Chapter 27

Identification of Protein-Protein Interactions by Standard Gal4p-Based Yeast Two-Hybrid Screening

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Abstract

Yeast two-hybrid (Y2H) screening permits identification of completely new protein interaction partners for a protein of interest, in addition to confirming binary protein-protein interactions. After discussing the general advantages and drawbacks of Y2H and existing alternatives, this chapter provides a detailed protocol for traditional Gal4p-based Y2H library screens in *Saccharomyces cerevisiae* AH109. This includes bait transformation, bait auto-activation testing, prey library transformation, Y2H evaluation, and subsequent identification of the prey plasmids. Moreover, a one-on-one mating protocol to confirm interactions between suspected partners is given. Finally, a quantitative α -galactosidase assay protocol to compare interaction strengths is provided.

Key words Protein-protein interactions, Gal4p, Yeast two-hybrid, α -galactosidase assay

1 Introduction

The development of the yeast two-hybrid (Y2H) system by Stanley Fields in 1989 [1] represented a major milestone in the study of protein-protein interactions, which play a crucial role in almost all biological processes. Although originally used to detect binary protein-protein interactions between known interaction partners, it is now clear that Y2H facilitates the identification of completely new protein interaction partners for a protein of interest.

1.1 Gal4p-Based Yeast Two-Hybrid

The standard Y2H system exploits the modular nature of eukaryotic transcription factors like Gal4p of *Saccharomyces cerevisiae*. This transcriptional activator has two functional domains: an amino terminal DNA binding domain and a carboxy terminal activation domain. While the former binds to the upstream activating sequence (UAS) of the galactose metabolism genes in *S. cerevisiae*, the latter activates their transcription by recruiting the RNA polymerase to the promoter region. Moreover, Keegan et al. [2] demonstrated that Gal4p remains functional even if the two

Fig. 1 The Y2H system. (a) Transcriptional activator Gal4p of *S. cerevisiae* is composed of two functional domains: the DNA binding domain (DBD) that binds to the upstream activating sequence (UAS) and the activation domain (AD) that recruits the RNA polymerase and activates transcription of a reporter gene. Both components are required to produce a selectable phenotype. Y2H uses the modularity of Gal4p. (b) The bait protein X is fused to the Gal4p DBD, which cannot activate transcription on its own. (c) The prey protein Y is fused to the Gal4p AD. Since this fusion protein is not recruited to the promoter, it is also unable to activate transcription on its own. (d) Only when X and Y interact, the DBD and AD are both present at the promoter site, restoring Gal4p function resulting in a detectable phenotype

domains are not covalently attached as long as they are both present at the promoter site. Fields [1] picked up this idea to fuse interacting proteins to the two Gal4p domains, which physically brings the two domains together and restores Gal4p function.

In Gal4p-based Y2H, the protein fused to the C-terminus of the Gal4p DNA binding domain is known as the bait, while the other interaction partner, fused to the C-terminus of the Gal4p activation domain, is called the prey. These constructs are both transformed into the same yeast cell. Finally, a third component of the Y2H system (Fig. 1) is the reporter construct in this bait- and prey-expressing yeast cell. This reporter gene is located downstream from a promoter with a UAS recognized by the Gal4p DNA binding domain. If bait and prey interact, the Gal4p activation domain is tethered to the promoter region and transcription of the reporter gene is activated. This results in a specific, selectable phenotype.

While Fields originally designed the system to investigate known interactions, Chien et al. [3] applied the technique 2 years later to unravel completely new interactions for a particular protein of interest. The known protein, fused to the Gal4p DNA binding domain, was used as a bait to screen for interaction partners in a whole library of potential interaction partners, fused to the activation domain. After Y2H interaction analysis, the prey plasmid was harvested from those yeast colonies able to activate all reporter genes. Finally, by sequencing the prey plasmid inserts, the interaction partner of the protein of interest was identified.

1.2 Drawbacks of the Yeast Two-Hybrid System and Possible Solutions

As for any other protein-protein interaction analysis technique, one should realize it is almost impossible to detect all interacting proteins using Y2H. In contrast, Y2H can also identify interactions that actually do not occur *in vivo*. These are called false negative and false positive results, respectively. These often arise due to the use of artificial fusion proteins in combination with an artificial reporter construct, the removal of the proteins from their natural biological context, their translocation to the yeast nucleus, or the production of the hybrid protein at a different level than its natural concentration in the cell [4].

One potential problem is the bait protein, which could independently lead to reporter gene activation (e.g., if the protein has transcription activation activity or interacts nonspecifically with the Gal4p activation domain). This is called auto-activation or self-activation and can lead to false positive results. While this activity can be related to the biological function of the protein, it can also be a consequence of the fusion with the DNA binding domain or the removal of the protein from its natural context [4]. Because of this phenomenon, auto-activation of the bait protein should always be checked before performing the YH2 screen. If this auto-activation test is positive, the bait as such is not suited for the screen. To resolve this problem, the transcription activation domain could be removed and the screen performed with the other domain, or another Y2H system could be used.

While only the *lacZ* reporter gene, encoding β -galactosidase, was originally used in a classical blue/white screening assay [1], current Y2H reporter yeast strains have several secondary auxotrophic reporter genes, which permit selection for interaction by monitoring yeast cell growth on selective media. For instance, Y2H strain *S. cerevisiae* AH109 [5] has the *HIS3* and *ADE2* reporter genes, encoding imidazole glycerol phosphate dehydratase and phosphoribosylaminoimidazole carboxylase, respectively. These enzymes are crucial in the biosynthesis of histidine and adenine. By plating on medium lacking histidine and/or adenine, interacting baits and preys are selected. In addition, *S. cerevisiae* AH109 contains the *MEL1* reporter gene, encoding α -galactosidase, a secreted enzyme enabling direct blue/white screening on X- α -gal indicator plates. The use of more than one reporter gene reduces the number of false positive results [5].

Moreover, when using *HIS3* as a reporter gene, as is the case with strain *S. cerevisiae* AH109, 3-amino-1,2,4-triazole (3-AT) should be added to the medium. *HIS3* is the most leaky construct of the three reporter genes in AH109, so there is always a low background transcription activation of this gene. 3-AT is a competitive inhibitor of the *HIS3* product and will reduce false positive results due to the background activation [6]. At the same time, the 3-AT concentration should not be too high to avoid losing identification of weaker interactions. The optimal concentration that

should be used in the Y2H screen is also determined in the auto-activation test.

