DupLEX-A™ Yeast Two-Hybrid System

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1. Introduction

The DupLEX-A™ system is a LexA-based version of the yeast two-hybrid system originally developed by Fields and Song (1). The yeast two-hybrid system has proven to be a powerful tool for identifying proteins from an expression library which can interact with one's protein of interest. The DupLEX-A™ system was developed as a more versatile and more accurate version of the yeast two-hybrid system (2).

The two-hybrid system of Fields and Song exploits the fact that a yeast transcriptional activator protein, GAL4, has a separable DNA binding domain and activation domain; neither domain can activate transcription on its own (3). Transcriptional activation is detected only when the binding domain is bound to its DNA recognition sequence and is also tethered to the activation domain. The two-hybrid system involves fusing the GAL4 binding domain with a protein "X" and the GAL4 activation domain with a protein "Y". If "X" and "Y" interact, then a functional GAL4 is restored and transcriptional activation can be detected. If binding sites for GAL4 are placed upstream of a reporter gene (such as *LacZ*), transcriptional activation can be monitored easily.

The DupLEX-A™ system utilizes the same basic idea except that the DNA binding protein is the *Escherichia coli* LexA protein while the activation protein is the acid blob domain B42. Neither LexA protein bound upstream of a reporter gene nor B42 alone can activate transcription of the reporter, but if brought together via fusions with two interacting proteins, reporter gene expression can be detected (2).

Advantages of the DupLEX-A™ system over other yeast two-hybrid systems include:

- Reduction in the number of false positives obtained since prokaryotic (LexA and B42) rather than eukaryotic (GAL4) proteins are used
- Ability to screen potentially toxic target proteins since their expression is galactose-inducible
- Ability to demonstrate a potential positive's interaction with bait is dependent upon expression of the potential positive
- Ease of doing a co-immunoprecipitation assay of bait and potential positive since antibodies to HA tag (fused downstream of B42) are available (if antibody to the bait protein is also available)
- Reporters with varying sensitivities are available so that baits which activate transcription on their own can potentially still be assayed simply by using a less sensitive reporter
- Ease of determining whether or not a particular bait protein will enter the yeast nucleus and bind LexA operators

1. Fields, S. and Song, O. (1989). *Nature* **340**, 245-247.
2. Gyuris, J., Golemis, E. A., Chertkov, H., and Brent, R. (1993). *Cell* **75**, 791-803.
3. Chien, C. T. et al. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 9578-9582.

2. Package Components and Storage Conditions

NOTE. All of the components listed below are required. Read the manual carefully to determine which components are best suited for a library screen.

The following components are included:

(i) Yeast strains

EGY48	**MAT**α	*trp1 his3 ura3 leu2*::6 LexAop-*LEU2* (high sensitivity)
EGY194	**MATa**	*trp1 his3 ura3 leu2*::4 LexAop-*LEU2* (medium sensitivity)
EGY188	**MATa**	*trp1 his3 ura3 leu2*::2 LexAop-*LEU2* (low sensitivity)
EGY40	**MAT**α	*trp1 his3 ura3 leu2*::0 LexAop-*LEU2* (negative control)
RFY206	**MATa**	*trp1*Δ*::hisG his3*Δ*200 ura3-52 lys2*Δ*201 leu2-3* (mating strain)

Streak on YPD plates and grow at 30°C for 2–3 days; start cultures from single colonies.

(ii) Reporter gene (LacZ) plasmids (2 µg each)

pSH18-34	*URA3*, 2 µ, Amp^r, 8 ops.-LacZ (high sensitivity)
pJK103	*URA3*, 2 µ, Amp^r, 2 ops.-LacZ (medium sensitivity)
pRB1840	*URA3*, 2 µ, Amp^r, 1 op.-LacZ (low sensitivity)
pJK101	*URA3*, 2 µ, Amp^r, GAL1-2 ops.-LacZ (used in repression assay)

(iii) Bait plasmids

pEG202: *HIS3*, 2 µ, Amp^r, (constitutive *ADH* promoter expresses LexA and is followed by a polylinker for making the bait fusion protein); 10 µg

pEG202-NLS: *HIS3*, 2 µ, Amp^r, (similar to pEG202 but with SV40 nuclear localization sequence between LexA and polylinker); 2 µg

pNLexA: *HIS3*, 2 µ, Amp^r, (similar to pEG202 except that the LexA sequence is 3' rather than 5' of the polylinker); 2 µg

(iv) Target plasmid

pJG4-5: *TRP1*, 2 µ, Amp^r, (inducible *GAL1* promoter expresses B42-HA tag and is followed by a polylinker for making target fusion protein expression libraries from cDNA); 10 µg

(v) Control plasmids (2 µg each)

pRHFM1: *HIS3*, 2 µ, Amp^r, (*ADH* promoter expresses LexA-Bicoid homeodomain fusion; used as a positive control in the repression assay and a negative control in the DupLEX-A™ screen)

pSH17-4: *HIS3*, 2 µ, Amp^r, (*ADH* promoter expresses LexA-GAL4 activation domain; used as a positive control in the DupLEX-A™ screen)

pEG202-Max: expresses LexA-Max fusion constitutively; used as a negative control when testing isolated target proteins or as a positive control in the repression assay

pBait: constitutively expresses a LexA-bait fusion protein that interacts with the fusion protein from pTarget (see below); can also be used as a negative control when testing isolated target proteins or as a positive control in the repression assay)

pTarget: expresses (galactose-dependently) a B42-target fusion protein that interacts with the fusion protein from pBait (see above)

(vi) Primers (10 µg each)

NOTE. The concentration of each oligonucleotide is approximately 80 µM.

