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Yeast Two-Hybrid Library Screening

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1. Introduction

The two-hybrid system was originally devised by Fields and Song *(1)* as a protein interaction detection system in yeast. Subsequently, it has been employed in many laboratories as a means of screening cDNA and genomic fusion libraries for protein interaction partners *(2–8)*. The method relies on the fact that transcription factors, such as the yeast GAL4 factor, consist of separable DNA-binding and transcriptional regulatory domains; the former being required to direct the latter to appropriate promoters where transcriptional activation is effected, usually by direct or indirect interaction of the activation domain with the general transcription machinery. The essence of the two-hybrid system is the in vivo reconstitution of a functional transcriptional activator from two interacting polypeptides, one fused to a sequence-specific DNA-binding domain and the other to a potent transcriptional activation domain. Interaction is detected when the reconstituted transcription factor activates a reporter gene (*see* Fig. 1). DNA-binding domain and activation domain fusion proteins are expressed from plasmid DNAs. The DNA-binding and transcriptional activation components are usually derived from the yeast GAL4 transcription factor (*see* Figs. 1 and 2), although alternatives have been used by some workers *(6,9,10)*. Where the GAL4 DNA-binding domain is used, yeast strains in which the wild-type GAL4 gene has been deleted are employed. For the purposes of identifying new protein partners, DNA encoding the polypeptide for which partners are sought (bait polypeptide) is ligated into a yeast shuttle vector to create a DNA-binding domain–bait fusion (Fig. 2A). Library cDNA is ligated into a second shuttle vector to create an activation domain-tagged cDNA library (*see* Fig. 2B). In the original method as proposed by Chien et al. *(2)*, the bait (GAL4 DNA-binding domain) construct and activation domain fusion library were cotransformed into a yeast strain containing an integrated *LacZ* gene

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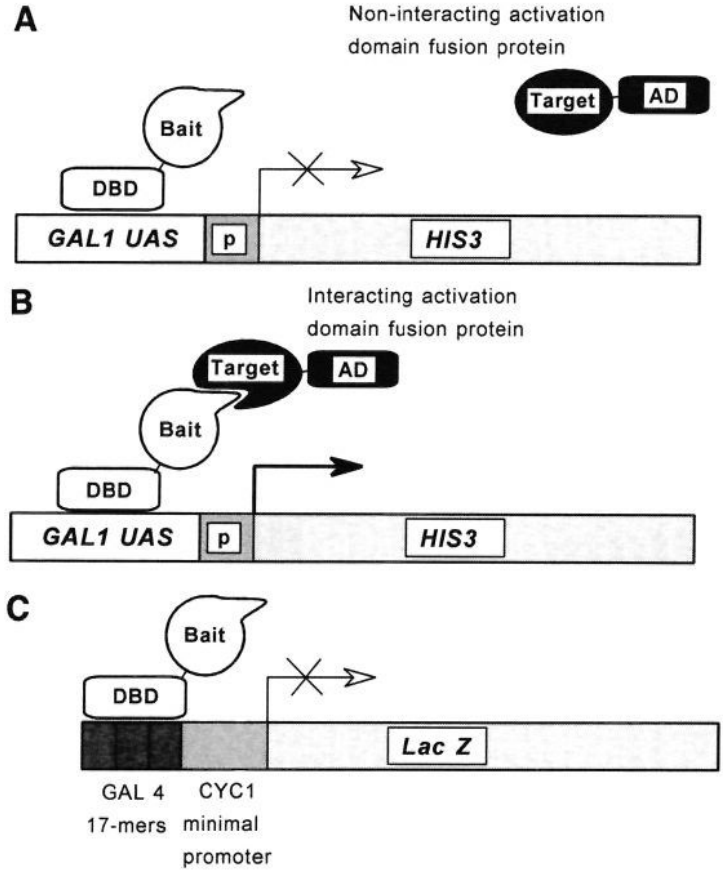

Fig. 1. The principle of the yeast two-hybrid system. The two-hybrid system is a method of detecting specific protein–protein interactions between two polypeptides in vivo. The "bait" and "target" polypeptides are fused to the DNA-binding and transcriptional activation domains of the transcription factor GAL4, respectively (other DNA-binding and activation domains have been used). In vivo interaction of "bait" and "target" polypeptides results in formation of a functional activator and activation of GAL4-responsive reporter genes. Yeast strains used for two-hybrid analysis, such as YPB2 and HF7c, contain two integrated GAL4-responsive reporter genes *HIS3* and *LacZ*. Activation of the *HIS3* reporter gene confers the ability to grow on media lacking histidine. **(A)** In the situation where bait and target proteins do not interact, the bait polypeptide is targeted to the promoter of the *HIS3* reporter gene through specific protein–DNA interactions between the GAL4 DNA-binding domain (DBD) and the GAL1 UAS, but the activation domain is not recruited to the promoter, resulting in no transcription of the *HIS3* gene. **(B)** Where there is a functional interaction between bait and target polypeptides, a functional activator is formed on the GAL1 UAS; *HIS3* expression ensues, resulting in histidine prototrophy. **(C)** The *LacZ* reporter gene in strains, such as YPB2 or HF7c, is driven by an artificial GAL4-dependent promoter. Interaction of bait and target polypeptides results in expression of β-galactosidase activity, which is easily assayed.