Although Y2H tests for interactions *in vivo*, the use of yeast as a host can be a potential disadvantage. It is possible that the fusion proteins will not be stable in the yeast cell or will require specific posttranslational modifications. Moreover, some proteins might be toxic to yeast. Bacterial two-hybrid [7] and mammalian two-hybrid [8] assays are possible alternatives to overcome these problems.

Targeting of non-nuclear proteins to the yeast nucleus can also be inefficient or could cause nonspecific interactions, which is likely for proteins with hydrophobic domains. For instance, membrane proteins forced into the nucleus tend to bind nonspecifically to other proteins [4]. To address this issue, the membrane yeast two-hybrid system, based on the split-ubiquitin assay [9], was developed.

Furthermore, interactions depending on more than two partners are not detected using Y2H. The yeast three-hybrid assay [10] provides a possible solution.

A last but common class of false positive results occurs because of spontaneous mutations in the yeast strain, bait or prey constructs causing reporter gene activation [11]. Hence, it is important to confirm the potential interacting proteins in an independent Y2H experiment using fresh yeast cells.

In addition to false positive results, the use of artificial fusion proteins can also lead to false negative results (e.g., if the expression levels are too low or when the fusion alters the actual conformation of the bait or prey protein). This could result in a reduced activity or the exposure of artificial surfaces that nonspecifically interact with other proteins. Moreover, the domain fused to the protein could occlude the natural interaction domain or cause steric hindrance [4]. To reduce the number of false negative results and to maximize the number of identified interactions, it is advisable to perform screens in more than just one Y2H vector system. Although the original system uses the modularity of Gal4p, other DNA binding domains like LexA [12], the λ repressor protein cI [13], or the human estrogen receptor protein [14] could be used. Similarly, different transcription activation domains like the herpes simplex virus protein VP16, or its alternative B42, have been applied [12].

Application of different Gal4p-based vector systems is also a possibility. As the expression level of the bait and prey fusion constructs influences the outcome of the Y2H analysis, vectors with varying promoters and copy numbers due to a different origin of replication (*ori*) could be exploited [15, 16]. For Y2H, our lab uses a combination of the pGBKT7g-pGADT7g (Gateway variants of the Clontech Matchmaker 3 vectors) and the pDEST32-pDEST22 (Life Technologies) plasmids that lead to different levels of expression (*see* Table 1). Hence, their results are complementary. Although one can easily use classical restriction enzyme cloning to construct bait plasmids and prey libraries, it is preferable to use the

Table 1
Summary of applied yeast two-hybrid vectors

Vector	Characteristics		Selection		Reference
	Promoter	Ori	<i>E. coli</i>	Yeast	
pGBKT7g (bait)	Truncated p _{ADH1}	2 μ	Kanamycin	<i>TRP1</i>	[17]
pGADT7g (prey)	Full-length p _{ADH1}	2 μ	Ampicillin	<i>LEU2</i>	[17]
pDEST32 (bait)	Full-length p _{ADH1}	CEN	Gentamicin	<i>LEU2</i>	Life Technologies
pDEST22 (prey)	Full-length p _{ADH1}	CEN	Ampicillin	<i>TRP1</i>	Life Technologies

Gateway system from Life Technologies to facilitate subcloning into different Y2H systems. By first constructing a prey library in an entry vector (e.g., pENTR1A, Life Technologies), one can easily shuttle the library to different prey vectors and use these different libraries in subsequent screens.

1.3 Advantages of the Yeast Two-Hybrid Approach

Despite its drawbacks, the Y2H system has some clear advantages over other methods. Firstly, it is clear that an *in vivo* assay more closely resembles cellular conditions. Furthermore, Y2H is able to detect weak and transient interactions since a continuous activation of the reporter genes is not necessary to see a signal. The fact that Y2H only detects binary interactions can also be an advantage. In contrast to methods such as affinity purification, which detect whole complexes, Y2H directly identifies the two interaction partners. Moreover, there is no need for recombinant proteins, which is a huge advantage. Finally, although it is labor intensive, Y2H has a relatively low investment cost. Only minimal laboratory equipment and low-cost microbial growth media are needed [4].

This chapter provides a detailed protocol for Gal4p-based Y2H screens using one protein as bait against a prey library. Assuming a bait and prey library has been constructed, this protocol will first describe small-scale yeast transformation of the bait plasmid. In addition, this chapter includes a bait auto-activation test, large-scale yeast transformation of the prey library, evaluation of the Y2H results, yeast plasmid isolation, and identification of the inserts by DNA sequencing. Since Y2H is prone to false positive results, it is important to confirm the potential interactions. A one-on-one protocol using mating between the two different *S. cerevisiae* strains AH109 (mating type a) [5] and Y187 (mating type α) [18] will also be discussed. This protocol can also be applied to directly confirm interaction between two suspected interaction partners. Finally, a quantitative α -galactosidase assay to compare different interaction strengths will be provided.

2 Materials

2.1 Bait Plasmid Transformation

1. *Yeast strain*: *S. cerevisiae* AH109 with genotype *MATa*, *trp1-901*, *leu2-3,112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ* (Clontech) [5].
2. *Bait plasmids*: Gene of interest cloned in bait vector (e.g., pGBKT7g [17] or pDEST32 (Life Technologies)).
3. *YPDA liquid medium*: 10 g yeast extract, 20 g peptone. Bring up to 880 ml with deionized water and autoclave. After sterilization, let the medium cool to room temperature. Add 100 ml of 20 % glucose stock solution and 20 ml of 2 mg/ml adenine stock solution for a final volume of 1 L. Store at room temperature.
4. *YPDA solid medium*: 10 g yeast extract, 20 g peptone, 20 g agar. Bring up to 880 ml with deionized water and autoclave. After sterilization, let the medium cool to 60 °C. Add 100 ml of 20 % glucose stock solution and 20 ml of 2 mg/ml adenine stock solution for a final volume of 1 L. Pour plates in a sterile hood and let the medium solidify. Store plates at 4 °C for 2–3 weeks.
5. *2 mg/ml Adenine stock solution*: 200 mg adenine hemisulfate salt. Bring up to 100 ml with deionized water and sterilize through a 0.22 μm filter.
6. *20 % Glucose stock solution*: Put 800 ml of ultrapure water in a beaker and stir. Weigh 200 g α-D (+)-glucose (anhydrous) and add it to the ultrapure water in small amounts to ensure that it is completely dissolved. Autoclave.
7. *Sterile water*: autoclave ultrapure water.
8. *1 M LiOAc*: 5.1 g lithium acetate dihydrate. Bring up to 50 ml with ultrapure water and autoclave. Store at room temperature.
9. *100 mM LiOAc*: 0.51 g lithium acetate dihydrate. Bring up to 50 ml with ultrapure water and autoclave.
10. *50 % (w/v) PEG3350*: Add 50 g polyethylene glycol (average molecular weight 3,350) to 50 ml of ultrapure water. Stir until completely dissolved. Filter sterilize and store at room temperature, securely capped to prevent evaporation. Water loss will increase the PEG concentration and severely reduce the yield of transformants. To avoid this, make fresh PEG3350 solution every few months.
11. *ssDNA*: 10 mg/ml single-stranded fish sperm DNA, MB grade. Store at –20 °C in aliquots.
12. *Selective liquid yeast medium*: 6.9 g yeast nitrogen base without amino acids. Bring up to 700 ml with ultrapure water and

