl should also be plated on SD/–L, SD/–H, SD/–A as control.
18. Incubate plates for 2 to 4 days at 30°C until yeast colonies appear (colonies should appear only in the SD/–W plate; if colonies appear also on other plates it means that the presence of the bait activates transcription of reporter genes and, therefore, the screening cannot be done).

Transformation of cells that contain the bait-plasmid with a cDNA library

19. Pick a single colony from the SD/–W plate (step 17) and inoculate it into a 500-ml flask containing 150 ml of SD/–W liquid medium. Incubate overnight at 30°C with vigorous shaking at 250 rpm.
20. Check the $OD_{600,}$ which ideally should be around 0.5 to 0.6. If the OD_{600} is 0.7 or higher, dilute the overnight culture with SD/–W liquid medium to bring the OD_{600}

up to 0.2 to 0.3 (for the calculation, see step 2) and incubate until OD_{600} is 0.5 to 0.6 at 30°C with shaking at 250 rpm. If the OD_{600} is lower than 0.5, keep the yeast growing until they reach $OD_{600} = 0.5$ to 0.6.

21. Centrifuge for 10 min at 1000 × *g*, room temperature.
22. Resuspend the cell pellet in 500 ml final volume of YPDA medium in order to have an OD_{600} of 0.2 to 0.3.
23. Incubate until the OD_{600} is 0.5 ± 0.1 at 30°C.

    The growth in YPDA medium for the time necessary for yeast cells to double their number (doubling time) greatly enhances transformation efficiency compared to cells grown only in –W synthetic medium).

24. Prepare the following:

    1× TE (260 ml; see step 4)
    1× TE/LiAc (10 ml; see step 4)
    PEG/LiAc (60 ml; see step 4)
    Carrier DNA (see recipe in Reagents and Solutions).

25. Centrifuge for 10 min at 1000 × *g*, room temperature.
26. Resuspend the cell pellet in 250 ml of 1× TE solution.
27. Centrifuge for 10 min at 1000 × *g*, room temperature.
28. Resuspend the cell pellet in 8 ml of TE/LiAc solution.
29. Transfer the 8 ml of cells in a 500-ml flask, then add 90 µg of cDNA library, 500 µl of carrier DNA, and 60 ml PEG/LiAc solution.

    It is important to add the cDNA library after adding the cells, in order to prevent DNA absorption to the glass flask.

30. Incubate for 30 min at 30°C with shaking at 250 rpm.
31. Add 7 ml of DMSO and incubate yeast cells for 15 min at 42°C in a water bath, gently shaking every 2 min to prevent sedimentation of the cells.
32. Put the cells immediately on ice for 1 to 2 min (thermal shock), then split the culture into two 50-ml tubes and centrifuge for 10 min at 1000 × *g* at room temperature.
33. Resuspend the first cell pellet in 2 ml of YPDA medium and plate 200 µl on 10 15-cm SD/–AHWL agar plates; resuspend the other cell pellet in 4 ml of 1× TE and plate 200 µl on 20 15-cm SD/–HWL agar plates.

    The yeast cells that have to be plated on a high-stringency medium (SD/-AHWL) are resuspended in YPDA so that they will have the possibility to recover after transformation; they are plated on a smaller number of plates because fewer clones are expected due to the high stringency.

34. To calculate transformation efficiency, spread 100 µl of 1:10, 1:100, 1:1000, and 1:10,000 dilutions of cells in 1× TE solution on 10-cm SD/–WL agar plates.
35. Incubate the plates until the colonies appear (2 to 6 days) at 30°C. In about 2 days, colonies on SD/–WL will appear, while colonies on SD/–HWL or on SD/–AHWL will appear after 3 to 4 or 4 to 6 days, respectively.
36. Calculate the transformation efficiency by counting the colonies growing on the dilution plates from step 34. If possible, to count colonies in order to establish transformation efficiency, use plates where there are about 30 to 300 colonies.

$$Colonies/\mu g\ of\ DNA = \frac{colonies \times total\ suspension\ volume\ (\mu l) \times dilution\ factor}{volume\ plated\ (\mu l) \times amount\ of\ DNA\ used\ (\mu g)}$$

For example, if 250 colonies are obtained on the 1:1000 dilution plate after plating 100 µl out of 2000 µl total suspension volume (diluted 1:1000 from the original yeast suspension), and if the amount of DNA used for the transformation is 50 µg, the transformation efficiency is 1×10^5 colonies/µg of DNA.

$$Colonies/\mu g\ of\ DNA = \frac{250\ colonies \times 2000\ \mu l \times 1000}{100\ \mu l \times 50\ \mu g} = 1 \times 10^5\ colonies/\mu g\ \text{of}\ DNA$$

Transformation efficiency should be at least 10^4 cfu (colony-forming units)/µg when transforming the cDNA library. This is important in order to screen an appropriate number of independent clones (about one million) to ensure good representation of the cDNA library. If the efficiency of transformation is lower, repeat the transformation procedure.

The yeast strain AH109 carries a mutation that inhibits the adenine synthesis pathway, causing the accumulation of a red pigment, so that colonies will appear from pink to reddish-brown. Instead, if the ADE2 gene is expressed, for instance, because of a strong interaction between bait and prey, the adenine pathway is unblocked and the red pigment will be absent or less present. Therefore, if ADE2 is expressed, yeast colonies will be from pale pink to white depending on the level of expression. Thus, the color of the colony can already give an indication of the expression of the ADE2 gene. However, when selecting colonies, although white or pale pink colonies indicate expression of ADE2, also pick pink colonies, as in these colonies there could be a weaker interaction that does not activate ADE2 expression.

ISOLATION OF PLASMIDS ENCODING PUTATIVE BAIT-INTERACTING PROTEINS

BASIC PROTOCOL 2

After yeast transformation with the bait plasmid and with the cDNA library (Basic Protocol 1), if the bait interacts with proteins encoded by some of the cDNAs of the library, some colonies will grow on the SD/–HWL and possibly also on the SD/–AHWL selective plates. In order to identify the putative interactors, the library plasmids must be extracted from the yeast clones and tested in the specificity assay (see Basic Protocol 3), and then the ones coding for bait-interacting proteins should be sequenced. As the amount of plasmid DNA that can be extracted from yeast cells is too low to be used for the next steps, after extraction from yeasts the plasmid DNA has to be transformed and amplified in *E. coli*. Thus, the first step is the extraction of the plasmids from the yeast colonies. Then, *E. coli* is transformed with the plasmids extracted from yeast cells. As the library plasmids carry the Amp^r gene (Fig. 2B), using ampicillin in the bacterial culture medium will allow selection of bacteria that have been transformed with the prey plasmid and not with the bait plasmid. Therefore, to isolate and amplify library prey plasmids, transformed bacteria are plated onto LB plates containing ampicillin. The next step is the extraction of the plasmid DNA from *E. coli* transformants by the boiling miniprep procedure (or, alternatively, by using any other method to extract plasmid DNA from *E. coli,* including commercial kits).

Materials

SD/–HWL and SD/–AHWL 15-cm-diameter agar plates (Basic Protocol 1, step 33)
SD/–HWL 10-cm-diameter agar plates (see recipe)
SD/–L liquid medium (–L medium, see recipe)
STET buffer (see recipe)
7.5 M ammonium acetate (see recipe)
100% and 70% (v/v) ethanol, prechilled at −20°C
Double-distilled water

Ultra-competent DH5α cells (Support Protocol 2)
LB medium (see recipe)
LB/ampicillin 10-cm-diameter agar plates (see recipe)
LB/ampicillin medium (see recipe)
25% (w/v) sucrose (see recipe)
Lysozyme (see recipe)
3 M sodium acetate, pH 5.2 (see recipe)
Isopropanol
TE/RNase A (see recipe)

Sterile inoculating loops
Incubators at 30°C and 37°C for plates
25-ml tubes Ø 25 × 90 mm (e.g., 2680/SG/CS, APTACA).
Shaking incubators at 30°C and 37°C for tubes and flasks
2-ml and 1.5-ml microcentrifuge tubes
Microcentrifuge
Glass beads acid-washed (e.g., Sigma-Aldrich, cat. no. G8772)
Sterile glass beads (e.g., Sigma-Aldrich, cat. no. 18406)
95°C thermomixer
42°C water bath
10-ml conical tube
Heat block
Beaker
Vortex mixer

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2001)

Yeast plasmid DNA extraction

This protocol describes the extraction of the DNA from yeasts using acid-washed glass beads that break the cell wall.