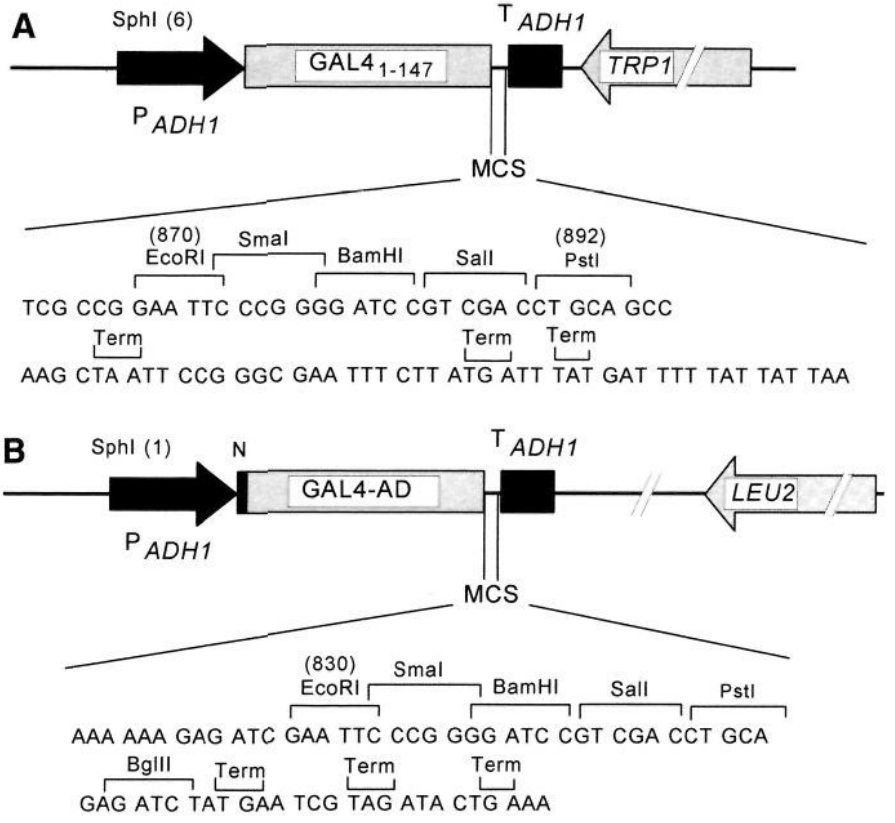

Fig. 2. Cloning sites in the DNA-binding domain fusion vector pGBT9 and activation domain vector pGAD424. **(A)** The region of pGBT9 around the segment encoding the DNA-binding domain of GAL4 (residues 1–147). P_{ADH1}, *ADH1* promoter; MCS, multicloning site; T_{ADH1}, *ADH1* transcriptional terminator. The *TRP1* auxotrophic marker gene is also indicated. The first TCG codon in the following sequence corresponds to residue 147 of GAL4. Restriction sites are indicated as are termination codons (Term). **(B)** The region of pGAD424 around the segment encoding the GAL4 activation domain (residues 768–881). The first GAG codon in the sequence corresponds to codon 881 of GAL4. The segment designated N indicates the SV40 large T nuclear localization sequence engineered into the N-terminus of GAL4 *(2)*.

driven by a GAL4-responsive promoter *(2)*. Yeast-containing plasmids encoding interacting polypeptides were scored by the presence of β-galactosidase activity (blue colonies in the presence of the chromogenic substrate X-Gal). The method was subsequently developed to facilitate the screening of large mammalian cDNA libraries *(11)*. This innovation employs a *his3* test yeast strain (that is auxotrophic for histidine) that contains, in addition to the *lacZ* reporter, an integrated *HIS3* gene that is transcribed at a significant level only

Table 1
10X Dropout Solution

Component	mg/L
L-Isoleucine	300
L-Valine	1500
Adenine hemisulfate	200
L-Arginine-HCl	200
L-Histidine-HCl	200
L-Leucine	1000
L-Lysine-HCl	300
L-Methionine	200
L-Phenylalanine	500
L-Threonine	2000
L-tryptophan	200
L-Tyrosine	300
Uracil	200

in the presence of the reconstituted activator (*see* Fig. 2). Yeasts transformed with the bait plasmid and the activator library are plated on minimal plates lacking histidine. Only *HIS3* colonies are then carried forward for further analysis. The genotype of two commonly used yeast reporter strains is given in Note 1.

2. Materials

2.1. Maintenance of Yeast Strains

1. A solution of 40% glucose (dextrose) in water Autoclave to sterilize
2. Yeast, peptone, dextrose (YPD) medium per liter: 20 g peptone, 10 g yeast extract. Adjust pH if necessary to 5.8–6.5. Add 15 g agar for plates Autoclave and add 50 mL of glucose (dextrose) from a sterile 40% stock
3. YPDA: Prepare YPD as in item 2, and after autoclaving add adenine sulfate to 40 mg/L from a filter-sterilized stock.
4. Yeast nitrogen base (Difco, Detroit, MI, cat no 0919-15-3).
5. Amino acids: L-tryptophan, L-histidine-HCl, L-arginine-HCl, L-methionine, L-tyrosine, L-leucine, L-isoleucine, L-lysine-HCl, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-valine, L-threonine, L-serine (all from Sigma, St. Louis, MO).
6. Adenine sulfate (Sigma, cat no. A2196).
7. Uracil
8. 10X dropout solution: Add the components in items 5 to 7 in the proportions given in Table 1, omitting the appropriate ingredient (*see* Section 3 1.) Dissolve in MilliQ water, and autoclave to sterilize. Store for up to 1 yr at 4°C
9. Synthetic (SD) medium and SD agar (per liter of medium): dissolve 6.7 g Difco yeast nitrogen base in 850 mL MilliQ deionized water, and add 20 g agar (omit