autoclave. Let the medium cool to room temperature and add 100 ml of 10× amino acid stock solution, 100 ml of 20 % glucose stock solution, and 100 ml of 10× amino acid dropout solution for a final volume of 1 L.

13. *Selective liquid yeast medium* (“synthetic defined” or “SD medium”): 6.9 g yeast nitrogen base without amino acids, 20 g bacto agar. Bring up to 700 ml with ultrapure water and autoclave. Let the medium cool to 60 °C and add 100 ml of 10× amino acid stock solution, 100 ml of 20 % glucose stock solution, and 100 ml of 10× amino acid dropout solution for a final volume of 1 L. If needed, add 3-AT and/or X-α-gal and pour the plates in a sterile culture hood. Store at 4 °C for up to 2–3 weeks.
14. *10× amino acid stock solution*: 500 mg L-arginine, 800 mg L-aspartic acid, 500 mg L-isoleucine, 500 mg L-lysine, 200 mg L-methionine, 500 mg L-phenylalanine, 1,000 mg L-threonine, 500 mg L-tyrosine, 1,400 mg L-valine, and 200 mg uracil. Bring up to 1 L with ultrapure water and sterilize with a 0.22 μm filter. Store at 4 °C for up to 1 month.
15. *10× amino acid dropout (DO) stock solutions*: Depending on the selective medium, add the remaining components not provided by the 10× amino acid stock solution.
 - SD-Trp medium (medium without tryptophan): add *10× DO-Trp*: 40 mg adenine hemisulfate salt, 20 mg L-histidine, 100 mg L-leucine dissolved in 100 ml of ultrapure water (filter sterilize).
 - SD-Leu medium (medium without leucine): add *10× DO-Leu*: 40 mg adenine hemisulfate salt, 20 mg L-histidine, 50 mg L-tryptophan in 100 ml of ultrapure water (filter sterilize).
 - SD-Trp-Leu medium (medium lacking tryptophan and leucine): add *10× DO-Trp-Leu*: 40 mg adenine hemisulfate salt, 20 mg L-histidine in 100 ml of ultrapure water (filter sterilize).
 - SD-Trp-Leu-His medium (medium lacking tryptophan, leucine, and histidine): add *10× DO-Trp-Leu-His*: 40 mg adenine hemisulfate salt in 100 ml of ultrapure water (filter sterilize).
 - SD-Trp-Leu-His-Ade medium: add *10× DO-Trp-Leu-His-Ade*, which is the same as sterile ultrapure water.
16. *1 M 3-AT stock solution*: 0.84 g 3-amino-1,2,4-triazole in 10 ml of ultrapure water. Filter sterilize. Store at 4 °C.
17. *X-α-gal stock solution*: 200 mg 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside dissolved in 10 ml of *N,N*-dimethylformamide. Store at –20 °C in the dark (e.g., a conical tube wrapped

in aluminum foil). Add 2 ml of stock solution to 1 L of SD medium. As X- α -gal is a heat-sensitive compound, let the medium first cool to 60 °C before adding.

2.2 Bait Auto-activation Test

1. *Yeast strain*: *S. cerevisiae* AH109 containing the bait plasmid.
2. *Prey plasmids*: empty prey vectors or prey vectors with an independent protein inserted (e.g., if the bait is constructed in pGBKT7g, use the empty pGADT7g vector as a control; if the bait is constructed in pDEST32, use the empty pDEST22 as a control).
3. *Solid media needed* (25 ml medium/90 mm petri dish):
 - 2 SD-Trp-Leu plates
 - 1 SD-Trp-Leu-His plate
 - 1 SD-Trp-Leu-His +1 mM 3-AT plate (25 μ l 1 M 3-AT/25 ml medium)
 - 1 SD-Trp-Leu-His +3 mM 3-AT plate (75 μ l 1 M 3-AT/25 ml medium)
 - 1 SD-Trp-Leu-His +5 mM 3-AT plate (125 μ l 1 M 3-AT/25 ml medium)
 - 1 SD-Trp-Leu-His +10 mM 3-AT plate (250 μ l 1 M 3-AT/25 ml medium)
 - 1 SD-Trp-Leu-His-Ade plate

2.3 Prey Library Transformation

1. *Yeast strain*: *S. cerevisiae* AH109 containing the bait plasmid.
2. *Prey library*: library cloned in prey vector (e.g., pGADT7g [17] or pDEST22 (Life Technologies)).
3. *2 $\times$ YPDA liquid medium*: 20 g yeast extract, 40 g peptone. Bring up to 760 ml with deionized water and autoclave. After sterilization, let the medium cool down. Add 200 ml of 20 % glucose stock solution and 40 ml of 2 mg/ml adenine stock solution for a final volume of 1 L.
4. *Solid media needed* (25 ml medium/90 mm petri dish; 80 ml medium/150 mm petri dish):
 - 12 SD-Trp-Leu 90 mm plates
 - 30 SD-Trp-Leu-His 150 mm plates + x mM 3-AT (concentration determined from auto-activation test)

2.4 Evaluation of Library Transformation

1. *Solid media needed* (40 ml medium/Nunc OmniTray dish or similar microtiter plate):
 - 1 OmniTray SD-Trp-Leu
 - 1 OmniTray SD-Trp-Leu-His + x mM 3-AT (concentration determined from auto-activation test)
 - 1 OmniTray SD-Trp-Leu-His-Ade + 40 mg/L X- α -gal

2.5 Yeast Plasmid Isolation

1. *0.67 mM K₂HPO₄ pH 7.5*: 0.0306 g K₂HPO₄. Dissolve in 150 ml of deionized water, adjust pH to 7.5, bring up to 200 ml with deionized water and autoclave.
2. *Zymolyase solution*: 12.5 mg zymolyase 20T (250 U) in 50 µl of 0.67 mM K₂HPO₄, pH 7.5, for each yeast colony. Prepare fresh before each use.