5' bait fusion primer: 5'-CGT CAG CAG AGC TTC ACC-3'
(used to determine the sequence of the junction between LexA and the bait)

5' target fusion primer: 5'-CTG AGT GGA GAT GCC TCC-3'
(used to determine the reading frame and identity of positive clones; also, can be used with 3' target primer to amplify clone by PCR)

3' target fusion primer: 5'-GCC GAC AAC CTT GAT TG-3'
(used to determine the identity of positive clones; also, can be used with 5' target primer to amplify clone by PCR)

(vii) Other items included in the kit:

pJG4-6: *TRP1*, 2 µ, Amp^r, (similar to pJG4-5 but without B42 activation domain; used to express an isolated target protein in yeast); 10 µg

sonicated salmon sperm DNA, 5 mg/mL (prepared specially for yeast transformation); 10 mg total [CARRIER DNA]

Escherichia coli strain KC8 (*pyrF, leuB600, trpC, hisB463)*

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(viii) Components required but not provided

NOTE. The specific materials listed below are ones tested by OriGene in the DupLEX-A™ system. Similar items from other sources may be interchangeable.

- Yeast growth media

	Vendor	Catalog #
peptone	BIO 101	4018-512
agar	BIO 101	4019-512
yeast extract	BIO 101	4018-012
yeast nitrogen base w/o amino acids	BIO 101	4027-012
raffinose	Sigma	R-0250
dextrose (glucose)	Fisher	D16-1
galactose (glucose-free)	Sigma	G-0750
dropout mix (–his –ura –trp –leu)	BIO101	4540-022
uracil	Sigma	U-0750
leucine	Sigma	L-5652
tryptophan	Sigma	T-0254
histidine	Fisher	BP382-100

- Bacterial growth media

	Vendor	Catalog #
LB broth	Difco	0402-07-0
magnesium sulfate	Fisher	BP213-1
potassium phosphate (monobasic)	Mallinckrodt	7100
potassium phosphate (dibasic)	Fisher	BP363-500
sodium citrate	Fisher	BP327-1

thiamine hydrochloride	Fisher	BP892-100
ammonium sulfate	Fisher	BP212R-1
kanamycin	Fisher	BP906-5
ampicillin	Boehringer Mann.	835 2697

- For yeast transformations

	Vendor	Catalog #
lithium acetate	Fisher	AC19984-2500
polyethylene glycol-3350	Sigma	P146-3
dimethyl sulfoxide	Fisher	D136-1
hydrochloric acid	Fisher	A144-500
tris base	Fisher	BP152-5
EDTA	Fisher	02793-500

- For bacterial transformations

	Vendor	Catalog #
glycerol	Sigma	G-5516
ElectroMax DH10B (competent cells)	Gibco/BRL	18290-015
SOC medium	Gibco/BRL	15544-018
electroporator	Gibco/BRL	11613-015

- For rescuing plasmids from yeast

	Vendor	Catalog #
glass beads, acid-washed	Sigma	G8772
Triton-X-100	Fisher	BP151-100
sodium acetate	Fisher	S209-500
ethanol	Aldrich	18,738-0
phenol	Fisher	BP1750I-400
chloroform	Fisher	BP1145-1
isoamyl alcohol	Fisher	BP1150-500
SDS	Sigma	L-4509

• Filter assay for β-galactosidase and yeast X-gal plates

	Vendor	Catalog #
sodium phosphate (monobasic)	Fisher	BP329-500
sodium phosphate (dibasic)	Mallinckrodt	7914
potassium chloride	Mallinckrodt	6858
β-mercaptoethanol	Fisher	03446I-100
nylon membrane filters	MSI	N04SP09025
X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)	Gold BioTech.	X4281C
N,*N*-dimethyl formamide	Fisher	BP1160-5008

3. Flow-Chart For Duplex-A™ Yeast Two-Hybrid Screen

A. Subclone gene for bait protein into pEG202; verify correct reading frame; go to step B.

B. Transform into all four yeast strains to test for autoactivation:

b1. If no autoactivation, then go to step C.

b2. If it autoactivates, then subclone only a portion of the bait gene into pEG202 and retest. If the truncated bait does not autoactivate, then go to step C.

C. Transform into appropriate strain along with pJK101 to test whether or not the bait fusion can enter the nucleus and bind LexA operators or not.

c1. If binding is detected, go to step D.

c2. If no binding is detected, then subclone into pEG202-NLS and retest. If binding is detected on the retest, then proceed to step D.

D. Transform into yeast strain, with reporter. A Western Blot to check for bait protein expression at this point is optional. Go to step E.

E. Transform with the library (large-scale). Go to step F.

F. Scrape colonies off plates and freeze in aliquots. Go to step G.

G. Titer the cells and replate, selecting for interactors ($LEU2^+$). Go to step H.

H. Grid positives to small plates, perform lacZ filter assay. Go to step I.

I. Rescue target plasmid from positives, transform back into strain along with reporter, and mate with opposite mating type strain containing different control baits. Go to step J.