1. Streak the clones that were grown on 15-cm SD/–HWL and SD/–AHWL agar plates (Basic Protocol 1, step 33) on 10-cm SD/–HWL agar plates. Incubate them approximately for 3 days (until the colonies are grown) at 30°C.
2. Next, inoculate a colony for each independent clone picked after the screening in 2 ml of –L medium in 25-ml tubes (Ø 25 × 90 mm), to increase surface for oxygen exchange, improving yeast growth. Incubate overnight at 30°C.

   This step allows enrichment of the prey plasmid.
3. Transfer the overnight culture to a 2-ml microcentrifuge tube and centrifuge for 30 sec at 13,400 × *g*, room temperature.
4. Resuspend the cell pellets in 100 µl of STET buffer, vortexing briefly.
5. Add 0.2 g of acid-washed glass beads and vortex each sample for at least 5 min.
6. Add 100 µl of STET buffer and incubate for 5 min at 95°C.
7. Put the samples on ice and centrifuge for 10 min at 13,400 × *g*, 4°C.
8. Transfer 100 µl of the supernatant in a new microcentrifuge tube containing 50 µl of 7.5 M ammonium acetate and mix well.
9. Incubate for 1 hr at −20°C.
10. Centrifuge for 10 min at 16,000 × *g*, 4°C.

11. Transfer 100 µl of the supernatant to a new microcentrifuge tube containing 200 µl of cold ethanol and mix.
12. Incubate for 30 min at −20°C in order to induce plasmid DNA precipitation.
13. Centrifuge for 15 min at 16000 × *g*, 4°C.
14. Remove the supernatant and add 300 µl of 70% cold ethanol to the DNA pellet.
15. Centrifuge for 10 min at 16000 × *g*, 4°C.
16. Resuspend the pellet in 20 µl of double-distilled water or 1× TE.

Transformation of DH5α E. coli ultra-competent cells with the plasmid DNA extracted from yeast cells

17. Thaw the DH5α competent cells on ice.
18. For each plasmid DNA sample, in a 1.5-ml microcentrifuge tube, mix 200 µl of competent cells and 10 µl of the plasmid DNA suspension extracted from yeast at step 16.
19. Incubate for 30 min on ice.
20. Incubate for 2 min at 42°C and then transfer immediately onto ice.
21. Add 800 µl of LB medium.
22. Incubate for 1 hr at 37°C with shaking at 500 rpm.
23. Centrifugate for 5 min at 800 × *g*, and discard the supernatant.
24. Resuspend the pellet in 100 µl of LB liquid medium and then plate on 10 cm LB/ampicillin agar plates using sterile (not acid-washed) glass beads.
25. Incubate overnight at 37°C.

Preparation of plasmid DNA from the E. coli transformants by the boiling-prep procedure

26. Inoculate one colony for each plate (step 25) in a 10-ml conical tube in 5 ml of LB/ampicillin medium in order to select for bacteria that have acquired the prey plasmid.
27. Incubate overnight at 37°C with shaking at 250 rpm.
28. Before harvesting the cells, turn on the heat block and boil water in a beaker.
29. Centrifuge 1.5-ml of culture grown overnight for 2 min at 15,700 × *g*, 4°C.
30. Discard the supernatant, resuspend the cell pellet in 50 µl of 25% sucrose, and vortex for 30 sec.
31. Add 300 µl of STET buffer and vortex for 10 sec.
32. Add 25 µl of lysozyme and incubate the tube for 45 sec at 100°C (hot water prepared at step 28).
33. Put immediately on ice and then centrifuge for 15 min at 15,700 × *g*, 4°C.
34. Remove the mucous pellet with the help of a toothpick and add 40 µl of 3 M sodium acetate, pH 5.2, and 270 µl of isopropanol at room temperature to the supernatant.
35. Mix by inverting five to six times and let the DNA precipitate for 1 min at room temperature.

36. Centrifuge for 15 min at 15,700 × *g* at room temperature.
37. Promptly discard the supernatant from each tube by inverting the tube, and keep it upside down on a piece of absorbent paper.
38. Wash the pellet with 250 µl of 70% cold ethanol by adding ethanol and immediately discarding it by inverting the tube and holding it upside down on a piece of absorbent paper. Dry the pellet.
39. Resuspend the dried pellet in 30 µl of TE/RNase.
40. Check the quality of the extracted DNA on agarose gel (Voytas, 2001).

This is a very quick and inexpensive method to prepare plasmid DNA from large number of colonies. Obviously, however, any other protocol can be used.

BASIC PROTOCOL 3

YEAST CO-TRANSFORMATION

This protocol describes simultaneous transformation with two plasmids, the bait plasmid and the prey plasmid. It can be used to test the interaction between two known proteins or to do specificity tests after a two-hybrid cDNA library screening in order to prove that activation of transcription of reporter genes in the isolated clones was actually due to the interaction between the bait protein and the prey protein (Fig. 5; Supporting Information Video 1). Thus, yeast co-transformation is useful for specificity tests to discriminate between true and false positive clones. Co-transforming two plasmids together yields a low transformation efficiency that, however, in this case it is not important as only few colonies are needed to test transcriptional activation of reporter genes.

Materials

Stock plate of AH109 yeast strain (Support Protocol 1)
YPDA liquid medium (see recipe)
10× TE buffer (see recipe)
Double-distilled water
1 M lithium acetate (see recipe)
50% (w/v) PEG (see recipe)
Carrier DNA (see recipe)
Bait plasmid DNA
Prey plasmid DNA
DMSO sterilized using a 0.22-µm pore filter
SD/–WL 10-cm-diameter agar plates (see recipe)
SD/–HWL 10-cm-diameter agar plates (see recipe)
SD/–AHWL 10-cm-diameter agar plates (see recipe)

Flasks
Incubator at 30°C for plates
Shaking incubator at 30°C for tubes and flasks
Cuvettes
Spectrophotometer
50-ml conical tubes
Thermomixer
Centrifuge (e.g., 5810R Eppendorf)
14-ml tubes with round bottom (e.g., Corning Falcon, cat. no. 352059)
Vortex mixer
Water bath 30°C and 42°C
Sterile glass beads (e.g., Sigma-Aldrich, cat. no. 18406-500G)
Sterile inoculating loops