the agar for liquid media). Autoclave at 120°C, at 15 lbs for no more that 15 min. Add 100 mL of the appropriate 10X dropout solution (Table 1) and 50 mL 40% glucose. For agar medium, pour plates on the thick side, and leave on the bench the right way up with lids on for 2–3 d to dry. Plates may then be wrapped and stored for up to several months at 4°C. If no short autoclave cycle is available for SD agar, make up the nitrogen base and agar in 425 mL of water each, autoclave separately, combine while still hot, and then add 100 mL 10X dropout solution and 50 mL 40% glucose.

2.2. Yeast Phenotype Verification

1 Two-hybrid yeast strains (*see* Note 1).
2. SD plates lacking trp, leu, his, or ura (*see* Sections 2.1. and 3.1.).
3 YPDA and YPD plates.
4 Disinfectant-free toothpicks

2.3. Yeast Transformation and Library Screening

2.3.1. Transformation with Bait Plasmid

1. Fresh plate of (verified phenotype) yeast, such as HF7c (*see* Note 1).
2 YPD (*see* Section 2 1)
3 Autoclaved 500-mL centrifuge pots, Beckman JA-10 or equivalent
4. Sterile water.
5. 50% Polyethylene glycol (PEG) 4000: Make 200–500 mL in water and autoclave.
6. 10X TE: 100 m*M* Tris-HCl, pH 7.8, 10 m*M* EDTA. Half fill a number of glass universal bottles and autoclave.
7 Lithium acetate: 1*M* prepared in water. Adjust to pH 7.5 with dilute acetic acid and autoclave.
8. 1X TE/LiAc: Mix 1 mL of 10X TE and 1 mL of 1*M* LiAc in a 30-mL sterile universal, and add 8 mL sterile water
9. PEG/LiAc: Mix 1 mL of 10X TE, 1 mL of 1*M* LiAc, 8 mL 50% PEG in a 30-mL sterile universal
10. Dimethyl sulfoxide (DMSO).
11. 10 mg/mL Sonicated salmon sperm DNA.
12. 9-cm SD plates without tryptophan (–T plates); *see* Section 2.1

2.3.2. Yeast Transformation with Activation Domain Library

1. Amplified activation domain library (*see* Note 3).
2. 2.5 L YPD medium (*see* Section 2.1.).
3. 1.5 L –TL synthetic medium (*see* Section 2.1. and 3.1.).
4. 250 mL –TLH synthetic medium (*see* Section 2.1. and 3.1.).
5. 20 mL 1X TE/LiAc (*see* Section 2.3.1.).
6. 140 mL PEG/LiAc. Prepare fresh by combining in a sterile container: 14 mL 10X TE, 14 mL 1*M* LiAc, 112 mL 50% PEG.
7. Sterile water.
8 Several 9-cm –TL plates
9. 10 mg/mL Sonicated salmon sperm DNA.

2.3.3. Library Plating

1 Several 9-cm –TL plates.
2. At least 50 15-cm –THL plates (*see* Note 4)
3 1*M* 3-Aminotriazole (3-AT) made up in water. Prepare 10–20 mL and filter sterilize (*see* Note 4).

2.4. Further Analysis and Elimination of False Positives

2.4.1. Gridding Out His$^+$ Transformants

1. 9-cm –THL plates.
2. 7-cm Filter paper circles (Whatman no. 1), imprinted with a grid of 1-cm squares. Number in pencil, wrap in an aluminum envelope, and autoclave to sterilize.
3 14-cm Sterile filter papers
4 Sterile toothpicks.

2.4.2. β-Galactosidase Filter Assays

1. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). dissolved in *N,N*-dimethylformamide (DMF) at 20 mg/mL. Store in 1-mL aliquots in 1.5-mL microfuge tubes at –20°C.
2. Z buffer. 16.1 g/L disodium hydrogen phosphate $7H_2O$, 5.5 g/L sodium dihydrogen phosphate, $1H_2O$, 0 75 g/L potassium chloride, 0.25 g/L magnesium sulfate · $7H_2O$. Make 1 L. Autoclave to sterilize.
3 Working X-Gal solution: 10 mL Z buffer, 167 µL X-gal stock, 27 µL β-mercaptoethanol.

2.4.3. Curing Transformant of the Bait Plasmid

1. 500 mL –L (SD) medium (*see* Section 2.1.)
2 –L plates (*see* Section 2.1)
3. –LT plates (*see* Section 2.1)

3. Methods

It is advisable for workers unfamiliar with yeast manipulations to read Sherman's article, "Getting Started with Yeast," in *Methods in Enzymology (12)*. This and other contributions in the volume provide additional information beyond the scope of this chapter.