J. Sequence positives, search sequence databases for homology.

4. DupLEX-A™ Yeast Two-Hybrid System Protocol

(i) Constructing the bait

Using standard recombinant DNA techniques, subclone the bait protein gene in the correct orientation into the polylinker of pEG202 (see Appendix F). Design the bait protein gene subcloning such that it fuses in-frame with LexA. It is strongly recommend that the sequence of the LexA-bait junction is verified to ensure that a LexA-cDNA fusion protein should be made.

NOTE. It is highly recommended that the bait fusion protein be tested in the assays below before performing a full-scale library screen.

(ii) Testing the autoactivation potential of the bait (LacZ)

Some bait proteins can activate reporter genes on their own, making a two-hybrid system library screen not possible. However, the DupLEX-A™ system offers some alternatives if this occurs. To test for autoactivation by the bait fusion, transform yeast strain EGY48 with the following combinations of plasmids:

1. pEG202-Bait + pSH18-34 (test)
2. pSH17-4 + pSH18-34 (strong activation)
3. pRHFM1 + pSH18-34 (no activation)

(a) Small-scale Yeast Transformation Protocol

- Grow a 5 mL culture of EGY48 in YPD at 30°C with shaking (overnight). Inoculate by picking a colony off of a streaked plate of EGY48.

- Measure the OD_{600} of a 1:10 dilution of the overnight culture. Calculate the OD_{600} of the 5 mL culture and use that to inoculate a 60 mL YPD culture to give an OD_{600} = 0.1. Grow at 30°C with shaking.

- When the OD_{600} = 0.5–0.7 (approximately 4–6 hours after inoculation), pellet the cells by spinning the culture at 1500 × *g* for 5 min. Resuspend in 20 mL of sterile distilled water, spin again, and resuspend the pellet in 0.3

mL of 1× TE/LiOAc. Pipette 100 µL into each of three sterile 1.5-mL microcentrifuge tubes.

• Boil the carrier DNA for 5 min and quickly chill on ice. This is necessary for obtaining a maximum efficiency of transformation; however, it should only be done every third or fourth time the carrier DNA is used.

• Add 100 ng of each plasmid DNA and 50 µg of denatured carrier DNA to each tube and mix.

• Add 0.3 mL of 1× TE/LiOAc/PEG, mix by inversion, and place the tubes at 30°C (with or without shaking) for 30 min.

• Add 70 µL of DMSO (dimethyl sulfoxide) to each sample, mix by inversion, and place at 42–45°C (without shaking) for 15 min.

• Spin at 10,000 rpm in a microcentrifuge for 10 sec, pour off the supernatant, and resuspend each pellet in 0.5 mL of sterile distilled water.

• Spread 50–100 µL of each sample onto separate YNB (glu) –his –ura plates. Incubate at 30°C for 2–3 days.

• Streak four colonies from each plate onto another YNB (glu) –his –ura plate. Inoculate at 30°C for 1–2 days. Perform a LacZ filter assay or replica to YNB(gal)-his-ura + X-gal plates and grow at 30°C overnight.

(b) Filter Assay

Cut a piece of Whatman 3M paper such that it just fits into a 100-mm petri dish. Place the paper in an empty dish and add 2 mL of 1 mg/mL X-gal (add 20 µL of X-gal in *N,N*-dimethyl formamide to 2 mL of Z buffer), making sure the paper is completely wet. Place a similarly-cut nitrocellulose filter on the surface of the plate containing the re-streaked yeast, then gently remove it off of the plate and place the filter in a –70°C freezer for 5 -10 min. Remove the filter, let thaw, and re-freeze it at –70°C. Remove the filter and place it yeast side up on the pre-wetted Whatman filter paper. Incubate at 30°C for 2 hr.

The colonies containing pSH17-4 should turn blue, the colonies containing pRFHM1 should not turn blue, and the colonies containing the pEG202-Bait plasmid may or may not turn blue. If pEG202-Bait-containing colonies do not turn blue, then the bait does not autoactivate reporter gene expression and can be used for screening in the yeast strain EGY48. If the

same colonies turn blue in the above assay, then the test should be repeated using either pJK103 or pRB1840 in place of pSH18-34 as the reporter plasmid. If the bait fails to autoactivate in the presence of either reporter plasmid, then either reporter can be used in a two-hybrid screen. If the bait continues to autoactivates with these reporter plasmids it cannot be used in a screen. To circumvent this problem, try subcloning only portions of the gene encoding the bait protein into pEG202 and repeat the autoactivation experiments.

***(iii) Testing the autoactivation potential of the bait* (LEU2)**

Since *LEU2* is the reporter used in the initial screen, it is important not to have a high background of colonies arising due to activation of the *LEU2* gene by the bait alone. Also, for some baits, the *LEU2* reporter in EGY48 is more sensitive than the lacZ reporter on pSH18-34. Therefore, the ability of the bait to autoactivate the *LEU2* reporter should be tested before performing a large screen.

• Using a sterile wooden applicator stick, transfer a colony of EGY48 containing the bait plasmid into 0.5 mL of sterile distilled water. Vortex. Dilute 100 µL into 1 mL of sterile distilled water. Vortex. This is Dilution 1. Perform three more serial 1:10 dilutions (Dilutions 2–4) such that if Dilution 1 is considered "undiluted", Dilution 2 = 1:10 diluted, Dilution 3 = 1:100 diluted, and Dilution 4 = 1:1000 diluted.