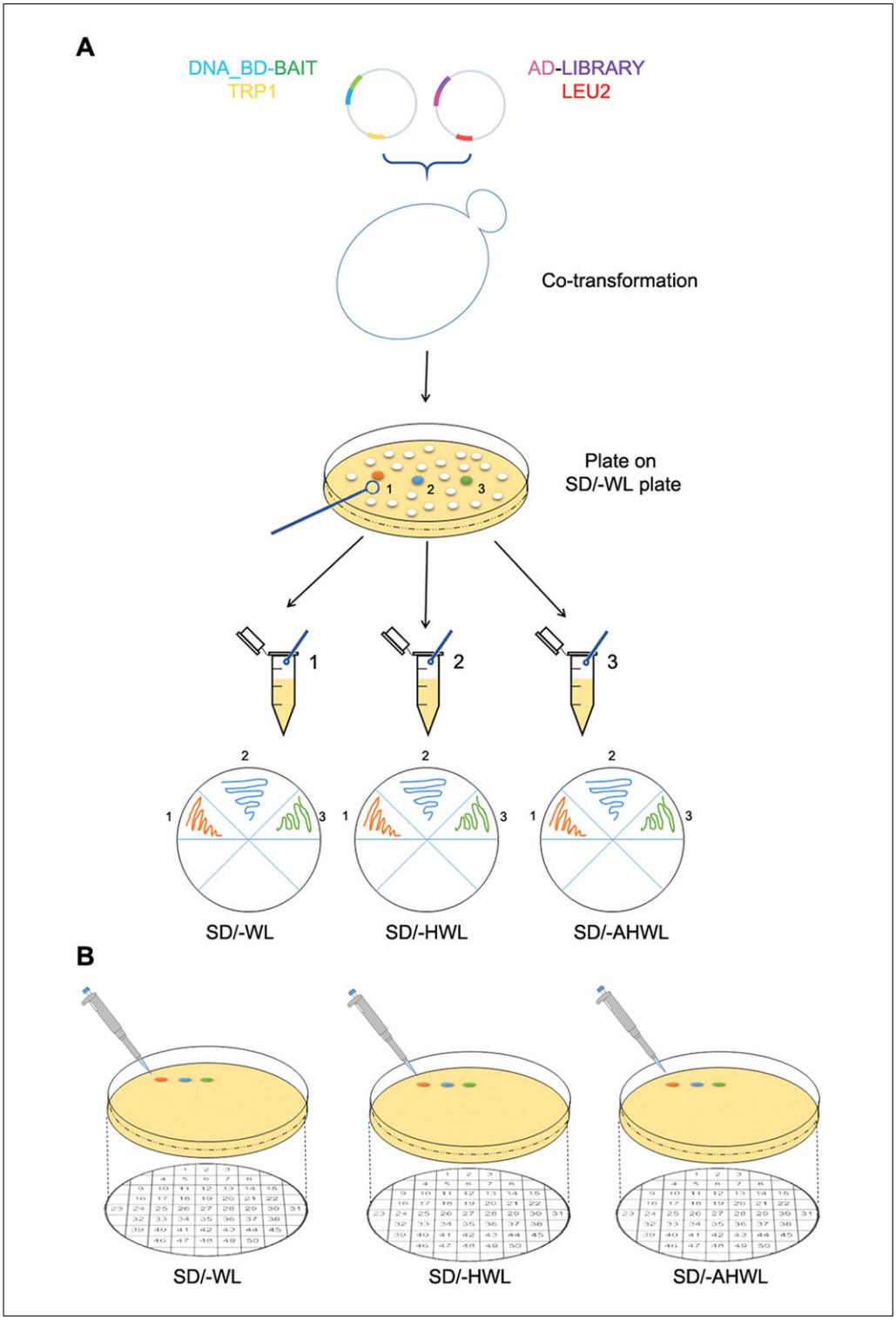

Figure 5 Yeast co-transformation for specificity assays. (**A**) AH109 yeast strain is transformed with BD-bait plasmid and AD-prey plasmid and plated on –WL agar plate for the selection of yeast cells co-transformed with both plasmids. Then, three colonies grown on SD/–WL agar plate are resuspended in 1× TE solution and each colony is streaked, with a sterile loop, on SD/–WL, –HWL, and –AHWL agar plates for the selection of weak and strong interactions, respectively. (**B**) An alternate method to streak the colonies is to spot a few microliters of the sample on agar plates; for this reason three colonies grown on SD/–WL agar plates are resuspended in 1× TE solution; 5 μl of each resuspension is spotted on SD/–WL, -HWL and –AHWL agar plates using, as a guide, a numbered grid positioned under the plate.

1. Inoculate a colony of AH109 yeast from the stock working plate into a 250-ml flask containing 50 ml of YPDA medium.
2. Incubate overnight at 30°C with shaking at 250 rpm.
3. Check the OD_{600}. For 15 transformations, transfer an appropriate volume of the overnight culture into a 1-liter flask containing 200 ml of YPDA in order to get an OD_{600} between 0.2 and 0.3 (for the calculation, see Basic Protocol 1, step 2).

   If you have to do more transformations, the volume of YPDA should be obviously increased accordingly.
4. Incubate until OD_{600} is about 0.5 (should take ~2 hr) at 30°C with shaking at 250 rpm.
5. Prepare the following (see step 4, Basic Protocol 1):

   1× TE/LiAc (30 ml)
   PEG/LiAc (15 ml)
   Carrier DNA.
6. Place cells in 50-ml tubes and centrifuge for 5 min at 1000 × *g*, room temperature.
7. Discard the supernatants and resuspend all four cell pellets in a total of 25 ml of 1× TE solution, pooling the cells in a single 50-ml tube.
8. Centrifuge for 5 min at 1000 × *g*, room temperature.
9. Discard the supernatant and resuspend the cell pellet in 1.5 ml of 1× TE/LiAc solution.
10. For each transformation, add 1 µg of bait plasmid and 1 µg of prey plasmid DNA plus 10 µl of carrier DNA to a 14-ml tube with a round bottom.

    In this co-transformation experiment, it is necessary to include a number of transformation controls.

    Positive control: Co-transformation with both a bait and a prey plasmid containing cDNAs encoding two proteins known to interact in the two-hybrid system. This control makes it possible to establish that the entire procedure has been performed correctly.

    Negative controls: (1) Co-transformation using the pGBKT7 plasmid containing the cDNA encoding the bait protein and the pGADGH vector; this control allows confirmation that expression of the bait protein alone does not activate reporter genes. (2) Co-transformation using the pGBKT7 vector and the pGADGH plasmid containing the cDNA encoding the prey protein; this control has to be done for each prey plasmid isolated from the library in order to check that expression of the prey does not activate reporter genes. If activation of reporter gene is detected, the prey plasmid should be discarded, as activation of transcription is not dependent on the interaction with the bait.
11. Add 100 µl of cells to each tube.
12. Add 750 µl of PEG/LiAc solution to each tube. Vortex for 10 sec to mix.
13. Incubate for 30 min at 30°C in a water bath and gently shake the samples every 5 min.
14. Add 100 µl of DMSO to each tube. Mix gently. Do not vortex.
15. Incubate for 20 min at 42°C in a water bath and gently shake the samples every 5 min.
16. Centrifuge for 5 min at 1000 × *g*, room temperature.
17. Discard the supernatant from each tube with a pipet in order to remove all DMSO.

18. Resuspend cells in 500 µl of 1× TE solution.
19. Plate 100 µl on 10-cm SD/–WL agar plates in order to select for yeast that have been transformed with both plasmids.
20. Incubate plates until colonies appear (it should take about 2 days) at 30°C.
21. Take three colonies from each plate and resuspend each colony in 50 µl of 1× TE solution. With a sterile 1-µl loop, streak each resuspended colony on one SD/–WL, one SD/–HWL, and one SD/–AHWL 10-cm agar plate in order to have the same colony streaked onto the three different selective plates (Fig. 5A; Supporting Information Video 2). An alternative to streaking is spotting 5 µl of each resuspension on an SD/–WL, SD/–HWL, and SD/–AHWL 10-cm agar plate (Fig. 5B; Supporting Information Video 2). Remember to shake the suspension before taking it with the loop for streaking or with the pipet for spotting.

At the end of this procedure, you will expect to have identified prey plasmids encoding bait-interacting proteins. Therefore, after these specificity assays, the insert contained in the selected prey plasmids should be sequenced and sequences subjected to BLAST (Basic Local Alignment Search Tool) analysis (http://www.ncbi.nlm.nih.gov/BLAST) in order to identify the interactors.

THE β-GALACTOSIDASE FILTER ASSAY

BASIC PROTOCOL 4

The β-galactosidase assay allows further checking and possibly quantification of two-hybrid interactions, as it detects β-galactosidase produced from an additional reporter gene. There are two different assays that can be applied. The filter assay (Basic Protocol 4), using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as a substrate for β-galactosidase, measures the activation of the LacZ reporter gene, in addition to HIS3 and ADE2. This is important to reduce the number of false positives, also considering that LacZ is a neutral marker not subject to selective pressure. The liquid assay uses a different substrate, o-nitrophenyl β-D-galactopyranoside (ONPG), which allows quantification of β-galactosidase activity that generates a chromogenic soluble product measurable with a spectrophotometer.

For the β-galactosidase liquid quantitative assay (Basic Protocol 5), the AH109 is not the ideal strain, as LacZ is under the control of a weaker promoter compared to other strains such as Y187 or L40. Therefore, for this assay, we used the L40 strain. For this strain, in order to activate the reporter gene under the control of LexA operators, which are an integral part of the promoter (Ebina, Takahara, Kishi, Nakazawa, & Brent, 1983), the cDNA sequence encoding the bait must be subcloned in the pLexA vector to generate a fusion protein in which the bait is fused to the DNA-BD provided by the LexA protein.

This assay is only qualitative and allows screening of a large number of yeast colonies; thus, it is used to check yeast colonies obtained after the interaction screening of Basic Protocol 1, after co-transformation in Basic Protocol 3, or after mating done in Alternate Protocol 1. The substrate used in this protocol is the colorless X-gal which, if cleaved by β-galactosidase, releases a chromogenic molecule of a characteristic blue color. Thus, cells containing functional β-galactosidase will appear blue.

Materials

SD (sucrose)/–WL medium (see recipe)
SD (sucrose)/–WL and –HWL 10-cm agar plates (see recipe)
β-mercaptoethanol
20 mg/ml X-gal (see recipe)
1× Z buffer (see recipe)

Liquid nitrogen

Sterile inoculating loops
Shaking incubator at 30°C for tubes
Incubator at 30°C for plates
3MM paper (e.g., GE Healthcare Life Sciences, cat. no. 3030917)
15-cm diameter plates
Forceps
Nitrocellulose membrane disks (e.g., RPN82D or RPN132D, GE Healthcare Life Sciences)

1. Resuspend a transformed colony (use only fresh colonies) in 200 µl of SD (sucrose)/–WL medium.
2. Spot 5 µl of each resuspension on a SD (sucrose)/–WL and –HWL 10-cm agar plates and incubate overnight at 30°C. Alternatively yeasts can be streaked directly on the plate.
3. Prepare:

   Rx buffer (10 ml)

β-mercaptoethanol	27 µl
X-gal (20 mg/ml)	200 µl
1× Z buffer	Up to 10 ml

4. Cut a piece of 3MM paper, place it in a sterile 15-cm plate, wet it in Rx buffer, and remove the excess buffer, avoiding formation of air bubbles underneath the paper.
5. Place a round piece of nitrocellulose membrane using forceps on the agar plate where yeast cells have been grown (as colonies, streaks or spots) and then, after few seconds, remove the filter carefully (yeast cells will be transferred to the filter).
6. Place the membrane with the yeast cells facing up on a piece of dry 3MM paper for few seconds.
7. Using forceps transfer the nitrocellulose membrane with the yeast cells facing up in a plate and immerse the plate in liquid nitrogen for 30 sec.
8. Remove the plate from the liquid nitrogen and allow the filter to thaw out.