3.1. Maintenance of Yeast Strains and Transformants

To select for and maintain yeast plasmid transformants, yeast strains with characteristic auxotrophic markers are grown on synthetic media lacking the cognate nutrient. The required enzymatic activity is then provided by a plasmid-borne marker gene. Common transformation markers include *trp1*, *leu2*, *his3*, and *ura3* affecting tryptophan, leucine, histidine, and uracil biosynthesis, respectively. SD consists of a minimal medium or nitrogen base plus glucose

combined with a "dropout solution," which contains amino acid and nucleotide supplements (*see* Table 1). One or more of the dropout solution components are omitted to make a selective medium. In this chapter, SD lacking leucine is referred to as –L medium, that lacking tryptophan as –T medium, that lacking both leucine and tryptophan as –TL medium, and so on.

3.2. Yeast Phenotype Verification

Before embarking on transformation and library screening, it is worthwhile checking the phenotype of the yeast strain to be used. The author has used the strain HF7c obtained commercially from Clontech (*see* Note 1), but a number of other strains have been used by other workers (*see* Note 1). HF7c is characterized by *trp1*, *leu2*, and *his3* auxotrophic markers and contains a second *HIS3* gene driven by the GAL4-responsive *GAL1* promoter. This strain also contains an inserted *LacZ* reporter gene driven by an artificial GAL4-responsive promoter.

1. Using a sterile inoculating loop, scrape some cells from the top of a frozen glycerol stock (*see* Note 5) and streak onto a YPDA plate. Incubate at 30°C for 2–3 d until colonies about 1–2 mm across have formed.
2. Take four or five well-isolated colonies, and using sterile toothpicks, streak some of the cells from each colony onto a sector of a second YPD or YPDA plate, and in parallel onto –Ura, –Trp, –Leu, and –His synthetic medium plates and on –H plates containing 5 m*M* 3-AT (*see* Note 4).
3. Incubate at 30°C for 3–4 d. Cells will grow on YPD plate and on –U plates (if the strain being used is Ura^+), but should not grow on either –T or –L plates. Some slow growth may occur on –His plates owing to leaky expression of the *HIS3* reporter gene, but no growth should occur on the plates containing 3-AT
4. Colonies displaying the expected phenotype should be used as the stock for library screening in Section 3.3.

3.3. Yeast Transformation and Library Screening

Construction of the bait plasmid encoding the DNA-binding domain fusion is not considered here in detail. DNA-binding domain fusion vectors are discussed in Note 2 and Table 2. The following are written assuming the use of a bait and activation library plasmids carrying *trp1* and *leu2* selection markers, respectively (*see* Tables 2 and 3), and a *trp1*, *leu2* reporter strain, such as HF7c (*see* Table 4) carrying a *HIS3* reporter gene, allowing preliminary screening for positive two-hybrid interaction by selection for histidine prototrophy.

3.3.1. Transformation with Bait Plasmid (see Note 2)

This protocol is derived from the methods of Schiestl and Gietz *(15)* and Gietz et al. *(16)*.

Table 2
DNA-Binding Domain Fusion Vectors Used in the Two-Hybrid System

Plasmid	Size	DNA-binding domain	Selection	Useful cloning sites	Other characteristics	Refs.
pMA424	12 kb	GAL4	*HIS3*	*Eco*RI, *Bam*HI, *Sal*I		*(9)*
pGBT9[a]	5 4 kb	GAL4	*TRP1*	*Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I		*(11)*
pAS2[b]	8.5 kb	GAL4	*TRP1*	*Nde*I, *Nco*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I	Contains CYH^S2 gene conferring cycloheximide sensitivity on transformed cells	*(4,13)*
pBTM116	5.4 kb	LexA	*TRP1*	*Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I		*(11)*

[a]Available commercially from Clontech, licensed from the Research Foundation of the State University of New York
[b]Available commercially from Clontech, licensed from Baylor College

Table 3
Activation Domain Fusion Vectors Used in the Two-Hybrid System

Plasmid	Size, kb	Activation domain	Selection	Useful cloning sites	Other characteristics	Refs
pGAD2F	13	GAL4[a]	*LEU2*	*Bam*HI	pGAD1F, 2F, and 3F contain the BamHI site in three different frames with respect to the activation domain	*(2)*
pGAD10	6.6	GAL4[a]	*LEU2*	*Bgl*II, *Xho*I, *Bam*HI, *Eco*RI		*(11)*
pGAD424[b]	6.6	GAL4[a]	*LEU2*	*Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Bgl*II		*(9,11)*
pJG4-5		B42	*TRP1*	*Eco*RI, *Xho*I, *Hin*dIII	Fusion protein expression is inducible with galactose, reducing potential toxicity problems	*(6)*
pVP16	8 1	VP16	*LEU2*	*Bam*HI, *Not*I		*(9)*
pACT	7.65	GAL4[a]	*LEU2*	*Bgl*II, *Eco*RI, *Bam*HI	Automatic excision from the λ vector λ-ACT	*(4)*

[a]A nuclear localization signal was engineered at the N termini of the GAL4 activation domain in the GAD series *(2)* and –Act *(4)*
[b]Available commercially from Clontech, licensed from the Research Foundation of the State University of New York

Table 4
Yeast Reporter Strains Used for Two-Hybrid Library Screening[a]