• Plate 100 µL of each of Dilutions 1–4 onto YNB (gal) –his plates and onto YNB (gal) –his –leu plates. Incubate at 30°C for 1–2 days. Colonies should be seen on the –his plates but not on the –his –leu plates. (Note: galactose plates are used in this experiment since that is the carbon source that will be used during the *LEU2* selection step of the large-scale screen.) If many colonies are obtained on the –his –leu plates, then the bait is autoactivating and the assays should be re-done using the bait in strains EGY194 and EGY188. Strain EGY40 is included as a negative control. In addition, three different sensitivity LacZ reporter plasmids are included:
pSH18-34 > pJK103 > pRB1840 (most sensitive > least sensitive).

Once convinced that the bait fusion can enter the nucleus and bind to LexA operators without autoactivating either of the two reporter genes, then a large-scale library screen can be performed. Note that for an unknown reason, some baits can autoactivate the reporter genes in a large-scale screen even when they did not autoactivate in small-scale tests. Therefore, it might be advantageous to perform a "medium-scale" screen, perhaps one-fifth the size of a large scale screen, first.

(iv) Testing the bait's ability to enter the nucleus and bind LexA operators

The plasmid pJK101 contains a *LacZ* reporter gene whose expression is driven by the yeast *GAL1* promoter. However, two LexA operators have been placed between the *GAL1* promoter and the *LacZ* gene; LexA fusion proteins will bind to these operators and decrease the level of *GAL1*-driven LacZ expression.

(c) Repression Assay

• Perform the following transformations into EGY48 (see protocol under section 4 (ii)):

1. pEG202-Bait + pJK101 (test)
2. pEG202-Max + pJK101 (repression)
3. pJK101 alone (no repression)

NOTE. Plate transformations 1 and 2 above onto YNB (glu) –his –ura and transformation 3 onto YNB (glu) –ura plates.

• Streak four colonies from each of plates 1 and 2 above onto YNB (gal) –his –ura + X-gal plates and four colonies from plate 3 onto YNB (gal) –ura + X-gal plates. After 12–24 hours at 30°C, it should be evident whether or not the bait fusion can bind to the LexA operators. If some level of repression is observed, then proceeding with the screen can be done. If no repression is seen, then the bait should be tested if it is even being synthesized (a Western blot can be performed if antibodies against the bait exist). If there is evidence that the protein is being made in yeast, then try re-cloning the bait into pEG202-NLS (formerly called pJK202; see Appendix H). The vector pEG202-NLS contains a nuclear localization sequence fused to LexA just upstream of the polylinker sequence.

(v) Performing a large-scale library transformation

• Grow a 20 mL overnight culture (at 30°C) of EGY48 (or EGY194 or EGY188) containing the LacZ reporter plasmid (pSH18-34, for example) and the bait plasmid. Grow in YNB (glu) –his –ura medium.

• The next morning, dilute 100 µL of culture into 0.9 mL of water, mix well, and immediately measure the OD_{600} of the dilution. Multiply by 10 to get the OD_{600} of the undiluted culture. Inoculate 300 mL of YPD medium (in a sterile 2 L flask) with enough of the overnight culture to give an OD_{600} = 0.1. For example, if the dilution of the overnight culture had an OD_{600} = 0.5, then the 20 mL undiluted overnight culture would have an OD_{600} = 0.5 × 10 = 5. Since the 300 mL culture should start at OD_{600} = 0.1, the amount of undiluted culture needed would be 0.1/5 × 300 mL = 6 mL. Therefore, add 6 mL of undiluted overnight culture to 300 mL of YPD. (Note: without agitation, yeast cells will settle to the bottom of a flask over time, therefore, always swirl the flask before removing any culture to ensure the culture is of uniform density.)

• Shake the culture vigorously at 30°C until the OD_{600} = 0.5–0.7. This should take 4–5 hours.

• Harvest the cells by spinning at 1,500 × *g* (3,000 rpm in a GSA rotor) for 5 min (room temperature). Pour off the supernatant.

• Resuspend in 30 mL of sterile distilled water, transfer to a 50-mL sterile conical tube, and spin at 1,500 × *g* (2,500 rpm in an HL-4 rotor) for 5 min (room temperature). Pour off the supernatant.

• Resuspend the cell pellet in 1.5 mL of 1× TE/LiOAc. Aliquot 50 µL portions into 30 sterile 1.5-mL microcentrifuge tubes. (Note: a higher transformation efficiency is obtained when several small transformations are performed rather than one large transformation.)

• Denature the carrier DNA by placing the tube in a boiling water bath for 5 min and then quickly placing the tube on ice (do this only if the carrier has not been denatured in the last two or three sets of transformations that have been done). Incubate on ice for five minutes.

• Add 50 µg (10 µL) of carrier DNA and 1 µg of pJG4-5-based plasmid library DNA to each microcentrifuge tube. Do not use more than 1 µg of library DNA per tube since this can lead to the introduction of multiple plasmids into a single yeast cell and subsequently give confusing results in later analyses.

• Add 300 µL of 1× TE/LiOAc/PEG to each tube and mix by inversion. Incubate (without agitation) at 30°C for 30 min.