   This freeze/thaw cycle allows the permeabilization of the cells.

9. Using forceps, transfer the membrane with the yeast cells facing up onto the wet 3MM paper prepared in step 3, avoiding the formation of air bubbles between the filter and the 3MM paper. Incubate at 30°C until the appearance of the blue color.

   Clearly besides the yeast colonies/co-transformants that have to be tested, be sure to include the appropriate controls: yeast co-transformed with a bait and a prey known to interact (positive control) and with a bait and a prey known not to interact (negative control).

BASIC PROTOCOL 5

LIQUID β-GALACTOSIDASE ASSAY

This protocol is used to quantify the strength of the interaction in a number of selected transformants. The assay determines the relative level of β-galactosidase activity in yeast cell extracts by measuring the appearance of a yellow color over time due to the hydrolysis of ONPG, an analog of lactose. Indeed, ONPG hydrolysis results in the release of galactose and a yellow chromogenic compound (Supporting Information Video 3).

Materials

L40 yeast strain grown on plate (Support Protocol 1)
SD (with sucrose)/–WL liquid medium (see recipe)
1× Z Buffer (see recipe)
β-mercaptoethanol (e.g., Sigma-Aldrich, cat. no. M6250)
ONPG (e.g., Sigma-Aldrich, cat. no. N1127)
Liquid nitrogen
1 M sodium carbonate (see recipe)

Sterile inoculating loops
Shaking incubator at 30°C for tubes
25-ml tubes (Ø 25 × 90 mm)
Vortex mixer
1.5 ml microcentrifuge tubes
Microcentrifuge (e.g., Eppendorf 5415 D)
Water bath
Cuvettes
Spectrophotometer

1. Resuspend a colony of selected transformants (use only fresh colonies, grown for 2 to 4 days and not stored at 4°C) in 5 ml of SD/–WL liquid medium in 25-ml tubes. Incubate overnight at 30°C with shaking (250 rpm).
2. Vortex the culture tubes and check the OD_{600} of each inoculum. Dilute the overnight culture, bringing the OD_{600} to 0.3 in a 6 ml final volume of SD with sucrose/–WL liquid medium (see Basic Protocol 1, step 2).
3. Incubate until the OD_{600} is 0.6 (approximatively 2 hr) at 30°C with shaking (250 rpm). Vortex the culture tube before measuring the OD_{600}.
4. Prepare:

   1× Z buffer/β-mercaptoethanol (10 ml)

β-mercaptoethanol	27 µl
1× Z buffer	9.973 ml

   Also prepare ONPG (4 mg/ml) in Buffer Z. Adjust to pH 7 and mix until it is dissolved (1 to 2 hr).

   These solutions must be prepared fresh before use

5. For each sample, centrifuge 1.5 ml of culture for 1 min at 15,700 × *g*, room temperature, and remove the supernatant.
6. Resuspend the cell pellet in 1.5 ml of 1× Z Buffer.
7. Centrifuge for 1 min at 15,700 × *g*, room temperature.
8. Remove the supernatant and resuspend the cell pellet in 0.3 ml of 1× Z Buffer.
9. Transfer 100 µl of the cell suspension to a 1.5-ml microcentrifuge tube.
10. Place the tube in liquid nitrogen for 0.5 to 1 min.
11. Place the tube in a water bath for 0.5 to 1 min at 37°C.
12. Repeat the steps 10 and 11 two more times.
13. Add 700 µl of 1× Z buffer/β-mercaptoethanol to each tube.

14. Prepare a blank sample in a 1.5-ml microcentrifuge tube containing:

1× Z buffer	100 µl
1× Z buffer/β-mercaptoethanol	700 µl

15. Immediately add 160 µl of ONPG/1× Z buffer to each tube from steps 13 and 14. This is the starting time (T0).
16. Incubate at 30°C in the dark until a yellow color develops. If the color develops too quickly (within seconds), repeat the assay using lower amounts of yeast cells. If the color does not develop, repeat the assay using higher amounts of yeast cells.
17. To stop the reaction, add 400 µl of 1 M sodium carbonate to each tube and write down the ending time (T1).
18. Record elapsed time from T0 to T1.
19. Centrifuge for 10 min at 15,700 × *g*.
20. Transfer 1 ml of each supernatants to a cuvette.
21. Measure the OD_{420} of the blank and then of the samples.
22. Calculate β-galactosidase units for each sample according to the following formula:

$$\beta\text{-galactosidase units} = 1000 \times OD_{420}/(T \times V \times OD_{600})$$

T= T1 – T0 (elapsed time of incubation expressed in decimal; for example, 24 min and 50 sec is equivalent to about 24.8 min, and this is obtained dividing seconds by 60).
V= volume of culture (in ml) added multiplied by the concentration factor. In this case, the volume of culture is 0.1 ml and the concentration factor is 5, as the initial yeast culture was concentrated 5 times (from 1.5 ml to 0.3 ml).
OD_{600} is the OD_{600} of 1 ml of culture measured at step 3.

ALTERNATE PROTOCOL 1

YEAST MATING

Yeast mating is an alternative method used for yeast co-transformation in two-hybrid systems (Kolonin, Zhong, & Finley, 2000; Soellick & Uhrig, 2001). This approach can be used in order to to do an interaction screening using a cDNA library, to test protein-protein interactions, or to do specific tests. Thus, it can be used as an alternative to Basic Protocol 1 or Basic Protocol 3, and is particularly useful to overcome problems in transformation efficiency. In yeast mating, the bait and prey plasmid are expressed in two different haploid yeast strains with opposite mating types. In particular, the bait plasmid can be expressed in Y187 cells and the prey plasmid can be expressed in AH109 cells. To test the interaction between bait and prey, a single transformant colony of Y187 can be combined with a single transformant colony of AH109 to allow diploid cells to form visible colonies that are then tested for activation of reporter genes (Fig. 6).

Materials

AH109 stock plate (see Support Protocol 1)
YPDA medium (see recipe)
Y187 stock plate (see Support Protocol 1)
SD/–L 10-cm-diameter agar plates
SD/–W 10-cm-diameter agar plates
Bait plasmid
Prey plasmid
SD/–WL, SD/–HWL, and SD/–AHWL 10-cm agar plates (see recipes)