2.6 Identification of Prey Inserts

1. *Competent Escherichia coli cells*: such as One Shot TOP10 chemically competent *E. coli* (Life Technologies).
2. *LB medium*: 10 g tryptone, 5 g yeast extract, 10 g NaCl (15 g agar). Bring up to 1 L with deionized water and autoclave. Let cool before adding antibiotics.
3. *Resuspension buffer*: 6.06 g tris base, 3.72 g ethylenediamine-tetraacetic acid disodium salt dihydrate (Na₂EDTA·2H₂O). Add approximately 800 ml of deionized water and adjust pH to 8. Bring up to 1 L with deionized water and autoclave. After autoclaving, add DNase- and proteinase-free RNase A to a final concentration of 100 µg/ml and store at 4 °C.
4. *Lysis buffer*: 8 g NaOH, 10 g sodium dodecyl sulfate (SDS). Bring up to 1 L with demineralized water. Filter sterilize and store in a plastic bottle at room temperature. When stored too cold, a white precipitate will appear. The precipitated SDS can be dissolved again by microwaving.
5. *Neutralization buffer*: 294.5 g potassium acetate. Dissolve in 500 ml of deionized water and adjust pH to 5.5 with glacial acetic acid (more than 100 ml will be needed). Bring up to 1 L and autoclave. Store at room temperature.
6. *70 % ice cold ethanol*: 70 ml ethanol, 30 ml deionized water. Store at −20 °C.
7. *Sequencing primers*: specific for the prey plasmids. E.g:
 pDEST22_F: 5'-TATAACGCGTTTGGAACTCACT-3'
 pDEST22_R: 5'-AGCCGACAACCTTGATTGGAGAC-3'
 pGADT7g_F: 5'-CTATTCGATGATGAAGATACCCAC-
 CAAACCC-3'
 pGADT7g_R: 5'-GTGAACTTGCGGGGTTTTTCAGTATC-
 TACGATT-3'

2.7 One-on-One Y2H Confirmation Test

1. *Yeast strains*:
S. cerevisiae AH109 containing the bait plasmid (generated in Subheading 3.1).
S. cerevisiae AH109 containing an empty bait vector or a bait vector with an independent gene inserted (this strain can be generated with the same protocol as in Subheading 3.1).

S. cerevisiae Y187 with genotype *MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4 Δ* , *met-*, *gal80 Δ* , *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ* (Clontech) [18].

2. *Solid media needed* (40 ml medium/Nunc OmniTray dish)
 - 1 OmniTray YPDA
 - 1 OmniTray SD-Trp-Leu
 - 1 OmniTray SD-Trp-Leu-His + *x* mM 3-AT
 - 1 OmniTray SD-Trp-Leu-His-Ade + 40 mg/L X- α -gal

2.8 Quantitative α -Galactosidase Assay

1. *Assay buffer*: 2 volumes of 1 $\times$ NaOAc buffer combined with 1 volume of 100 mM PNP- α -Gal solution. Mix well. Prepare fresh before each use.
2. *100 mM PNP- α -Gal solution*: 30.13 mg p-nitrophenyl- α -D-galactopyranoside in 1 ml of deionized water. Filter sterilize. Prepare fresh before each use.
3. *1X NaOAc*: 2.05 g sodium acetate in 50 ml of deionized water (0.5 M NaOAc). Adjust pH to 4.5.
4. *10X Stop solution*: 10.6 g Na₂CO₃ in 100 ml of deionized water (1 M Na₂CO₃).

3 Methods

3.1 Bait Plasmid Transformation

This protocol is adapted from the lithium acetate/single-stranded carrier DNA/polyethylene glycol method by Gietz and Woods [19], which permits high-efficiency transformation of yeast. This method generally yields 10⁵ transformants per μ g of DNA.

1. Streak *S. cerevisiae* strain AH109 on a fresh YPDA agar plate starting from a 25 % glycerol cell stock (stored at -80°C). Incubate for 2–3 nights at 30°C (*see Note 1*).
2. Inoculate two 5 ml YPDA cultures (in 50 ml flasks) with one big (3 mm) or two to four small (1–2 mm) colonies. Inoculate different numbers of colonies for the two cultures to ensure different cell densities the next day. Incubate overnight in a shaking incubator at 30°C . The next morning, measure the optical density at 600 nm (OD₆₀₀). A preculture with a value below 1 is preferred as these cells are still not stationary and will have a shorter lag phase. Use this overnight culture to inoculate a fresh yeast culture.
3. Inoculate 50 ml of YPDA (in a 500 ml flask) until an OD₆₀₀ value of approximately 0.120–0.140 is achieved (*see Note 2*). Incubate at 30°C until the OD₆₀₀ reaches ± 0.480 – 0.560 (this usually takes 4–5 h). At least two cell divisions are needed for a good transformation efficiency. This efficiency remains constant during the next three to four cell divisions.

4. From this point forward, all steps are performed at room temperature. Centrifuge the cells for 5 min at $3,500 \times g$.
5. Discard the medium and resuspend the cells in a half volume of sterile water (25 ml). Centrifuge again for 5 min at $3,500 \times g$ to wash the cells.
6. Discard the water and resuspend the cell pellet in 1 ml of 100 mM LiOAc (*see Note 3*). Transfer to a 1.5 ml microcentrifuge tube.
7. Pellet the cells ($14,000 \times g$, 15 s) and remove the LiOAc with a micropipette. Resuspend the cells in 400 μ l of 100 mM LiOAc. The final volume is now approximately 500 μ l. This is sufficient for ten transformations (*see Note 4*). Divide the cells into 50 μ l aliquots.
8. Centrifuge ($14,000 \times g$, 15 s) and remove the LiOAc. The cells are now ready for transformation. The transformation mixture is added in the following order (*see Note 5*):
 - 240 μ l PEG3350 (50 % w/v)
 - 36 μ l 1 M LiOAc
 - 10 μ l ssDNA (10 mg/ml)
 - x μ l (1 μ g) plasmid DNA
 - 74 – x μ l sterile water
 - Total 360 μ l
9. Vortex for 1 min and incubate the transformation mixture in a water bath set at 42 °C for 30 min. Invert tubes every 5–10 min (*see Note 6*).
10. Centrifuge the mixture for 15 s at $3,500 \times g$ and remove the supernatant with a micropipette. Resuspend the cells in 1 ml of sterile water.
11. After resuspension, plate the cells on selective medium (10 μ l, 100 μ l, remaining volume) (e.g., SD-Trp for pGBKT7g and SD-Leu for pDEST32) and incubate at 30 °C for 3 nights (*see Note 7*).