• Add 40 µL of DMSO to each tube, mix by inversion, and heat shock by incubating at 42–45°C for 20 min.

• Add 0.6 mL of sterile distilled water to each tube and mix by inversion. Dilute 10 µL of one sample into 990 µL of sterile distilled water, vortex, and plate 100 µL of the dilution onto a 100-mm YNB (glu) –his –ura –trp plate. Plate all samples from each tube onto 30 separate 24 cm × 24 cm YNB (glu) –his –ura –trp plates (or 300 µL onto each of 100 150-mm YNB (glu) –his –trp –ura plates. Incubate all plates at 30°C for 2–3 days, until colonies appear.

• Calculate the number of transformants obtained by counting the number of colonies on the 100-mm plate. For example, 100 colonies corresponds to an efficiency of 10^5/µg, therefore, the total number of transformants is 30 µg × 10^5/µg = 3 × 10^6 total transformants. Similarly, 50 colonies corresponds to an efficiency of 5 × 10^4/µg, or 1.5 × 10^6 total transformants. A saturating screen of a mammalian library requires at least 2 × 10^6 transformants.

• Harvest the transformants as follows:

• Soak a microscope slide in ethanol, then let air-dry.

• Pipet 10 mL of sterile distilled water onto each 24 cm × 24 cm colony plate, scrape off the colonies with the long edge of the microscope slide (taking care to use good sterile technique), and pipet the slurry into a sterile disposable centrifuge tube.

• Centrifuge for 5 min at 1,500 × *g* (2,500 rpm in an HL-4 rotor) at room temperature. Pour off the supernatant, resuspend the pellet in a total of about 75 mL of sterile distilled water, spin again as above, and pour off the supernatant. Resuspend the pellet in an equal volume of sterile distilled water. Estimate the total volume, add half a volume of sterile 50% (v/v) glycerol, mix, and freeze 1 mL aliquots at –70°C. Frozen stocks will remain good for at least 1 year when stored at –70°C.

(vi) Screening for potential positive transformants

(a) Titer the number of viable cells

• Thaw an aliquot of the frozen yeast transformants and dilute 1:10 with YNB (gal) –his –ura –trp medium. Shake at 30°C for 4 hr to induce the *GAL1* promoter.

• Perform serial dilutions of the transformants in YNB (gal) –his –ura –trp and plate 100 µL of each dilution onto separate YNB (gal) –his –ura –trp plates. Incubate at 30°C for 2–3 days, until colonies are visible.

• Calculate the number of colony-forming units (cfu) per frozen aliquot of yeast transformants.

(b) Screen for Leu^+ colonies

• To fully screen all the transformants, about 5–7 times the number of original transformants obtained should be screened. (Therefore, if 3×10^6 transformants were originally obtained , then about 2×10^7 cfu should be plated. Thaw enough of the frozen transformants equal to that number of cfu.

• The number of cfu per 1 mL frozen aliquot can be used to estimate an OD_{600} of the aliquot since 1 $OD_{600} = 2 \times 10^7$ yeast cells. That is, the number of cfu / $2 \times 10^7 = OD_{600}$ of the aliquot. Dilute the appropriate amount of frozen transformants with YNB (gal) –his –ura –trp –leu medium, down to approximately 1×10^7 cells / mL ($OD_{600} = 0.5$). Shake at 30°C for 4 hr.

• Plate 100 µL aliquots onto 100-mm YNB (gal) –his –ura –trp –leu plates. This corresponds to a screen of about 1×10^6 cfu per plate (do not exceed this number). Incubate at 30°C for 2 days, until colonies appear.

• Pick colonies onto a YNB (gal) –his –ura –trp –leu master plate and incubate at 30°C for a few days, until new colonies appear. Refer back to the plates from the step above and create another master plate for colonies which arise three days after plating and another master plate for colonies which arise four days after plating. Colonies from the first two master plates should definitely be characterized further. Colonies that do not appear until about a week after plating are likely to be artifactual and should not be characterized further, unless they are the only colonies obtained.

(c) Test galactose growth dependence and LacZ expression of potential positive transformants

• Since the expression of the target protein is dependent on galactose, any colonies which can activate *LEU2* and grow on –leu medium in the presence of glucose are false positives and should not be further characterized. Colonies that can grow on -leu medium containing

galactose but that cannot grow on -leu medium containing glucose are potentially true positives and should be tested for LacZ expression. Re-streak colonies from the master plates onto the following types of plates:

YNB (glu) –his –ura –trp –leu
YNB (gal) -his –ura –trp –leu
YNB (glu) -his –ura –trp + X-gal
YNB (gal) -his –ura –trp + X-gal

• Incubate at 30°C for 1–2 days, until growth occurs. Potential positive transformants will grow on the –leu (gal) plate but not the –leu (glu) plate, and will turn blue on the X-gal (gal) plate but not on the X-gal (glu) plate.

• If the number of potential positives is small (<50), then all should be recovered and further characterized. If >50 potential positives are obtained, the first 50 that arise should be characterized and the rest frozen at –70°C in 20% glycerol.

(vii) Recovering plasmids from yeast

• To isolate DNA from the potential positives, grow each one in 2 mL of YNB(glu)-trp medium (or any other -trp medium you have available) at 30°C overnight.