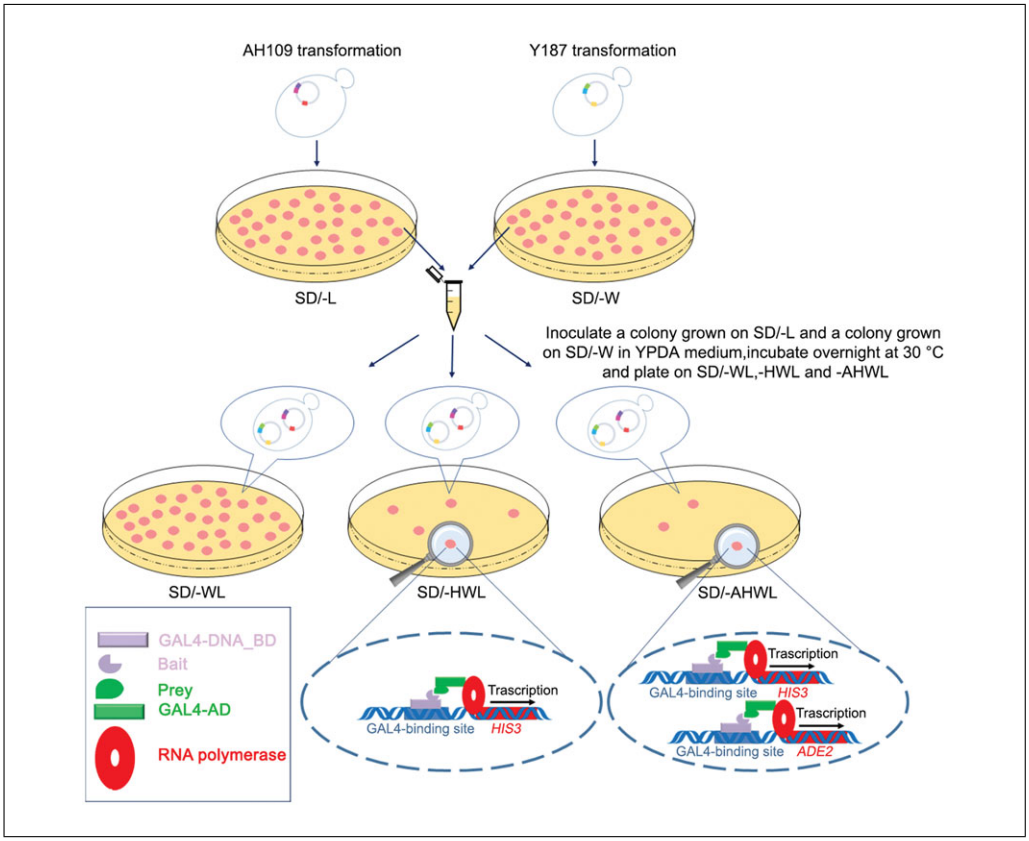

Figure 6 Yeast mating. AH109 yeast strain is transformed with AD-prey plasmid and plated on SD/–L agar plate; the Y187 yeast strain is transformed with BD-bait plasmid and plated on SD/–W agar plate. A colony grown on an SD/–L plate and a colony grown on an SD/–W agar plate are resuspended together in YPDA medium and incubated overnight at 30°C. The next day, the culture is streaked on SD/–WL, SD/–HWL, and –AHWL agar plates.

Sterile inoculating loops
Flasks
Shaking incubator at 30°C for tubes and flasks
Cuvettes
Spectrophotometer
Incubator at 30°C for plates
1.5 ml microcentrifuge tubes
Vortex mixer
Sterile glass beads (e.g., Sigma-Aldrich, cat. no. 18406)

1. Inoculate a colony of AH109 yeast from the stock plate into a 250-ml flask containing 50 ml of YPDA medium.
2. Inoculate a colony of Y187 yeast from the stock plate into a 250-ml flask containing 50 ml of YPDA medium.
3. Incubate overnight at 30°C with shaking at 250 rpm.
4. Check the OD_{600} of both overnight cultures and transfer a sufficient volume of them into 1-liter flasks containing 160 ml of YPDA each (for 10 transformations). Check the OD_{600} of the diluted cultures, which must be between 0.2 and 0.3 (for the calculation, see Basic Protocol 1, step 2).
5. Incubate until the OD_{600} is 0.5 ± 0.1 (approximately for 3 hr) at 30°C with shaking at 250 rpm.

6. Transform the AH109 cells with the following plasmids and plate on SD/–L agar plate to select the transformed colonies:

   pGAD GH plasmid
   AD/library and/or prey pGAD GH plasmids

7. Transform the Y187 cells with the following plasmids and plate on SD/–W agar plate to select the transformed colonies:

   pGBKT7 plasmid
   DNA-BD/bait pGBKT7 plasmid

8. Incubate until colonies grow (2 to 4 days) at 30°C.
9. Inoculate a colony grown on SD/–L plate and a colony grown on SD/–W plate in one 1.5-ml microcentrifuge tube containing 500 µl of YPDA medium, and vortex.
10. Incubate overnight at 30°C at 250 rpm.
11. Spread 100 µl of the mating culture on SD/–WL, SD/–HWL and SD/–AHWL plates using glass beads and incubate until colonies appear (3-5 days) at 30°C.

SUPPORT PROTOCOL 1

PREPARATION OF YEAST STRAIN WORKING PLATE

AH109, L40, and Y187 yeast strains are stored in YPDA medium with 25% glycerol at – 80°C. This protocol describes how to streak the yeast on a plate from a –80°C stock.

Materials

AH109 yeast strain glycerol stock stored at –80°C
L40 yeast strain glycerol stock stored at –80°C
Y187 yeast strain glycerol stock stored at –80°C
10-cm YPDA agar plate

Sterile inoculating loop
Incubator at 30°C for plates

1. Take a small portion of the yeast strain stock stored at –80°C and streak it onto a 10-cm YPDA agar plate.
2. Incubate for 3 to 4 days, until yeast colonies grow, at 30°C. Store the working stock plate at 23°C and use for 2 weeks at maximum.

SUPPORT PROTOCOL 2

PREPARATION OF ULTRA-COMPETENT CELLS (INOUE METHOD)

This protocol describes how *E. coli* cells, which are not naturally transformable, can be induced to take up plasmid DNA from the environment by a procedure described by Inoue in 1990 (Inoue, Nojima, & Okayama, 1990).

Materials

DH5α cells (Thermo Fisher Scientific) glycerol stock stored at –80°C
10-cm LB agar plate (see recipe)
LB liquid medium (see recipe)
SOB medium (see recipe)
Ice-cold TB buffer (see recipe)
DMSO, filtered with a 0.22 µm pore filter
Liquid nitrogen

Sterile inoculating loops
Incubator at 37°C for plates

10-ml cylindrical tubes, Ø 16 × 100 mm (e.g., 1009/TE/SG, APTACA)
Shaking incubator at 37°C for tubes and flasks
1-liter flask
Spectrophotometer
Cuvettes
50-ml conical tube
Centrifuge
1.5-ml microcentrifuge tubes

1. Using a sterile loop, streak a small portion of the DH5α bacteria from the frozen glycerol stock stored at −80°C on a LB agar plate and incubate overnight at 37°C.
2. Inoculate a single colony grown on the plate in a 10-ml cylindrical tube in 5 ml of LB and incubate overnight at 37°C with shaking at 250 rpm.
3. The next day, inoculate 1 ml of the overnight culture in 250 ml of SOB medium in a 1-liter flask.
4. Incubate the culture with shaking until the OD_{600} is 0.6 at 20°C (150 to 200 rpm).
5. Dispense the culture in centrifuge tubes and put them on ice for 10 min.
6. Centrifuge the cells for 10 min at 1800 × *g*, 4°C.
7. Discard the supernatant, resuspend the pellet in 80 ml of cold TB buffer, and divide the culture in two 50-ml tubes (40 ml per tube).
8. Put the tubes on ice for 10 min.
9. Centrifuge for 10 min at 1800 × *g*, 4°C.
10. Discard the supernatant, resuspend the pellet in 20 ml of cold TB buffer (10 ml for tube), and transfer all into a single tube.
11. Add 1.4 ml of DMSO (to a final concentration of 7%) and put on ice for 10 min.
12. Aliquot in 1.5-ml microcentrifuge tubes (consider 200 µl for each transformation and make aliquots for two to three transformation each).
13. Freeze the competent cells in liquid nitrogen, then store at −80°C until needed.

REAGENTS AND SOLUTIONS

Adenine, 0.2% (w/v) (250 ml)

To a glass beaker add:

Adenine (e.g., Sigma-Aldrich, cat. no. A9126)	0.5 g
Double-distilled water	up to 250 ml

Stir until completely dissolved and sterilize by passing through a 0.22-µm pore filter. Store up to 3 months at 4°C.

Ammonium acetate, 7.5 M (200 ml)

Ammonium acetate	115.62 g
Double-distilled water	up to 200 ml

Sterilize by passing through a 0.22-µm pore filter. Store at room temperature.

Ampicillin (50 mg/ml) (50 ml)

Ampicillin (e.g., Sigma-Aldrich, cat. no. A9518)	2.5 g
Double-distilled water	up to 50 ml

Dissolve by stirring. Sterilize by passing through a 0.22-µm pore filter. Aliquot in sterile 1.5-ml microcentrifuge tubes and store at –20°C.

Carrier DNA (10 mg/ml) (20 ml)

To a sterile glass beaker containing a sterile stir bar, add:

Salmon sperm DNA, sodium salt (e.g., Sigma, cat. no. D1626)	200 mg
Sterile double-distilled water	20 ml

Stir for at least 2 to 4 hr to dissolve the DNA. To reduce the viscosity of the solution, pass the DNA solution rapidly approximately 20 times through a 17-G needle or a couple of times through a 23-G needle. If sonication is used, the DNA is sonicated until it is no longer extremely viscous.

Dispense the DNA in 1-ml aliquots into sterile 1.5-ml microcentrifuge tubes and store at – 20°C.