Strain	Mating type	Transformation markers	Reporter genes	Refs
YPB2	*MATa*	*trp1, leu2*	$GAL1_{UAS}$-$LEU2_{TATA}$-*HIS3*, $(GAL\ 17\text{-}mers)_3$-$CYC1_{TATA}$-*LacZ*	*(11)*
CTY1	*MATα*	*trp1, leu2*	*GAL1-LacZ, GAL1-HIS3*	*(11)*
				(11)
Y185	*MATa*	*trp1, leu2*	*GAL1-LacZ, GAL1-HIS3*	*(4)*
Y190	*MATa*	*trp1, leu2*	*GAL1-LacZ, GAL1-HIS3*	*(13)*
HF7c[b]	*MATa*	*trp1, leu2*	$GAL1_{UAS\text{-}TATA}$-*HIS3*, $(GAL\ 17\text{-}mers)_3$-$CYC1_{TATA}$-*LacZ*	*(14)*
CG-1945[b,c]	*MATa*	*trp1, leu2*	$GAL1_{UAS\text{-}TATA}$-*HIS3*, $(GAL\ 17\text{-}mers)_3$-$CYC1_{TATA}$-*LacZ*	
L40	*MATa*	*trp1, leu2*	$(LexAop)_4$-*HIS3*, $(LexAop)_8$-*LacZ*	*(7,9)*
Y187d	*MATα*	*trp1, leu2, his3*	*GAL1-LacZ*	*(13)*

[a]Only strains with a two-hybrid activated reporter gene conferring a means of selection for positive interaction are listed.

[b]Available commercially from Clontech, licensed from the Research Foundation of the State University of New York

[c]Used in conjunction with DNA-binding domain vector pAS2 encoding the CYC^S2 gene, loss of the bait construct can be selected for by plating on plates containing cycloheximide.

[d]Available commercialy from Clontech, licensed from Baylor University, used in yeast mating, *see* Note 12

1. Use a single colony of phenotype-verified reporter strain, such as HF7c (*see* Table 4), to inoculate 10 mL of YPD. Shake overnight at 30°C.
2. Measure the OD_{600} of the culture, which should be about 1.5–2, and dilute the overnight culture into 50 mL YPD at 30°C to give a final OD_{600} of 0.2.
3. Incubate for a further 3 h at 30°C.
4. Pellet the cells by centrifuging at 1000*g* for 3 min using a bench top centrifuge.
5. Resuspend the cells in 25 mL of sterile water, and pellet as above.
6. Gently resuspend the cells in 0.6 mL of TE/LiAc.
7. For each transformation, add to a sterile 1.5-mL microfuge tube: 0.5 µg plasmid DNA, and 100 µg sonicated salmon sperm DNA. Mix and add 100 µL of yeast cell suspension from step 6
8. Mix gently, but thoroughly, and add 600 µL of PEG/LiAc solution. Vortex to mix, and place the tubes in a 30°C incubator for 30 min. Mix gently several times during this incubation.
9. Add 70 µL of DMSO to each tube, mix gently, and place the tubes in a 42°C water bath for 15 min.
10. Briefly chill on ice, and pellet cells by spinning for 5 s in a microfuge.
11. Aspirate the supernatant, resuspend the cells in 500 µL of sterile TE, and spread the cells onto appropriate selective plates (Trp minus, –T for most bait plasmid/reporter strain combinations). Spread 100–200 µL/9-cm plate or 200–400 µL/15-cm plate.
12. Incubate at 30°C for 2–4 d for colonies to appear.
13. Keep stocks of the transformed strain on selective medium plates. Use one of the Trp^+ colonies to prepare competent cells for large-scale transformation as described in Section 3.3.2. It is also advisable to make a glycerol stock of the transformed strain (*see* Note 4).

Before going any further, it is advisable to carry out some preliminary tests with the bait construct as described in Note 6.

3.3.2. Yeast Transformation with Activation Domain Library

This protocol is derived from the methods of Schiestl and Gietz *(15)* and Gietz et al. *(16)*.

1. Inoculate one of the Trp^+ colonies from Section 3.3.1. into 5 mL selective –T medium. Incubate at 30°C overnight with shaking.
2. Inoculate the whole 5-mL culture into 300 mL of prewarmed –T medium in a 1-L flask, and shake for 24 h at 30°C (*see* Note 7).
3. Measure the OD_{600}, which should be approx 1.2–1.5, and add sufficient culture to each of two 2.5-L flasks each containing 500 mL of YPD prewarmed to 30°C to attain an OD_{600} of 0.3.
4. Shake at 30°C for 4 h.
5. Pellet the cells by centrifugation at 2000*g* for 5 min using a Beckman JA-10 or similar rotor.
6. Decant the supernatant and resuspend the cell pellet in 500 mL water.
7. Pellet the cells as in step 5, decant the supernatant, resuspend the washed cells in 20 mL TE/LiAc solution, and transfer to a sterile 250-mL flask.

8 Add, premixed, 1 mL denatured salmon sperm DNA (10 mg/mL) and 500 µg of activation library plasmid DNA, and mix (*see* Note 3).
9. Add 140 mL of PEG/LiAc solution and mix again
10 Incubate at 30°C for 30 min with shaking.
11. Transfer to a sterile 2-L flask, and add 17.6 mL of DMSO Swirl to mix while adding the DMSO.
12 Stand the flask in a 42°C water bath for 6 min to heat-shock the cells, swirling occasionally.
13 Transfer the flask to a water bath at 20°C to cool
14. Pellet the cells at 2000*g*. Decant the supernatant and gently resuspend the cells in 200 mL YPD
15. Pellet the cells as in step 14, and resuspend in 1 L of the same medium prewarmed to 30°C, and shake at that temperature for 1 h
16. Remove 1 mL of the cell suspension from step 15, wash the cells once in –TL medium, and resuspend in 1 mL of the same medium Plate 1, 10, and 100 µL of the cell suspension on –TL plates to determine the primary transformation efficiency (*see* Note 8).
17. Pellet the remainder of the cells as in step 5, and wash with 500 mL –TL medium
18. Resuspend the cell pellet in 1 L warm –TL medium, and incubate with gentle shaking for 4 h.
19 Pellet the cells as in step 5, and wash twice in 100 mL –TLH medium.
20. Resuspend the final pellet in 10 mL of the same medium.