• Spin down 1.5 mL of each in microcentrifuge tubes for 10 sec at maximum speed. Pour off the supernatant, vortex to resuspend the pellet in the residual liquid, and add 200 µL of plasmid rescue solution.

• Add 100 µL of phenol (tris-saturated, pH 8.0) and 100 µL of 24:1 chloroform:isoamyl alcohol. Add about 0.3 g of acid-washed glass beads and vortex vigorously for 2 min.

• Spin at 14,000 rpm in a microcentrifuge for 5 min at room temperature. Carefully remove 200 µL from the top (aqueous) layer and transfer it to a clean tube. Add 20 µL of 3 M NaOAc, vortex, and add 440 µL of 95% ethanol. Vortex, spin for 20 min at 14,000 rpm in a microcentrifuge, pipet off and discard the supernatant, wash with 70% (v/v) ethanol, carefully pipet off and discard the supernatant, vacuum dry the pellet, and resuspend the pellet in 5 µL of sterile distilled water. Use 1 µL to transform *E. coli* KC8 cells (see below).

(viii) Obtaining potential positive target plasmids in bacteria

The pellet recovered from the yeast cells in the above procedure consists mostly of yeast RNA and genomic DNA. Very little of it is library plasmid DNA. Also, the *trp*⁻ *E. coli* strain used (KC8) is not very amenable to transformation. Therefore, electroporation of KC8 cells is recommended in order to recover the library plasmid.

Preparation of electrocompetent KC8 cells:

- Streak KC8 onto a LBK plate and grow at 37°C overnight.

- Pick a single colony and inoculate into 5 mL of LBK medium. Grow at 37°C overnight (with shaking).

- Use all 5 mL to inoculate a 500 mL culture in LBK medium. Grow with shaking at 37°C until the OD_{600} = 0.5.

- Chill the cells on ice for 30 min. Spin at 5,000 rpm for 10 min at 4°C.

- Pour off the supernatant and resuspend the pellet in 300 mL of ice-cold 10% glycerol. Spin again as above, pour off the supernatant, and resuspend the pellet in 150 mL of ice-cold 10% glycerol.

- Spin again as above, pour off the supernatant, resuspend the pellet in 10 mL of ice-cold 10% glycerol, spin again as above, carefully pipette off and discard the supernatant, and resuspend the pellet in 2 mL of ice-cold 10% glycerol.

- Aliquot into 75 µL portions and store at –70°C.

- Electroporate 1 µL of the recovered yeast plasmid DNA into 75 µL of competent KC8 cells following the instructions of the particular electroporator being used. Plate onto LBA plates and grow at 37°C overnight. Colonies arising at this stage contain either the bait, target, or LacZ reporter plasmid.

- To select only those colonies which contain the target plasmid, re-streak (or replica) the colonies from the LBA plate to a minimal (-trp) plate. Grow at 37°C overnight.

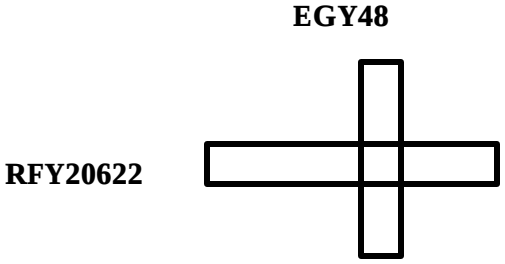
- Prepare plasmid DNA from at least two colonies from each plate since more than one target plasmid can be present in a particular yeast cell. Digest the DNA with *Eco*RI and *Xho*I to release the insert and separate the sample by agarose gel electrophoresis to determine the insert size.

(ix) Determining the specificity of the interaction using mating tests

To determine whether the potential positives are specific for the particular bait used, test the positive clones against other baits with which it should not interact. This is most easily done by transforming the recovered library plasmid back into the yeast strain that was used in the screening (EGY48, for example), transforming pSH18-34 plus a test bait plasmid into a strain of the opposite mating type, and mating the two strains.

If the library plasmid was transformed into EGY48, then the reporter plasmid and bait plasmid should be transformed into RFY206. If the library plasmid was transformed into either EGY194 or EGY188, then the reporter plasmid and bait plasmid should be transformed into EGY40. Alternatively, the strain used for the screening can be transformed with two of the plasmids, selected, and then transformed with the third plasmid.

Example: using the small-scale transformation protocol, transform EGY48 with the isolated potential positive target plasmid. Similarly, transform RFY206 with pLexA-Max + pSH18-34. Mate some transformants from each plate by streaking the two in a "+" pattern on a YPD plate.



Incubate the plate at 30°C overnight and replica onto a YNB (gal) –his –ura –trp plate the next day. Grow at 30°C for 1–2 days. The only cells that should grow are the ones at the intersection of the two streaks. Perform LacZ filter assays to test for LacZ expression.

The vectors pLexA-Max, pBait, and pRHFM1 are all bait plasmids that encode proteins that should not interact with the isolated target protein(s). The target protein should be tested for interaction against these bait proteins. In addition, the original bait should be re-tested with the target protein to verify the original interaction.