Dropout solution, 10× (1 liter)

To a 1-liter bottle, add:

L-Arginine HCl (e.g., Sigma, cat. no. A6969, 200 mg/liter final concentration)	200 mg
L-Isoleucine (e.g., Sigma, cat. no. I7403, 300 mg/liter final concentration)	300 mg
L-Lysine HCl (e.g., Sigma, cat. no. L5626, 300 mg/liter final concentration)	300 mg
L-Methionine (e.g., Sigma, cat. no. M9625, 200 mg/liter final concentration)	200 mg
L-Phenyalanine (e.g., Sigma, cat. no. P5482, 500 mg/liter final concentration)	500 mg
L-Threonine (e.g., Sigma, cat. no.T8625) (2000 mg/liter final concentration)	2000 mg
L-Tyrosine (e.g., Sigma, cat. no.T3754, 300 mg/liter final concentration)	300 mg
L-Uracil (e.g., Sigma, cat. no.U0750, 200 mg/liter final concentration)	200 mg
L-Valine (e.g., Sigma, cat. no.V0513, 1500 mg/liter final concentration)	1500 mg
Double-distilled water	up to 1 liter

Autoclave 15 min at 121°C, put on ice immediately, and store up to 1 year at 4°C.

EDTA, 0.5 M (500 ml), pH 8

In a glass beaker combine:

Disodium EDTA	93.05 g
Double-distilled water	up to 400 ml

Stir vigorously on a magnetic stirrer. Adjust the pH to 8 with NaOH. Adjust the total volume of the solution to 500 ml with water. Dispense the solution in a bottle and autoclave 20 min at 121°C. Store up to 1 year at room temperature.

The EDTA will not be completely dissolved until the pH of the solution is adjusted to approximately 8 with NaOH.

Glucose, 40% (w/v) (500 ml)

In a glass beaker combine:

Glucose (e.g., Sigma, cat. n. G7528	200 g
Double-distilled water	up to 400 ml

Stir until completely dissolved and adjust the total volume to 500 ml with water. Sterilize by passing through a 0.22-µm pore filter. Store up to 1 month at 4°C.

Histidine, 1% (w/v) (50 ml)

L-Histidine HCl (e.g., Sigma, cat. no. H8125, Sigma-Aldrich)	0.5 g
Double-distilled water	up to 50 ml

Dissolve by stirring and sterilize by passing through a 0.22-µm pore filter. Store up to 3 months at 4°C.

LB medium (liquid and solid) (500 ml)

Bacto Tryptone (e.g., Oxoid, cat. no. LP0042) (10 g/liter final concentration)	5 g
Yeast Extract (e.g., Oxoid, cat. no. LP0021 (5 g/liter final concentration)	2.5 g
NaCl (10 g/liter final concentration)	5 g
Double-distilled water	up to 500 ml

Autoclave at 121°C for 20 min. Store up to 1 month at room temperature.

For LB agar plates: Before autoclaving add 7.5 g agar (e.g., Oxoid, cat. no. LP0011) (15 g/liter final concentration). Autoclave at 121°C for 20 min. Allow medium to cool down to 55°C in a water bath and dispense the warm medium into sterile plates. When solidified, store plates upside down up to 1 month at 4°C (for each 10-cm plate approximately 25 ml of medium is required).

For LB agar plates supplemented with ampicillin (75 µg/ml final concentration): After autoclaving, put the flask in a water bath at 55°C in order to cool it down, and just before dispensing the medium into sterile plates, add 750 µl of ampicillin stock solution (50 mg/ml; see recipe). It is important to add ampicillin only when the medium has cooled down to 55°C in order to avoid ampicillin inactivation.

Leucine, 0.2% (w/v) (50 ml)

Leucine (e.g., Sigma, cat. no. L8000)	0.1 g
Double-distilled water	up to 50 ml

Dissolve by stirring and sterilize by passing through a 0.22-µm pore filter. Store up to 3 months at 4°C.

Lithium acetate (LiAc), 1M, pH 7.5 (100 ml)

To a glass beaker, add:

Lithium acetate (e.g., Sigma, cat. no. L6883)	10.2 g
Double-distilled water	up to 100 ml

Dissolve by stirring. Adjust to pH 7.5 with diluted acetic acid. Dispense in glass bottle, autoclave 20 min at 121°C, and store up to 1 year at 4°C.

Lysozyme, (10 mg/ml) (20 ml)

Lysozyme (e.g., Sigma, cat. no. L6876)	200 mg
1× TE (see recipe)	up to 20 ml

Dissolve by vortexing, aliquot in 1.5-ml microcentrifuge tubes, and store up to 1 year at –20°C.

PEG, 50% (w/v) (250 ml)

To a glass beaker, add:

PEG (e.g., Sigma, cat. no. P3640)	125 g
Double-distilled water	up to 200 ml

Dissolve completely by stirring, adjust the total volume to 250 ml and sterilize by passing through a 0.45-µm pore filter. Store up to 3 months at 4°C.

SD (synthetically defined) liquid medium (250 ml)

To a bottle add:

Yeast nitrogen base w/o amino acids (e.g., Sigma, cat. no. Y0626 or BD Biosciences) (6.7 g/liter final concentration)	1.67 g
Double-distilled water	up to 210 ml

Autoclave 25 min at 121°C and add:

10× dropout solution (see recipe)	25 ml
40% glucose (see recipe) or 40% sucrose (see recipe) (2% final concentration each)	12.5 ml

For SD/–L, after autoclaving add also:

0.2% adenine (see recipe; 0.002% final concentration)	2.5 ml
1% histidine (see recipe; 0.002% final concentration)	0.5 ml
1% tryptophan (see recipe; 0.002% final concentration)	0.5 ml

Store up to 1 month at 4°C.

For SD/–W, after autoclaving add also:

0.2% adenine (see recipe; 0.002% final concentration)	2.5 ml
1% histidine (see recipe; 0.002% final concentration)	0.5 ml
0.2% leucine (see recipe; 0.002% final concentration)	2.5 ml

Store up to 1 month at 4°C.

For SD/–WL, after autoclaving add also:

0.2% adenine (see recipe; 0.002% final concentration)	2.5 ml
1% histidine (see recipe; 0.002% final concentration)	0.5 ml

Store up to 1 month at 4°C.

SD medium, 3× (1 liter) (for plates)

In a glass beaker combine:

Yeast nitrogen base without amino acids (e.g., Sigma, cat. no. Y0626) (20 g/liter final concentration)	20 g
40% (w/v) glucose (see recipe) or 40% sucrose (see recipe) (6% final concentration)	150 ml
10× dropout solution (see recipe)	300 ml
Double-distilled water	up to 800 ml

Add stir bar. First, adjust to pH 5.8 to 6.0 with NaOH and then adjust the total volume to 1 liter with water. Sterilize by passing through a 0.22 µm pore filter. Store at 4°C up to 1 month.

For SD agar plates (600 ml):

In a 1-liter flask combine:

Bacto Agar (e.g., BD Biosciences, cat. no. 214010) (15 g/liter final concentration)	9 g
Double-distilled water	up to 400 ml

Autoclave 20 min at 121°C. Allow medium to cool down to 55°C.

For SD/–AHWL plates, after autoclaving add:

3× SD medium (see above)	200 ml

For SD/–HWL agar plates after autoclaving add:

0.2% adenine (see recipe; 0.002% final concentration)	6 ml
3× SD medium (see above)	200 ml

For SD/–WL agar plates, after autoclaving add:

0.2% adenine (see recipe; 0.002% final concentration)	6 ml
1% histidine (see recipe; 0.002% final concentration)	1.2 ml
3× SD medium (see above)	200 ml

For SD/–L agar plates, after autoclaving add:

0.2% adenine (see recipe; 0.002% final concentration)	6 ml
1% histidine (see recipe; 0.002% final concentration)	1.2 ml
1% tryptophan (see recipe; 0.002% final concentration)	1.2 ml
3× SD medium (see above)	200 ml

For SD/–A agar plates, after autoclaving add:

0.2% leucine (see recipe; 0.002% final concentration)	6 ml
1% histidine (see recipe; 0.002% final concentration)	1.2 ml
1% tryptophan (see recipe; 0.002% final concentration)	1.2 ml
3× SD medium (see above)	200 ml

For SD/–H agar plates, after autoclaving add:

0.2% adenine (see recipe; 0.002% final concentration)	6 ml
0.2% leucine (see recipe; 0.002% final concentration)	6 ml
1% tryptophan (see recipe; 0.002% final concentration)	1.2 ml
3× SD medium (see above)	200 ml

For SD/–W agar plates, after autoclaving add:

0.2% adenine (see recipe; 0.002% final concentration)	6 ml
1% histidine (see recipe; 0.002% final concentration)	1.2 ml
0.2% leucine (see recipe; 0.002% final concentration)	6 ml
3× SD medium (see above)	200 ml

Dispense the warm medium into sterile plates (for each 10-cm plate, approximately 25 ml of medium are required). Store plates, when solidified, upside down, for up to 1 month at 4°C.