3.2 Bait Auto-activation Test

This protocol also uses the LiOAc/ssDNA/PEG method. The important differences are highlighted in bold.

1. Streak *S. cerevisiae* AH109 containing the bait plasmid (obtained in Subheading 3.1) on a fresh *selective agar* plate. Incubate for 2–3 nights at 30 °C (*see Note 8*).
2. Inoculate two 5 ml *SD medium* cultures (in 50 ml flasks) with one big (3 mm) or two to four small (1–2 mm) colonies. Incubate overnight in a shaking incubator at 30 °C. The next morning, determine the OD₆₀₀ and use the overnight culture to inoculate a fresh yeast culture.

3. **Steps 3–10** are exactly the same (*see* **Note 9**). In **step 8**, use 1 μg of empty prey vector or an independent prey construct to transform the *S. cerevisiae* AH109 containing the bait plasmid.
4. After resuspension in 1 ml of sterile water, 10 μl and 100 μl are plated on SD-Trp-Leu to check the transformation efficiency. The remaining volume (890 μl) is divided between SD-Trp-Leu-His +0, 1, 3, 5 and 10 mM 3-AT (100 μl on every plate) and SD-Trp-Leu-His-Ade (the remaining volume) plates. All plates are incubated at 30 °C for 5 days.
5. Evaluation: a background of very small colonies, due to leaky expression of the *HIS3* reporter, can usually be observed. Only large colonies on the SD-Trp-Leu-His plates (or maybe even on the SD-Trp-Leu-His-Ade plate) are an indication of auto-activation. Out of the different concentrations of 3-AT, choose one concentration that will be used in the Y2H screen. This concentration should not be too low to reduce the number of false positive “interactions” but also not too high, to reduce the chance of missing weaker interactions (*see* **Note 10**).

3.3 Prey Library Transformation

1. Streak *S. cerevisiae* AH109 containing the bait plasmid (obtained in Subheading 3.1) on a fresh selective agar plate. Incubate for 2–3 nights at 30 °C (*see* **Note 11**).
2. *Day 1*: Inoculate the freshly streaked strain in 5 ml of selective SD medium in a 50 ml flask (use several small colonies). Incubate overnight at 30 °C.
3. *Day 2*: Inoculate two 100 ml cultures of selective SD medium in 1 L baffled flasks with the overnight culture (use two different volumes: 1 and 2 ml) (*see* **Note 12**). The next day, one of the 100 ml cultures should have an OD_{600} below 1 (still exponentially growing, but preferably also close to 1 to have enough inoculum for a 600 ml culture). Prewarm 600 ml 2 $\times$ YPDA in a 2 L baffled flask at 30 °C and autoclave two additional empty 2 L baffled flasks and a 250 ml graduated cylinder.
4. *Day 3*: Measure the OD_{600} . Centrifuge the culture with the value the closest to, but still below, 1 (divide between two 50 mL tubes) (3,500 $\times g$, 5 min). Discard the supernatant and resuspend the cell pellets in 1 ml of 2 $\times$ YPDA. Use this concentrated cell solution to inoculate the 600 ml 2 $\times$ YPDA culture to an OD_{600} of approximately 0.120–0.140. Divide the 600 ml culture between the three 2 L baffled flasks using the sterile graduated cylinder. Incubate the three cultures at 30 °C in a shaking incubator until the OD_{600} reaches ± 0.480 –0.560. This will take 4–5 h.
5. Divide the culture in twelve 50 ml sterile tubes. Centrifuge (3,500 $\times g$, 5 min).

6. Discard the supernatant and wash the cells in a half volume (300 ml) of sterile water (50 ml/2 cell pellets) (*see Note 13*). Centrifuge again.
7. Discard the supernatant and wash the cells a second time with 300 ml of sterile water. Collect the cells by centrifugation. Resuspend all cell pellets in 4 ml of sterile water.
8. Prepare the 120× transformation mixture (*see Note 14*):
 - 28.8 ml PEG3350 (50 % w/v)
 - 4.32 ml 1 M LiOAc
 - 1.2 ml ssDNA (10 mg/ml)
 - x ml (120 µg) prey library DNA
 - 4.88 – x ml sterile water
9. Vortex the transformation mixture vigorously and add it to the competent cells which were resuspended in 4 ml of water. Vortex the entire mixture for 10 min (*see Note 15*).
10. Divide the solution into six 15 ml conical tubes and incubate for 30 min at 30 °C followed by 45 min at 42 °C in a water bath (*see Note 16*). Invert the tubes every 5–10 min.
11. Collect all cells in one 50 ml tube and centrifuge for 5 min at $3,500 \times g$. Discard the transformation solution and resuspend the cells in 12 ml of sterile water.
12. Plate three dilution series on SD-Trp-Leu to calculate the transformation efficiency. For each series, 10 µl of cells are diluted in 990 µl of sterile water (10^{-2}). 100 µl from this dilution is further diluted in 900 µl water (10^{-3}). Repeat for a 10^{-4} and 10^{-5} dilution. Mix every dilution again right before plating 100 µl on SD-Trp-Leu.
13. Divide the remaining volume (± 400 µl per plate) over 30 SD-Trp-Leu-His + x mM 3-AT 150 mm plates (use the appropriate concentration of 3-AT determined from the auto-activation test) (*see Note 17*).
14. After 3 nights at 30 °C, the dilution series can be counted. Calculate the average. The transformant yield is the average $\times 10$ (100 µl plated) $\times$ dilution factor $\times 12$ (total volume). The minimum number of transformants needed depends on the library used (*see Note 18*). Incubate the 150 mm SD-Trp-Leu-His plates at 30 °C for 5–7 nights.

3.4 Evaluation of Library Transformation

1. Check all 30 plates and select a maximum of 96 yeast colonies to analyze (*see Note 19*).
2. Pick the selected colonies with a toothpick and transfer them to a microtiter plate containing 100 µl of sterile water.
3. Shake for 15 min on a shaker to resuspend all cells.