Once a positive has been obtained that passes all the specificity tests, then the target plasmid DNA should be re-transformed into a new *E. coli* strain, re-purified and have the insert partially sequenced. A database search can then be performed to determine the origin of the target sequence. It is imperative to realize that even a clone that passes all the above tests could still be a false positive. For example, in some instances clones have been obtained that appear to interact specifically with a certain bait, but it was already known that the two molecules are located in different parts of the cell; therefore, they are not real interactors. That is why the yeast two-hybrid system should be considered a relatively quick and easy method of obtaining the cloned gene for a protein which may interact with a protein of interest. Once the clone is isolated, additional assays must still be done to show that the interaction is biologically relevant. Note that pJG4-6 is included in the kit; it is similar to pJG4-5 except that it does not contain LexA. It can be used to express a target as a non-hybrid protein in yeast.

5. Appendix

A. Yeast Growth Media

- YPD (rich medium)

  20 g peptone
  10 g yeast extract
  20 g glucose
  one pellet (0.1 g) NaOH (for plates)
  20 g agar (for plates)

  Add 1 L of distilled water and autoclave for 20 min.
  For plates, cool to 50°C before pouring.

- YNB –ura –his –leu –trp (selective medium)

  1.7 g yeast nitrogen base w/o amino acids*
  5 g ammonium sulfate*
  0.6 g –his –ura –trp –leu dropout mix
  20 g glucose (or 20 g galactose + 10 g raffinose for gal/raff media)
  20 g agar (for plates)

  Add 1 L of distilled water and autoclave for 20 minutes.
  For plates, cool to 50°C before pouring.

**NOTE. yeast nitrogen base is sold either with or without ammonium sulfate. The particular one listed in this manual does not contain ammonium sulfate, so it must be added separately. If the label on the bottle of yeast nitrogen base instructs to add 6.7 g/L to make media, then it already contains ammonium sulfate .*

- Other YNB (selective) media

Add the following amounts of reagents to the YNB –ura –his –leu –trp medium described above (before autoclaving) to make the appropriate medium. Filter sterilize and store at 4°C; briefly heat in a microwave oven if a precipitate forms.

trp = 10 mL of 4 mg/mL stock per liter of medium (0.04 mg/mL final conc.)
ura = 5 mL of 4 mg/mL stock per liter of medium (0.02 mg/mL final conc.)
leu = 15 mL of 4 mg/mL stock per liter of medium (0.06 mg/mL final conc.)
his = 5 mL of 4 mg/mL stock per liter of medium (0.02 mg/mL final conc.)

For example, to make medium lacking only leucine, add 5 mL of 4 mg/mL uracil, 10 mL of 4 mg/mL tryptophan, and 5 mL of 4 mg/mL histidine to 1 L of YNB –ura –his –leu –trp medium.

- Yeast selective X-gal media

amino acid solution as per above
0.6 g –his –ura –trp –leu dropout mix
1.7 g yeast nitrogen base without amino acids*
5.0 g ammonium sulfate*
20 g glucose (or 20 g galactose + 10 g raffinose for gal media)
20 g agar
900 mL distilled water

Autoclave and cool to 65°C. In a separate bottle, autoclave 7 g of sodium phosphate (dibasic) and 3 g of sodium phosphate (monobasic) in 100 mL of distilled water. Mix the two solutions,

add 0.8 ml of 100 mg/ml X-gal (in *N*,*N*-dimethyl formamide), and pour plates.

*SEE NOTE ON PREVIOUS PAGE

B. Bacterial Growth Media

- LB medium

Dissolve 20 g of dry LB Broth (Lennox; from Fisher) in 1 L of distilled water and autoclave for 15 min. For plates, add 15 g of agar per liter of medium before autoclaving and cool to 50°C before pouring.

- LBA medium (ampicillin selection)

Cool the LB medium above to 50°C and add 2 ml of 50 mg/mL ampicillin (in distilled water, filter-sterilized) per liter of medium. Mix.

- LBK medium (kanamycin selection)

Cool the LB medium above to 50°C and add 5 mL of 10 mg/mL kanamycin sulfate (in distilled water, filter-sterilized) per L of medium. Mix.

- Minimal (–trp) medium

Prepare the following stocks (autoclaved):

i. 20% magnesium sulfate
ii. 4 mg/mL uracil
iii. 4 mg/mL histidine
iv. 4 mg/mL leucine
v. 20% glucose

Prepare the following filter-sterilized solutions:

vi. 50 mg/mL kanamycin sulfate
vii. 1% thiamine hydrochloride

Autoclave the following two solutions separately:

viii. 15 g agar in 800 mL distilled water
ix. 10.5 g potassium phosphate (dibasic)
4.5 g potassium phosphate (monobasic)
1 g ammonium sulfate
0.5 g sodium citrate
160 mL distilled water

Mixing: cool solutions viii and ix to 50°C, mix, and quickly add 1 mL of solution i, 10 mL of solution ii, 10 mL of solution iii, 10 mL of solution iv, 10 ml of solution v, 1 mL of solution vi, and 0.5 ml of solution vii. Mix well and pour plates immediately.

C. Solutions

- 10× TE

  50 mL of 1 M tris (pH 7.5) [0.1 M]
  10 mL of 0.5 M EDTA [0.01 M]
  Add 440 mL distilled water and autoclave for 20 min.

- 10× LiOAc

  51 g of lithium acetate [1 M]

  Bring volume up to 500 mL with distilled water, mix until dissolved, and autoclave for 20 min.

- 50% PEG-3350

  250 g polyethylene glycol-3350

  Bring volume up to 500 mL with distilled water, mix until dissolved, and autoclave for 20 min.