SOB medium (500 ml)

Tryptone (e.g., Oxoid, cat. no. LP0042) (20 g/liter final concentration)	10 g
Yeast Extract (e.g., Oxoid, cat. no. LP0021) (5 g/liter final concentration)	2.5 g
NaCl (10 mM final concentration)	290 mg
KCl (2.5 mM final concentration)	92 mg
$MgCl_2 \cdot 6H_2O$ (10 mM final concentration)	1 g
$MgSO_4 \cdot 7H_2O$ (10 mM final concentration)	1.23 g
Double-distilled water	up to 350 ml

Dissolve by stirring. Adjust the pH to 6.7 with 10 N NaOH; adjust the total volume to 500 ml and autoclave for 20 min at 121°C. Store up to 3 months at room temperature.

Sodium acetate, 3 M, pH 5.2 (500 ml)

Sodium acetate·$3H_2O$	204 g
Double-distilled water	400 ml

Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 500 ml with double-distilled water and sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

Sodium carbonate (Na_2CO_3), 1M (100 ml)

Na_2CO_3	10.6 g
Double-distilled water	up to 100 ml

Sterilize by passing through a 0.22-µm pore filter. Store at room temperature.

STET buffer (100 ml)

Triton X-100 (5% final concentration)	5 ml
0.5 M EDTA, pH 8 (50 mM final concentration)	10 ml
1 M Tris·Cl, pH 8 (see recipe; 50 mM final concentration)	5 ml
Sucrose (e.g., Riedel-de Haën, cat. no. 16104) (8% w/v final concentration)	8 gr
Double-distilled water	up to 100 ml

Dissolve by stirring. Store up to 1 year at 4°C.

Sucrose, 25% (w/v) (100 ml)

Sucrose	25 g
Double-distilled water	up to 80 ml

Stir until completely dissolved and adjust the total volume to 100 ml with double-distilled water. Sterilize by passing through a 0.22-µm pore filter. Store up to 1 month at 4°C.

Sucrose, 40% (w/v) (200 ml)

Sucrose	80 g
Double-distilled water	up to 160 ml

Stir until completely dissolved and adjust the total volume to 200 ml with double-distilled water. Sterilize by passing through a 0.22-µm pore filter. Store up to 1 month at 4°C.

TB buffer (200 ml)

PIPES (10 mM final concentration)	0.6 g
$CaCl_2$ (15 mM final concentration)	0.44 g
KCl (250 mM final concentration)	3.7 g
Double-distilled water	up to 150 ml

Adjust to pH 6.7 with 5 N KOH, then add:

$MnCl_2 \cdot 4H_2O$ (55 mM final concentration)	2.17 g
Double-distilled water	up to 200 ml

Sterilize by passing through a 0.22-µm pore filter. Store up to 1 year at 4°C.

TE + RNase A (100 µg/ml) (10 ml)

RNase A (e.g., Sigma, cat. no. R6513)	1 mg
1× TE (see recipe)	up to 10 ml

Aliquot in microcentrifuge tubes and store at –20°C.

TE, 10× (200 ml)

To a bottle, add:

1 M Tris·Cl, pH 7.5 (see recipe; 100 mM final concentration)	20 ml
Disodium EDTA (10 mM final concentration)	0.9 g
Double-distilled water	up to 100 ml

Adjust to pH 7.5 with HCl and adjust the total volume to 200 ml with water. Autoclave 20 min at 121°C. Store up to 1 year at 4°C.

TE, 1× (20 ml)

To prepare 20 ml, combine:

10× TE (see recipe)	2 ml
Double-distilled water	18 ml

Tris·Cl, 1 M (500 ml), pH 7.5 or 8

In a glass beaker combine:

Tris base (e.g., Sigma, cat. no. T1503)	60.55 g
Double-distilled water	up to 400 ml

Dissolve by stirring. Adjust the pH to 7.5 or 8 with HCl. Adjust the total volume of the solution to 500 ml with water. Dispense the solution in a bottle and autoclave 20 min at 121°C. Store up to 1 year at room temperature.

Tryptophan, 1% (w/v) (50 ml)

L-Tryptophan (e.g., Sigma, cat. no. T0254)	0.5 g
Double-distilled water	up to 50 ml

Dissolve by stirring and sterilize by passing through a 0.22-µm pore filter. Store up to 3 months at 4°C.

X-gal, 20 mg/ml (10 ml)

X-gal (BD Biosciences, cat. no. 8060-1)	200 mg
N,*N*-dimethylformamide (Carlo Erba, cat. no. 444921)	up to 10 ml

Store in the dark up to 1 year at –20°C.

YPDA agar medium (300 ml)

To one 200 ml flask, add:

Bacto Yeast Extract (e.g., BD Biosciences, cat. no. 212750) (1% final concentration)	3 g
Bacto Peptone (e.g., BD Biosciences, cat. no. 211677) (2% final concentration)	6 g
Double-distilled water	up to 80 ml

To a second 500 ml flask, add:

Bacto agar (e.g., BD Biosciences, cat. no. 214010) (1.5% final concentration)	4.5 g
Double-distilled water	up to 200 ml

Autoclave both flasks at 121°C for 20 min. Allow medium to cool down to 55°C

To the first flask, add:

40% glucose (see recipe; 2% final concentration)	15 ml
0.2% adenine (see recipe; 0.004% final concentration)	4.5 ml

Allow medium to cool down to 55°C. Then, pour the contents of the first flask into the second flask and dispense the warm medium into sterile plates. Store plates when solidified upside down up to 1 month at 4°C.

YPDA liquid medium, 1× (1 liter)

To a 1-liter bottle add:

Bacto Yeast Extract (e.g., BD Biosciences, cat. no. 212750) (1% final concentration)	10 g
Bacto Peptone (e.g., BD Biosciences, cat. no. 211677) (2% final concentration)	20 g
Double-distilled water	up to 935 ml

Autoclave 20 min at 121°C and then, when the solution is cold, add:

40% glucose (see recipe; 2% final concentration)	50 ml
0.2% adenine (see recipe; 0.004% final concentration)	15 ml

Store up to 1 month at room temperature.

Z buffer, 1× (200 ml)

$Na_2HPO_4 \cdot 7\ H_2O$ (16.1 g/liter final concentration)	3.22 g
$NaH_2PO_4 \cdot H_2O$ (5.5 g/liter final concentration)	1.1 g
KCl (0.75 g/liter final concentration)	0.15 g
$MgSO_4 \cdot 7\ H_2O$ (0.246 g/liter final concentration)	0.05 g
Double-distilled water	up to 150 ml

Adjust to pH 7 and adjust the total volume with water. Autoclave 20 min at 121°C. Store up to 1 year at room temperature.

COMMENTARY

Background Information

The yeast two-hybrid assay, developed in 1989, is a very powerful technique for discovering and investigating protein–protein interactions *in vivo* and also for understanding biological processes crucial for cell functions (Fields & Song, 1989). Several techniques are available to analyze protein–protein interactions. Biochemical approaches, such as co-immunoprecipitation, affinity

chromatography followed by mass spectrometry, and the phage or virion display technique have been used in recent years, although these techniques are usually time consuming and laborious. Instead, the yeast two-hybrid assay allows rapid identification of several putative interacting proteins *in vivo* (Chien, Bartel, Sternglanz, & Fields, 1991; Fields & Song, 1989). In addition, the two-hybrid system has provided an important contribution for interactome maps. Indeed, the combination with mass spectrometry allows development of a complete and reliable map of interactions, as the two-hybrid system mainly identifies direct binary interactions while mass spectrometry identifies members of a complex.

The great success of this technique can be attributed also to the versatility of the system, demonstrated by the fact that numerous modifications of the system have been developed in recent years. These approaches allow the identification and analysis of different types of interactions, such as interactions involving membrane proteins, DNA-binding proteins, and RNA binding proteins (Stasi, De Luca, & Bucci, 2015). Moreover, this technique is suitable to high-throughput screening, which represents the future of the method, and it will probably contribute to identifying new interaction networks and characterizing new molecular mechanisms underlying cellular processes.

Critical Parameters and Troubleshooting

False positives and false negatives

An important critical aspect of the two-hybrid assay is the generation of false positives and false negatives. False negatives are protein-protein interactions that are not detectable in the system. False negatives may occur for different reasons: for instance, (1) the fusion proteins could be toxic for the yeast cells; 2) the interaction between two proteins could be associated with post-translational modifications that the yeast cells are incapable of performing correctly; (3) the construction of the fusion protein could result in a change in conformation of the interacting proteins that alters or hides the interacting domains, preventing interaction between the prey and the bait.