4. Spot in parallel 2 μ l on an SD-Trp-Leu, SD-Trp-Leu-His + x mM 3-AT, and SD-Trp-Leu-His-Ade + 40 mg/L X- α -gal OmniTray plate (*see* **Note 20**).
5. Incubate at 30 °C for 5–7 nights.
6. Add 100 μ l of sterile water +30 % glycerol to the remaining volume of resuspended cells. This backup cell stock can be stored at –80 °C.
7. Evaluation: everything should grow on the SD-Trp-Leu and the SD-Trp-Leu-His + x mM 3-AT plates. Interactions should also be strong enough to activate not only the *HIS3* reporter but also the *ADE2* and *MEL1* reporter genes, so colonies of interest are those that can grow on the SD-Trp-Leu-His-Ade + X- α -gal plate and have a blue halo around them caused by the α -galactosidase activity. A maximum of 24 blue colonies should be selected for further analysis (*see* **Note 21**).

3.5 Yeast Plasmid Isolation

1. Inoculate the colonies in 5 ml of selective SD medium and incubate for 2 nights at 30 °C (*see* **Note 22**). It is only necessary to select for the prey plasmid.
2. Centrifuge for 5 min at $3,500 \times g$ and discard the supernatant. Resuspend the cell pellet in 250 μ l of 0.67 mM K_2HPO_4 , pH 7.5. Transfer to a 1.5 ml tube.
3. Add 50 μ l of zymolyase solution and incubate in a water bath at 35 °C. Invert regularly.
4. Collect cells by centrifugation for 5 min at $3,500 \times g$. Discard the supernatant.
5. The zymolyase will degrade the yeast cell wall and create spheroplasts; from now on a standard bacterial miniprep kit can be used to isolate the yeast plasmid.

3.6 Identification of Prey Inserts

Because of its low yield and impurity, the isolated yeast prey plasmid cannot be directly used as a template in DNA sequencing analysis. To identify the prey inserts, the plasmid should first be transformed back to *E. coli*. A direct PCR is not advisable, as there is still some genomic yeast DNA present that may cause nonspecific products and there can be more than one type of prey plasmid present.

1. For each blue colony, transform 5 μ l of yeast plasmid DNA to competent *E. coli* cells. Plate cells on LB medium selecting for the prey plasmid.
2. For each transformation, four colonies (derived from one yeast colony) are selected for further analysis (*see* **Note 23**). Grow the yeast overnight in a 4 ml selective LB culture and isolate the prey plasmid using a standard miniprep kit. In the case of a larger number of yeast colonies, it is better to use a microtiter plate scaled plasmid isolation method. Pick four colonies per

transformation in a microtiter plate with 100 μ l selective LB and incubate for 2 h at 37 °C without shaking.

3. First inoculate 15 μ l of the cells in a microtiter plate containing 150 μ l of selective LB. Close the lid and seal the plate with parafilm around the borders. Grow overnight at 37 °C on a microtiter plate (MTP) shaker. To the remaining culture, add 100 μ l of LB with 40 % glycerol. This backup glycerol cell stock can be stored at –20 °C.
4. The next day, collect the cells by centrifugation at $4,000 \times g$ for 10 min. Discard the supernatant (*see Note 24*).
5. Add 35 μ l of resuspension buffer, shake for approximately 5 min on an MTP shaker until everything is resuspended.
6. Add 35 μ l of lysis buffer, shake for about 2 min until the solution is clear again. Do not allow lysis to proceed for longer than 5 min.
7. Add 49 μ l of neutralization buffer, shake for another 5 min. A white precipitate of cell debris and genomic DNA will form.
8. Centrifuge for 20 min at $4,000 \times g$. Transfer the supernatant containing the plasmid DNA to a new round-bottomed microtiter plate (approximately 110 μ l).
9. Add 60 μ l of isopropanol and mix by pipetting up and down. Incubate for 15 min without shaking at room temperature.
10. Centrifuge 30 min at $4,000 \times g$ to precipitate the plasmid DNA. Remove the supernatant by quickly inverting the plate above a sink.
11. Add 70 μ l of 70 % ice cold ethanol to wash the pellet. Centrifuge 10 min at $4,000 \times g$ at 4 °C. Discard supernatant by quickly inverting the plate above the sink and tapping the plate on paper tissues (*see Note 25*). Let the DNA dry for about 15 min at room temperature.
12. Resuspend the DNA pellets in 20 μ l of ultrapure water by pipetting up and down.
13. Digest the plasmid DNA with a restriction enzyme cutting out the insert (for miniprep on a column: use 2 μ l of plasmid DNA as template; for miniprep in MTP format: use 6 μ l of plasmid DNA as template) and visualize using DNA agarose gel electrophoresis.
14. Evaluation: this digest checks whether all prey plasmids originated from one blue yeast cell contain the same insert (based on length alone). As yeast can contain more than one prey plasmid, in contrast to *E. coli* that can only harbor one, it is possible to see different insert lengths. If this is the case, both types of inserts have to be analyzed. One construct per insert length

should be chosen for DNA sequencing analysis using prey plasmid-specific primers to identify the potential interaction partners for the protein of interest.

3.7 One-on-One Y2H Confirmation Test

All the observed interactions could be false positive results and should be confirmed using an independent Y2H experiment. Choose one prey construct (the plasmid isolated from *E. coli* in Subheading 3.6) for each different potential interaction partner. These prey constructs will be transformed to the α -mating type yeast strain Y187 [18] and will be mated with an AH109 strain containing the bait construct and another strain containing an empty bait vector as a negative control. An independent protein inserted in the bait vector could also be used. If there are not too many different prey plasmids, the same protocol provided in Subheading 3.1 can easily be followed to transform the plasmids into *S. cerevisiae* Y187. After transformation, one can proceed with **step 15** from this protocol. In the case of a higher number of different preys or different Y2H screens confirmation tests combined in one experiment, the protocol given in this section, which is adapted for up to 96 yeast transformations in parallel, can be followed.