- 1× TE / LiOAc

  Right before use, mix 1 part 10× TE, 1 part 10× LiOAc, and 8 parts sterile distilled water.

- 1× TE / LiOAc / PEG

Right before use, mix 1 part 10× TE, 1 part 10× LiOAc, and 8 parts 50% PEG-3350.

- Z buffer

  16.1 g of sodium phosphate (dibasic) [60 mM]
  5.5 g of sodium phosphate (monobasic) [40 mM]
  0.75 g of potassium chloride [10 mM]
  0.246 g of magnesium sulfate [1 mM]
  2.7 mL of β-mercaptoethanol [50 mM]

  Dissolve in 1 L of distilled water. DO NOT AUTOCLAVE!

- 100 mg / mL X-gal

  Dissolve 100 mg of X-gal in 1 ml of N,N-dimethylformamide and store at –20°C.

- Plasmid rescue solution

  85 mL of distilled water
  2 mL of triton-X-100 [2 %]
  10 mL of 10% SDS [1 %]
  2 mL of 5 M sodium chloride [0.1 M]
  1 mL of 1 M tris (pH 8.0) [0.01 M]
  0.2 mL of 0.5 M EDTA [0.001 M]

  Mix and store at room temperature.

- 3 M sodium acetate

  40.8 g of sodium acetate trihydrate

  Bring volume up to 100 mL with distilled water, mix until dissolved, and autoclave for 20 min.

- 70% (v / v) ethanol

  Mix 350 mL of absolute ethanol with 150 mL of distilled water and store at –20°C.

- 10% glycerol

  Mix 100 mL of glycerol with 900 mL of distilled water and autoclave for 20 min.

- 50 % glycerol

  Mix 250 mL of glycerol with 250 mL of distilled water and autoclave for 20 min.

D. Library information

All DupLEX-A™ Yeast Two-Hybrid System libraries are made in the B42 activation domain-HA tag expression vector pJG4-5. The cDNA is made by oligo d(T) priming and is cloned unidirectionally between the *Eco*RI and *Xho*I sites of pJG4-5 (see Appendix H for details). The libraries are provided as ready-to-use plasmid DNA and also as plasmid-containing bacterial cells.

E. Working with yeast

The budding yeast *Saccharomyces cerevisiae* is very amenable to genetic and molecular biological methodologies due to its ability to be transformed by foreign DNA, its highly efficient system of homologous recombination, and its relatively rapid rate of growth. Whereas *E. coli* has a generation time of 30–45 minutes, most yeast strains can double in 90–120 minutes. As with *E. coli*, yeast can be grown on plates or in liquid culture. However, antibiotics which work on *E. coli* do not work on yeast, making good sterile technique mandatory when working with yeast. Finally, the optimum growth temperature for yeast is 28–32°C

F. Polylinker sequences

(a) pEG202 polylinker sequence: (unique sites shown)

LexA *Eco*RI *Bam*HI *Nco*I *Not*I
5'-CTG GAA TTC CCG G GG ATC C GT CGA CCA TGG CGG CCG CTC GAG TCG ACC TGC AGC-3'

(b) pJG4-5 polylinker sequence:

*Eco*RI *Xho*I
5'-CCC GAA TTC GGC CGA CTC GAG AAG-3'

(c) pNLexA polylinker sequence:

5'-G AAT TCG CGG CCG CCT CGA GGG ATC CAA TTC ATG AAA GCG-3'

(d) pEG202-NLS polylinker sequence:

5'- GTG GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC-3'

G. Common false positives

Although the yeast two-hybrid system has proven to be a powerful tool for detecting protein-protein interactions *in vivo*, all versions of it to date have been plagued with the problem of false positives. Many clones isolated in a library screen will interact with a variety of control baits, suggesting that they are simply artifacts of the screening process and do not represent biologically relevant partners to the particular bait used in the screen. To help researchers better recognize these false positives, Dr. Erica Golemis and Dr. Ilya Serebriiskii of the Fox Chase Cancer Center did a survey of 100 yeast two-hybrid library screens that were performed and what results were obtained. (The results listed here are found on the Web at http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html and have been reprinted here with the consent of Dr. Golemis.) Of the 100 screens done, 54 appeared to produce biologically relevant interactors, 30 were still in progress, 13 gave no real interactors, and three were aborted due to transactivation of reporter gene expression by the bait or because of instability in the bait fusion protein. Excluding the 30 screens that were still in progress, the results show that 77% of the screens produced real

interactors, 19% produced no real interactors, and 4% were aborted due to the reasons mentioned above. Even for the 77% of the screens that produced real interactors, false positives were still a problem. More than 90% of the screens that produced real interactors also gave false positives. The most common types of false positives obtained were heat shock proteins, ribosomal proteins, ferritin, and ubiquitin. Other false positives reported were cytochrome oxidase, mitochondrial proteins, proteasome subunits, tRNA synthase, collagen-related proteins, zinc finger proteins, vimentin, inorganic pyrophosphatase, PCNA, lamins, elongation factors, and cytoskeletal proteins. Of course, some baits will be involved in real, biologically relevant interactions with one or more of the above listed proteins, so use the list as a guide, not a rule. Remember — even clones that seem to be real interactors should be considered to be putative interactors until a biological relevance for the interaction can be established.