3.3.3. Library Plating

1. To estimate the final transformation and plating efficiency, dilute 10 µL of the cell suspension from Section 3.3 2. into 1 mL of TE, and spread 1, 10, and 100 µL onto single 9- or 15-cm –TL plates (*see* Note 8). This represents one millionth, one one hundred thousandth, and one ten thousands of the transformation, respectively.
2 Spread 100 µL of the cell suspension from step 20 in Section 3 3 2 on each of a series of –THL plates (or –THL plates containing 3-AT; *see* Note 4). This requires at least 50 plates to screen enough colonies to cover all of the primary transformants (*see* Note 9)!
3. Leave the plates right side up on the bench with lids on for 1–2 h for any surplus liquid to dry Transfer plates, lid-side-down to a 30°C incubator
4 His^+ colonies should appear after 2–4 d, although up to 10 d may be required for weak positives to appear.

3.4. Further Analysis and Elimination of False Positives

False positives are a big problem in two-hybrid screening. Assuming the bait construct has already been tested to check that it lacks activation potential alone (*see* Note 6), false positives may arise through fortuitous binding of the activation domain fusion to the test promoter, nonspecific binding to the bait, or through one of a number of less well-understood routes. The first step after His^+ colonies appear is to test them for β-galactosidase activity (*see* Section

3.4.3.). The *LacZ* reporter in strain HF7c, for example, is driven by a different GAL4-responsive promoter than the *GAL1-HIS3* reporter. His$^+$, β-galactosidase$^+$ colonies are then carried forward for further analysis. Since it is possible for more than one activation domain fusion plasmid to be present in each β-galactosidase-positive colony, such colonies should be streaked onto –TL plates to allow segregation and then retested for β-galactosidase activity. The next stage is to check that reporter gene activity requires both activation domain and DNA-binding domain constructs. This is achieved by curing the tentative positives of the DNA-binding domain plasmid and retesting the resulting Trp$^-$ cells for β-galactosidase activity and/or His prototrophy. Only transformants that are Trp$^-$, Leu$^+$, and β-galactosidase$^-$ are candidates for genuine positives. A rapid method for curing of the bait plasmid has been devised by Elledge and colleagues *(13)* using the bait vector pAS2 (*see* Table 2), which carries a gene conferring cycloheximide sensitivity on resistant reporter strains, such as Y190 *(13)* or CG-1945. Plating on –L plates containing cycloheximide then selects for bait plasmid loss (*see* Note 12).

Further testing then involves retransformation of the Trp$^-$, Leu$^+$, and β-galactosidase$^-$ activation domain fusion-containing strain with the original bait plasmid along with a series of controls, including the original bait vector and one or more different fusion constructs, which would not be expected to interact with the same target. An ideal control would be a mutant bait domain, known to be inactive in some way. The retransformed strains would then be tested again for β-galactosidase activity or His prototrophy. As a faster alternative to transformation, these plasmids can be introduced using yeast mating methods (*see* Note 12). Those positives that are β-galactosidase-positive when retransformed with the bait plasmid, but not with the controls, are candidates for real positives! The final step is to isolate the activation domain plasmid DNA and to confirm the protein–protein interaction by independent means.

3.4.1. Transferring Colonies to Filter Papers for β-Galactosidase Assays

For β-galactosidase filter assays, colonies are transferred onto filter paper circles, leaving most of each colony in place for later analysis. If there are a large number of positives, it is easier to transfer cells from each colony directly onto sterile filter papers as described in Section 3.4.1.1. Otherwise, it is probably best to grid the colonies out as described in Section 3.4.1.2.

3.4.1.1. Preparing Replica Lifts of Positive Colonies

1. Lay a sterile 14-cm Whatman filter paper directly on top of each 15-cm plate from the two-hybrid screen, and make sure there are no air bubbles Make several orientation marks through the filter paper and agar using a syringe needle dipped in India ink

2. Using blunt-ended forceps, carefully remove each filter and place directly onto a new –THL plate, colony side up
3 Incubate the original plates at 30°C for 24 h to allow regeneration of the colonies, and then store the plates at 4°C until required
4. Incubate the new plates at 30°C until colonies are clearly visible on the filter papers
5. Proceed with β-galactosidase filter assays as described in Section 3.4 2.