1. Streak *S. cerevisiae* Y187 on a fresh YPDA agar plate starting from a 25 % glycerol cell stock (stored at -80°C). Incubate for 2–3 nights at 30°C .
2. Inoculate two tubes of 4 ml of YPDA with several small colonies. Shake overnight at 30°C .
3. Dilute the overnight culture with an OD_{600} value below 1 in 100 ml of YPDA (in a 1 L baffled flask) until an OD_{600} of ± 0.120 – 0.140 . Incubate at 30°C until the OD_{600} reaches ± 0.480 – 0.560 .
4. Divide the culture between two conical tubes and centrifuge ($3,500 \times g$, 5 min).
5. Discard the medium and resuspend the cells in 2×25 ml sterile water. Centrifuge again.
6. Discard the supernatant and resuspend the cells in 2×1 ml of 100 mM LiOAc. Transfer to 1.5 ml microcentrifuge tubes.
7. Pellet the cells ($14,000 \times g$, 15 s) and remove the LiOAc with a micropipette. Resuspend each cell pellet in 400 μl of 100 mM LiOAc. The final volume is now around 2×500 μl . This is enough for 96 small-scale transformations. Centrifuge ($14,000 \times g$, 15 s) and remove the LiOAc.
8. Resuspend the two pellets in 1,480 μl of sterile water and transfer to a 15 ml conical tube. The transformation mixture is now added in the following order to the cells:
4,800 μl PEG3350 (50 % w/v)

720 μ l 1 M LiOAc

200 μ l ssDNA (10 mg/ml)

9. Vortex for 10 min. Prepare a PCR plate by adding to each well 100 ng of plasmid DNA (a different plasmid per well).
10. Divide the transformation mixture in the PCR plate by adding 25 μ l to each well. Pipette up and down a few times to mix. Place a lid on the plate during incubation (*see Note 26*).
11. Incubate for 10 min at 30 °C followed by 30 min at 42 °C to heat shock the cells. This step can be done on a PCR block.
12. Centrifuge the plate at $3,500 \times g$ for 1 min and discard the supernatant by pipetting. Resuspend the cells in 100 μ l of sterile water.
13. Plate the entire cell mixture on selective SD medium and incubate at 30 °C for 3 nights (*see Note 27*).
14. On the same day as **step 13**, freshly streak the different AH109 strains on selective agar plates. Incubate for 3 nights at 30 °C.
15. Fill a microtiter plate with 50 μ l of sterile water and resuspend the different *S. cerevisiae* Y187 and AH109 strains in different wells (pick several colonies).
16. Mating: spot 2 μ l a-yeast on a YPDA agar plate. Let dry. Spot 2 μ l α -yeast on the same position as the a-yeast. Do this for all different combinations (e.g., each prey in *S. cerevisiae* Y187 is mated with *S. cerevisiae* AH109 containing the bait construct and *S. cerevisiae* AH109 containing the empty bait vector). There will be two positions for each prey which has to be tested. Incubate overnight at 30 °C.
17. The next day, mating has occurred. To select for diploid cells, pick some cell material from every position in 50 μ l of sterile water and spot the different wells (2 μ l) on SD-Trp-Leu. Incubate for two nights at 30 °C.
18. Resuspend the diploid cells again in 50 μ l of sterile water and spot 2 μ l on SD-Trp-Leu-His + x mM 3-AT. Incubate for 5–7 nights at 30 °C.
19. Pick the different cells in 50 μ l of sterile water and spot 2 μ l on SD-Trp-Leu-His-Ade +40 mg/L X- α -gal. Incubate for 5–7 nights at 30 °C.
20. Evaluation: those prey plasmids that only generate colonies with a halo for the bait-prey combination but not when combined with the empty bait vector are confirmed interactions.

3.8 Quantitative α -Galactosidase Assay

To compare different interaction strengths, a quantitative assay to measure the α -galactosidase activity (encoded by the *MEL1* reporter gene) should be performed. The stronger the interaction between bait and prey, the more transcription of *MEL1*, and consequently the more α -galactosidase is produced. This enzyme is

secreted in the medium, so there is no need to lyse the yeast cells in this protocol (adapted from ref. 23).

1. Inoculate 3 ml of liquid SD-Trp-Leu-His medium with a bait- and prey-expressing yeast cell (*see Note 28*). Do this in triplicate for every type of yeast colony that needs to be analyzed. Incubate overnight at 30 °C in a shaking incubator.
2. Vortex each culture for 30 s to disperse cell clumps, then transfer 1 ml to a cuvette to measure the OD₆₀₀ (should be between 0.5 and 1; if higher, dilute the cell suspension).
3. Transfer 1 ml of the vortexed cell suspension to a 1.5 ml microcentrifuge tube. Centrifuge (14,000 × g, 2 min). Immediately proceed to **step 4** to reduce loss of enzyme activity.
4. Prepare enough assay buffer for all samples and controls, and let it equilibrate to room temperature. For each assay 48 µl of buffer is needed (*see Note 29*).
5. Transfer 16 µl of the supernatant (of **step 3**) into a well of a clear 96-well flat-bottom microtiter plate. Also include one well containing sterile SD-Trp-Leu-His. This blank will be used to zero the spectrophotometer in **step 8**.
6. Add 48 µl of assay buffer to each well and close the plate. Incubate at 30 °C for 1 h.
7. Stop the reaction by adding 136 µl of 10× stop solution to each well.
8. Measure the OD at 410 nm (OD₄₁₀) relative to the blank sample using a spectrophotometer that can read microtiter plates.
9. Calculate the α-galactosidase units. One unit is defined as the amount of enzyme that hydrolyzes 1 µmol of *p*-nitrophenyl-α-D-galactoside to *p*-nitrophenol and D-galactose in 1 min at 30 °C in acetate buffer, pH 4.5 [20].

In the following formula, the OD₆₀₀ of the overnight culture is used to normalize the OD₄₁₀ to the amount of cells. Remember to take into account the dilution factor from **step 2**, if necessary.

$$\begin{aligned} &\alpha\text{-galactosidase in milliunits per ml per cell} \\ &= (\text{OD}_{410} \times V_f \times 1,000) / (\epsilon \times b \times t \times V_i \times \text{OD}_{600}) \end{aligned}$$

t = elapsed time of incubation (in min) (60 min)

V_f = final volume of the assay (200 µl)

V_i = volume of supernatant added (16 µl)

ϵ = *p*-nitrophenol molar absorptivity at 410 nm

b = the light path (in cm)