False positives could be caused by protein-protein interactions that occur in the yeast system but have no biological relevance. An example would be an interaction between the prey and the bait that occurs in the context of the two-hybrid assay but does not happen in other experimental systems because the two proteins are normally expressed in different tissues, are localized to different cellular compartments, or are expressed at different stages of development and therefore never come in contact with each other. False positives could also be caused by nonspecific activation of the reporter gene transcription by the prey protein (i.e., if the prey protein alone is able to bind DNA and activate reporter gene transcription).

To minimize false positives, more than one reporter gene has to be used. Importantly, an interaction identified through the two-hybrid assay must be validated with alternative techniques such as co-immunoprecipitation and pull-down.

Transformation efficiency

The transformation efficiency of yeast cells is crucial for the success of the two-hybrid assay. It should be at least 10^4 cfu/μg for transformation with a single plasmid and at least 10^3 cfu/μg in the co-transformation with two plasmids. If the transformation efficiency is lower than expected, it could be due to several causes.

- Make sure that the reagents used have been properly prepared and are not contaminated. Remake the reagents and repeat the transformation.
- Make sure to have used the right amount of plasmid. Repeat the transformation using more or less DNA. If the co-transformation efficiency is lower than 10^3 cfu/μg, calculate transformation efficiency of plasmids individually. If the efficiency for a single transformation is $\geq 10^4$ cfu/μg, repeat the experiment, making a sequential transformation (transform the yeast cells with a plasmid and then transform the selected transformants with the second plasmid).
- Make sure to have optimal carrier DNA. Otherwise, repeat the transformation using a new aliquot of carrier DNA stored at –20°C, or prepare new carrier DNA.
- Make sure that the density of yeast culture is correct. The yeast culture must be in log-phase growth when it is used for the transformation. If the yeast culture grows slowly, prepare a fresh overnight culture or prepare a new yeast stock working plate from the glycerol stock and inoculate a fresh colony.

Anticipated Results

The correct execution of the procedures should guarantee the achievement of typically expected results for each protocol.

In Basic Protocol 1, after steps 18, 33, and 35, growth of several colonies would be appropriate. The presence of colonies after step 18 indicates that the transformation procedure has been performed correctly and that the bait plasmid has been introduced in yeast cells. Selection on SD/–W agar plates allows selection of transformed yeast cells. Also, growth of yeasts after step 35 on SD/–WL agar plates shows that prey plasmid has been introduced into yeast transformed previously with the bait plasmid. Transformation efficiency calculated at step 36 is expected to be 10^4 cfu/μg or higher for library screenings. This transfection efficiency guarantees that the number of independent clones screened is representative, and that putative positive clones in which transcription of reporter genes has been activated will appear after plating on SD/–HWL and SD/–AHWL agar plates in step 33. These clones then have to be tested in order to establish that growth on SD/–HWL and SD/–AHWL is actually due to the interaction between bait and prey using Basic Protocol 3 (specificity assay). In order to do this, DNA extraction and amplification from yeast clones should be performed.

In Basic Protocol 2, step 36 should provide a pellet indicating the correct extraction of plasmid DNA, while after step 25 several bacterial colonies should appear, indicating the success of the DNA isolation from yeasts, the quality of the plasmid used for bacterial transformation, and the successful transformation of *E. coli* cells. Step 40 will ensure the quality of the DNA extracted from bacteria that will be used in specificity assay.

In Basic Protocol 3, after step 20, several colonies should grow if the transformation procedure has been conducted correctly. At the end of step 21, growth on SD/–HWL is expected if interaction between bait and prey proteins occurs, while growth on SD/–AHWL should happen only in case of strong interaction, so fewer colonies are expected. Positive and negative controls should give the expected results, allowing selection of true positives. In fact, if colonies will also grow on SD/–HWL or SD/–AHWL when only prey proteins are expressed, this means that reporter gene activation does not depend on the interaction between bait and prey, and therefore those prey plasmids should be discarded. Specificity tests should be done also looking at the β-galactosidase reporter gene using the filter assay (Basic Protocol 4). In this case, blue colonies should appear after step 9 of the assay, again only in the presence of both bait and prey. Prey plasmids that will pass the specificity tests for all reporter genes (including the gene coding for β-galactosidase) should then be sequenced. BLAST analysis of the insert sequence will allow the identification of the prey protein interacting with the bait. Then, the interaction can be further investigated to evaluate its strength with the liquid β-galactosidase assay of Basic Protocol 5.

In this case, a yellow color should develop at step 16, making it possible to evaluate the strength of the interaction based on the intensity of the color. Yellow color indicates the hydrolysis of ONPG substrate, and the values of β-galactosidase units at step 22 should be appropriate for controls.

Finally, colonies should grow at steps 8 and 11 of Alternate Protocol 1. Growth after step 8 indicates that the single transformation occurred properly, while growth on SD/–WL at step 11 proves the mating of the two yeast strains. Colonies on SD/–HWL and SD/–AHWL are to be expected if there is interaction between the bait and prey proteins.

Time Considerations

If the plasmid encoding the bait and the cDNA library are ready, the cDNA library screening (Basic Protocol 1) should take 10 days at maximum. Then, the putative positive clones are collected by streaking them on appropriate agar plates (at least 2 days are needed for yeast growth). Subsequently, library plasmids will be extracted (2 days) and used to transform *E. coli* in order to amplify and purify them (3 days). Clearly, however, the time spent for these procedures will vary depending on the number of clones resulted from the screening. Plasmids prepared from *E. coli* are then used in specificity assays in order to discard false positives, and this procedure requires about a week, but again the time necessary can increase if a high number of putative positive clones were initially obtained after the screening. Once true positives have been selected, the inserts will be sequenced in order to identify the interactors.

Thus, the procedure indicated in Basic Protocol 1 requires 7 to 10 days, while Basic Protocol 2 can be performed in around 2 weeks. Yeast co-transformation for specificity assays (Basic Protocol 3) requires about 1 week, while performing a β-galactosidase assay (Basic Protocols 4 and 5) may require from a few days to 1 week depending on which

assay is chosen and on the number of clones. Finally, yeast mating (Alternate Protocol 1) requires 7 to 10 days.

Preparation of necessary materials, such as making a yeast stock working plate, are not included in this time assessment and should be evaluated separately.

Supporting Information

The supporting information text file discussed in this article can only be accessed from the online version of this article.

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Literature Cited

Causier, B. (2004). Studying the interactome with the yeast two-hybrid system and mass spectrometry. *Mass Spectrometry Reviews*, *23*, 350–367. doi: 10.1002/mas.10080.

Chien, C. T., Bartel, P. L., Sternglanz, R., & Fields, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(21), 9578–9582. doi: 10.1073/pnas.88.21.9578.

Ebina, Y., Takahara, Y., Kishi, F., Nakazawa, A., & Brent, R. (1983). LexA protein is a repressor of the colicin E1 gene. *World Journal of Biological Chemistry*, *258*, 13258–13261.

Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, *340*, 245–246. doi: 10.1038/340245a0.

Inoue, H., Nojima, H., & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, *96*, 23–28. doi: 10.1016/0378-1119(90)90336-P.

James, P., Halladay, J., & Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*, *144*(4), 1425–1435.

Kolonin, M. G., Zhong, J., & Finley, R. L. (2000). Interaction mating methods in two-hybrid systems. *Methods in Enzymology*, *328*, 26–46. doi: 10.1016/S0076-6879(00)28388-2.

Ma, J., & Ptashne, M. (1988). Converting a eukaryotic transcriptional inhibitor into an activator. *Cell*, *55*, 443–446. doi: 10.1016/0092-8674(88)90030-X.

Parrish, J. R., Gulyas, K. D., & Finley, R. L. J. (2006). Yeast two-hybrid contributions to interactome mapping. *Current Opinion in Biotechnology*, *17*, 387–393. doi: 10.1016/j.copbio.2006.06.006.

Soellick, T. R., & Uhrig, J. F. (2001). Development of an optimized interaction-mating protocol for large-scale yeast two-hybrid analyses. *Genome Biology*, *2*(12). RESEARCH0052. doi: 10.1186/gb-2001-2-12-research0052.

Stasi, M., De Luca, M., & Bucci, C. (2015). Two-hybrid-based systems: Powerful tools for investigation of membrane traffic machineries. *Journal of Biotechnology*, *202*, 105–117. doi: 10.1016/j.jbiotec.2014.12.007.

Voytas, D. (2001). Agarose gel electrophoresis. *Current Protocols in Molecular Biology*, *51*, 2.5A.1–2.5A.9.