3.4 1.2. Gridding Positive Colonies

1 Lay a series of 7-cm gridded filter papers (*see* Section 2.4.1) directly onto a set of –THL plates
2 Using sterile toothpicks, grid His^+ colonies from Section 3.3 onto the plates from step 1, and onto a second set of –THL plates, using the same pattern for both sets The second set of plates will be the master set of tentative positives
3 Incubate at 30°C for 2–4 d until colonies or small blobs of cells have appeared on the filter papers

3.4.2. β-Galactosidase Filter Assay

1 Using flat-ended forceps, carefully lift the filters from the agar plates one at a time, and float colony-side-up in a pool of liquid nitrogen After a few seconds, submerge the filter for 10 s, and then remove from the liquid nitrogen and lay colony side up on a tissue paper to thaw (*see* Note 10).
2 Once thawed, carefully place the filter colony-side-up on a second filter paper soaked in freshly prepared Z buffer containing X-Gal (*see* Section 2.4.2).
3 Lift the filter sandwich colony-side-up into a 9-cm Petri dish, replace the lid, and incubate at 30°C for 30 min to several days (*see* Note 11)
4. β-Galactosidase-positive colonies can be matched with the colonies on the master plates from their grid positions or orientation marks, and can be carried forward for further analysis

3.4.3. Curing of Bait Plasmid (see also *Ref.* 13 *and Note 12)*

1. For each β-galactosidase-positive transformant, set up an overnight culture in 2 mL –L medium. Use 1 drop of the overnight culture to inoculate a further 2 mL of –L medium, and incubate overnight again.
2. Take 100 µL of the second overnight culture, dilute to 1 mL with sterile water, and plate out 50–100 µL of the culture onto a series of –L plates.
3. Using sterile toothpicks, grid out 20–30 colonies from each plate onto –L and –TL plates
4. Transformants that grow on –L, but fail to grow on –LT plates are cured of the bait plasmid (which carries a *TRP* marker gene) These transformants should be retested for β-galactosidase activity, and those that are now negative in this assay should be saved for further verification (*see* Note 12)

3.4.4. Recovery of Plasmid DNA from Transformed Yeast

Further analysis and identification of cDNA inserts require isolation of activation domain plasmid DNA. Only plasmid DNA heavily contaminated

with genomic DNA and other impurities can easily be isolated from yeast. Therefore, plasmid DNA is shuttled into a suitable *Escherichia coli* strain for preparation. Usefully, *E. coli* strains, such as HB101, are leucine auxotrophs owing to the *leuB* mutation, which can be complemented by the yeast *LEU2* gene, and so activation domain plasmids with *LEU2* as their transformation marker can be selected for by plating transformed *E. coli* cells on minimal plates lacking leucine. A method for the recovery from yeast of plasmid DNA suitable for transformation of *E. coli* can be found in ref. *17*. Electroporation or high-efficiency chemical means should be used for *E coli* transformation with this material.

4. Notes

1. Two of the most commonly used reporter strains for two-hybrid library screening are YPB2 *(11)* (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-109, leu2-3, 112, gal4-542, gal80-358, LYS2· GAL1*$_{UAS}$*-LEU2*$_{TATA}$*-HIS3, URA3 (GAL4 17-mers)*$_3$*-CY1C*$_{TATA}$*-LacZ*) and HF7c *(14)* (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-109, leu2-3, 112, gal4-542, gal80-358, LYS2··GAL1*$_{UAS,TATA}$*-HIS3, URA3 (GAL4 17-mers)*$_3$*-CYC1*$_{TATA}$*-LacZ*) (*see* Table 4) both constructed for use with GAL4 DNA-binding domain bait plasmids. The *HIS3* reporter gene in HF7c reportedly has a lower level of background expression (*see also* Note 4). For bait constructs using the LexA DNA-binding domain, the strain L40 has been described *(7,9)* (partial genotype. *MATa, trp1, leu2, his3, LYS::lexA-HIS3, URA3::lexA-LacZ*). Some of the other yeast strains that have been used for two-hybrid library screening are listed in Table 4 and ref. *11*.
2. Table 2 lists some DNA-binding domain fusion plasmids (bait plasmids). The important features of each of these plasmids are as follows:
   a. A yeast transformation marker (*TRP1* in most cases),
   b A DNA segment encoding sequence-specific DNA-binding domain (the DNA-binding domain from the yeast transcription factor GAL4 in most cases) driven by a constitutive promoter, such as the ADH1 promoter, and placed immediately upstream of a series of restriction sites for cloning of insert cDNAs,
   c. The origin of replication from the yeast 2 μ plasmid; and
   d. The *Bla* gene for ampicillin resistance in *E. coli* and an *E coli* origin.

   The plasmid pAS2 *(13)* possesses a number of additional features (*see* Table 2). The sequence of the multiple cloning sites in pGBT9 *(11)* is shown in Fig. 2A; the multicloning sites of pGBT9 and pAS2 *(13)* are identical between the *Sma*I and *Pst*II sites, but the pAS2 MCS also contains *Nde*I and *Nco*I sites (containing in-frame ATG codons) just upstream of the *Sma*I site (*see* ref. *13*).
3. The quality of the activation domain-tagged cDNA library is obviously of central importance for successful two-hybrid screening. It is outside the scope of this chapter to describe in detail the construction of such libraries beyond the following points. First, two approaches can be made in the construction of activation domain-tagged plasmid libraries. The first approach is to generate a relatively large amount of cDNA from the target tissue or cell line. Sufficiently large libraries may then be generated by direct ligation into the activation domain vector