$\epsilon \times b$ = 10.5 ml/µmol for the microtiter plate format

4 Notes

1. Always use freshly streaked cells for your transformations. This will give the best transformation efficiency. The plate used to inoculate the overnight culture can be up to 2 weeks old for routine transformations, but for the prey library transformation, it is definitely advisable to use fresh colonies.
2. The fresh culture in the morning should have 5×10^6 cells/ml because at the start of transformation 2×10^7 cells/ml are needed. For strain AH109, this start density corresponds to an OD₆₀₀ of ± 0.130 . To reach this, first vortex the overnight culture to disperse all cell clumps, add the cells in smaller steps, and measure the OD₆₀₀ in between until an OD₆₀₀ of 0.130 is reached. It is critical that all cell clumps are dispersed to obtain a healthy liquid culture. If the culture does not reach the final OD₆₀₀ in the expected 4–5 h, it is best to start over with an overnight culture and do the transformation the following day, especially when the efficiency should be high (e.g., for the library transformation).
3. It is not required to prepare a separate 100 mM stock of LiOAc, a 1 M stock suffices. In this case, first resuspend the cells in sterile water and then add the required LiOAc. For instance, if the cells need to be resuspended in 400 μ l of 100 mM LiOAc, first resuspend them in 360 μ l of sterile water and then add 40 μ l of 1 M LiOAc.
4. When less than ten transformations are needed, all washing volumes can be adapted. Five milliliters of yeast culture is needed for one transformation. For every 5 ml of exponentially growing cells, the washing volumes are subsequently 2.5 ml of water, 100 μ l of 100 mM LiOAc, and 40 μ l of 100 mM LiOAc.
5. Adding the PEG3350 before the LiOAc is important. LiOAc is harmful for the cells if the concentration is too high. The PEG will first make a shield around the cells. When transforming a higher number of different plasmids, it can be useful to resuspend the cells in batches in the transformation mixture without the plasmids and then divide the cells over different tubes each containing a different plasmid. This does not affect the transformation efficiency. For instance, for ten transformations, stop in **step 7** when the amount of cells is 500 μ l. Centrifuge and remove the LiOAc. Then resuspend the cells in $740 - y$ μ l of sterile water (where y is the total amount of plasmid DNA) and transfer to a small conical tube. Add 2.4 ml of PEG3350 and vortex. Add 360 μ l of 1 M LiOAc and 100 μ l of ssDNA and vortex again for 2 min. Add $360 - x$ μ l of transformation mixture to each tube with x μ l of plasmid DNA.

6. The vortexing step is crucial for a good transformation efficiency. Cells have to be fully resuspended and surrounded by all the compounds to take up the plasmid DNA. Cells that are not vortexed sufficiently will settle faster to the bottom of the tube and the transformant yield will be lower.
7. Usually the 100 μ l plate will have a sufficient number of single colonies. Once you have become familiar with the protocol, only plate 100 μ l and keep the rest of the transformation mixture at 4 °C. Check the plate after 2 days and when more colonies are needed, the remaining cells can be plated.
8. It is possible to directly use the colonies obtained in Subheading 3.1 if you directly proceed. Then there is no need to streak them again before performing the auto-activation test.
9. Also use YPDA for this culture. Growth in SD medium selecting for the bait would take too long.
10. Most of the time, 1 or 3 mM 3-AT is sufficient.
11. For the library transformation, one should always use fresh cells to obtain a high transformant yield.
12. One overnight culture would not give enough cells to inoculate 600 ml of culture, which is needed for the library transformation. Hence, two steps are needed. Inoculation of the second overnight culture (100 ml culture) early in the afternoon (2–3 PM) is usually successful to get an OD close to 1 the next morning.
13. First add ± 2 ml of water per pellet to resuspend them by pipetting up and down. Then adjust to the final volume by pouring.
14. The transformation mixture can be made and vortexed during the washing steps. As the PEG3350 is viscous, pipette it slowly to obtain the correct volume.
15. Vortexing for 10 min can easily be done by taping the tubes on the vortex machine.
16. After dividing the mixture in the small conical tubes, make sure the level is below the water level. If this is not the case, divide over more tubes.
17. It is recommended to prepare the selective plates only 2–3 days before your planned experiment, especially for the library transformations. In that case, they can be kept at room temperature, so the plates will be dried, allowing the transformation mixtures to dry much faster after plating.
18. The number of transformants needed depends on the library. For random genomic libraries, this can be calculated using the Clarke and Carbon formula [21] that indicates that the actual number of clones required equals $\ln(1 - P)/\ln(1 - (f/G))$

where P is the probability, f is the mean fragment size of the plasmid inserts, and G is the genome size (e.g., for *E. coli* K-12 [22] with a genome size of 4,639,221 bp and a mean fragment size of 600 bp, 35,605 transformants are needed to cover the whole genome once with a probability of 0.99). Knowing that in Y2H fusion proteins the fragment orientation and reading frame has to be correct, the actual total number of yeast transformants needed is 6×35605 or 2.14×10^5 .

19. The total number of colonies growing on the SD-Trp-Leu-His plates depends on the bait, the 3-AT concentration, and the efficiency. The higher the transformation efficiency, the more colonies will be growing. In this case the library is covered multiple times, so there will be colonies that contain the same prey plasmid. Because it is too labor intensive to analyze all of the colonies in this case, it is important to make a selection. Limit the selection to 96 colonies, which is still an easy format to work with. Careful inspection of all plates and numbering and sorting the colonies in groups of different sizes is one way to remove duplicates (e.g., 1, 2, 3, >3 mm). Although size might reflect interaction strength, there is no reason to assume that weak interactions have less biological significance, so always select different sizes of colonies from all different plates. When picking the colonies to array them in a microtiter plate, check the consistency of the colony. Based on our experience, granular colonies that fall apart after picking generally contain false positive interactions. So it is preferable to only pick smooth and creamy colonies.
20. Other single well microtiter plates or a small petri dish in the case of a smaller number of colonies can be used.
21. Limit this selection to 24 colonies again by carefully inspecting and sorting the colonies in groups of different colors (e.g., white, pale blue, dark blue, dark blue with a blue halo).
22. It is easier to use glass culture tubes than flasks. Yet, when using glass tubes, the cells will settle more easily to the bottom of the tube. To grow a culture sufficiently dense for plasmid yield, vortex after inoculation, shake the tubes vigorously (250 rpm), and vortex them again after 1 day of incubation.
23. Different *E. coli* colonies derived from the same yeast colony are selected because yeast can contain more than one prey plasmid. As *E. coli* can only contain one, this step will show the different inserts.
24. Supernatant removal can be performed using a vacuum pump. The microtiter plate method to recover plasmids requires some practice but is an inexpensive and efficient solution when screening large numbers of colonies.

25. To remove the supernatant, invert the plate very quickly to avoid well-to-well contamination.
26. Use a microtiter plate lid on top of the PCR plate. When using strips to close the plate, it is too difficult to remove the strips after incubation which will cause well-to-well contamination.
27. To reduce the amount of medium and to make the plating more convenient, QTray bioassay trays from Genetix can be used for plating. These contain a 48-position grid, so only two plates are needed to plate 96 transformation mixtures.
28. Inoculate the cells starting from an SD-Trp-Leu or SD-Trp-Leu-His plate. Use fresh cells (maximum 2 weeks old).
29. When the number of samples is smaller or a spectrophotometer to read microtiter plates is not available, a 1 ml assay can also be performed. Refer to [23]) for a detailed protocol.

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