(*see* ref *3*, for example). Following plasmid transformation by electroporation, transformed *E. coli* are plated onto a large number of 15-cm LB-amp plates (approx 50–100) ideally at about 1×10^5 colonies/plate. After overnight incubation, cells are collected by scraping them from the plates and plasmid DNA prepared by standard alkaline lysis *(18)* In the second method, described by Durfee et al. *(4)*, a library is constructed in the λ-phage vector λ-ACT, which contains an embedded copy of the two-hybrid activation domain-tagged plasmid pACT In vivo excision allows the direct generation of the pACT activation domain library, which may be amplified as described. Whichever method is used for activation domain-tagged library construction, it is argued that random primed first-strand cDNA synthesis is preferable, since this eliminates biases toward C-terminal sequences. Rather than generating libraries themselves, many workers will obtain libraries from other workers or from commercial sources (Clontech has on catalog a number of activation domain libraries constructed largely in pGAD10) Plasmid libraries obtained commercially or from other workers will usually require amplification as described above prior to yeast transformation (for which 500 μg of plasmid DNA are required/transformation) Caution should be exercised here, and *E. coli* transformation should aim to generate at least as many colonies as the estimated original complexity of the library. A high-efficiency transformation procedure, such as electroporation, should be used, plating the results of several independent transformations containing 10–50 ng of library DNA each on a large number of 15-cm plates

4. The *HIS3* reporter gene in such strains as YPB2 (*see* Table 4) is leaky, resulting in slow growth on –HTL plates in the absence of GAL4 activity. This can produce a significant background when the transformed two-hybrid library is plated Background growth can be eliminated by the addition of 5–20 m*M* 3-aminotriazole (3-AT) to plates The reporter strain HF7c *(14)* contains a slightly different *HIS3* reporter (*see* Table 4) and is reported not to suffer in this way.
5. Glycerol stocks of yeast strains are prepared by adding 0 5 mL of YPDA or appropriate SD medium containing 20% glycerol to 0.5 mL of a midlog culture. The glycerol-containing culture can then be stored in cryogenic storage vials at –80°C. Glycerol stocks remain viable in excess of 1 yr at this temperature
6. Prior to library screening, the bait construct should be tested for fortuitous transcriptional activation properties in the absence of interacting activation domain-tagged plasmid. Perform a series of small-scale transformations as in Section 3.3.1. using:
   a. The bait construct alone;
   b. The bait construct and the activation domain vector from which the library to be screened was constructed; and
   c. The empty bait vector alone.

   Plate out on appropriate selective plates, and test for β-galactosidase activity as in Sections 3.4.2. and 3.4.3. In addition, grid the transformants on plates lacking histidine to check for fortuitous activation of the HIS3 reporter gene. None of the transformants should give significant reporter activity.
7. The cells should be at approximately midlog phase at this point. If the OD_{600} is below 1.2, continue incubation until the optical density falls into this range.

8. The primary transformation efficiency using this method should be in excess of 1×10^4 colonies/μg of plasmid DNA. The protocol given here should therefore give >5 million transformants The protocol includes a 4-h recovery period. This time can be optimized for different strains. Since some growth will occur during this period, a second series of aliquots should be plated after this recovery period to determine the plating efficiency
9. This is a large number of plates, but do not be tempted to plate more cells per plate, since growth of His^+ colonies is inhibited by too dense plating. Even at 100 μL/plate, each plate will receive 50,000 transformants
10 Freeze thawing allows the cells to be permeable.
11. Strong signals may appear in hours, whereas weak positives will take at least overnight for β-galactosidase activity to be apparent.
12. Two significant enhancements to the basic method as described here that speed up false-positive elimination have been described by Harper et al. *(13)*. First, curing of putative positive colonies of bait plasmid is facilitated by using the DNA-binding domain vector pAS2 for the bait construct and a tetracycline-sensitive yeast strain, such as Y190 *(15)* or CG-1945 (*see* Table 4). Plasmid pAS2 carries the *CYH2* gene conferring cycloheximide sensitivity on suitable resistant strains. Using pAS2-derived bait plasmids and one of the aforementioned strains, segregants that have spontaneously lost the pAS2– derivative can be selected by picking Leu^+, Trp^+, His^+, $LacZ^+$ colonies into 200 μL of sterile water and spreading 100 μL of the cell suspension onto each of two –L plates containing cycloheximide at 1 μg/mL. The resulting Trp^+, leu^-, colonies can be tested for *LacZ* expression as in Section 3.4.2 The second enhancement involves mating the **a** strain Trp^+, leu^- colonies described above with α mating type yeast, such as Y187 *(13)* carrying either the original DNA-binding domain bait plasmid or one of a number of controls, such as the empty DNA-binding domain vector or unrelated DNA-binding domain fusions. Resulting **a**/α diploids selected on –TL plates now carrying both plasmids are again tested for β-galactosidase activity and/or ability to grow on –TLH plates. Original positives that produce diploid colonies on both –TL and –TLH plates (and that are positive for β-galactosidase) only when mated with α cells containing the DNA-binding domain bait plasmid and not any of the controls are strong candidates for genuine positive interactions This procedure eliminates the necessity of isolating plasmid DNAs and retransforming to test for false positives caused by nonspecific interactions of the activation domain fusion (*see* Section 3.4.). To produce the diploids, pick a single colony of each cured Trp^+, Leu^- two-hybrid positive into a microfuge tube containing 500 μL of YPD, and add a single colony of the appropriate α mating type transformant. Incubate overnight with vigorous shaking, and plate 50 μL of each culture on –TL and –TLH plates.

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