User Manual

Matchmaker™ Random Peptide Library User Manual

Clontech

See List of Components for storage conditions

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Cat. No.: 638853
PT3039-1 (PR19344)
Published 05 October 2001

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I. Introduction

Table I provides a list of abbreviations used throughout this User Manual.

TABLE I. LIST OF ABBREVIATIONS

AD	Activation domain
DNA-BD	DNA-binding domain
AD vector	Vector encoding the GAL4 AD
DNA-BD vector	Vector encoding the GAL4 DNA-BD
AD/library peptide	A hybrid protein comprised of the GAL4 AD fused to a peptide encoded by a synthetic $(NNK)_{16}$ oligonucleotide
AD/library plasmid	A hybrid plasmid encoding a fusion of the GAL4 AD and a peptide library insert
DNA-BD/target protein	A hybrid protein (the "bait") comprised of the GAL4 DNA-BD fused with your target protein
DNA-BD/target plasmid	The hybrid plasmid encoding the "bait" (i.e., the DNA-BD/target protein)
Yeast Phenotypes:	
Trp^-	Requires tryptophan (Trp) in the medium to grow, i.e., is a Trp auxotroph
Leu^-	Requires leucine (Leu) in the medium to grow, i.e., is a Leu auxotroph
His^-	Requires histidine (His) in the medium to grow, i.e., is a His auxotroph
$LacZ^+$	Expresses the lacZ reporter gene, i.e., is positive for β-galactosidase activity
His^+	Expresses the His3 reporter gene, i.e., does not require His in the medium to grow
Yeast Growth (Selection) Media:	
SD	Synthetic, minimal medium
SD/–Trp	SD medium lacking Trp
SD/–Leu	SD medium lacking Leu
SD/–Trp/–Leu	SD medium lacking Trp and Leu
SD/–Trp/–Leu/–His	SD medium lacking Trp, Leu, and His

I. Introduction *continued*

A system for screening a GAL4 activation domain library to identify peptides that interact with a target protein

The Matchmaker™ Random Peptide Library contains 10^7 independent random peptide clones to identify novel peptide-protein interactions in a two-hybrid screening in yeast (Yang *et al.*, 1995). Each clone expresses a different 16-residue random peptide fused to the activation domain (AD) of the yeast GAL4 transcriptional activator. The combinatorial random peptides are encoded by synthetic, random oligonucleotides having an open reading frame for 16 amino acids followed by an in-frame stop codon. The oligonucleotides are flanked by BamH I and EcoR I recognition sites and are directionally cloned in pGAD GH (Hannon *et al.*, 1993; see Appendix A). The stop codon ensures that, in the case of concatemers, only the first oligonucleotide sequence will be expressed as a GAL4 AD/peptide fusion. Note that the random peptides in this library are not dependent on species-, tissue-, or developmental stage-specific expression.

This Matchmaker Random Peptide Library can be used to identify novel peptide-protein interactions in a simple two-hybrid screening in yeast, such as that described by Stanley Fields and coworkers (Yang *et al.*, 1995). This *in vivo* screening assay is highly sensitive (Li & Fields, 1993; van Aelst *et al.*, 1993) and has been shown to correlate in most cases with the level of binding affinity determined using biochemical methods (Yang *et al.*, 1995; Estojak *et al.*, 1995). Thus, once an interacting peptide has been identified, additional two-hybrid tests may be performed to pinpoint single amino acid residues critical for the interaction and to evaluate peptide variants for the relative strength of their interaction. Potential applications of this fusion library include the identification of peptides that discriminate between wild-type and mutant forms of a target protein, and of peptides that can inhibit specific protein-protein interactions. These applications may facilitate the search for drugs or other ligands that have affinity for selected proteins.

The Matchmaker Random Peptide Library is designed for use with the Matchmaker Two-Hybrid System 3 (Cat No. 630303) or any other GAL4-based two-hybrid system that uses LEU2 to select for the AD vector. This library is compatible with the Hollenberg system (Hollenberg *et al.*, 1995; Vojtek *et al.*, 1993), **but** you must provide the appropriate DNA-BD vector and yeast reporter strain.

Experimental overview

In the Matchmaker Two-Hybrid System, the target gene is cloned into a DNA-BD vector such that a fusion between the target protein and the GAL4 DNA-BD is generated. The Matchmaker Random Peptide Library is constructed in an AD vector to generate fusions between peptides and the GAL4 AD. The two types of hybrid plasmids are then cotransformed into one of the yeast host strains provided. Both fusion proteins are targeted to the yeast

I. Introduction *continued*

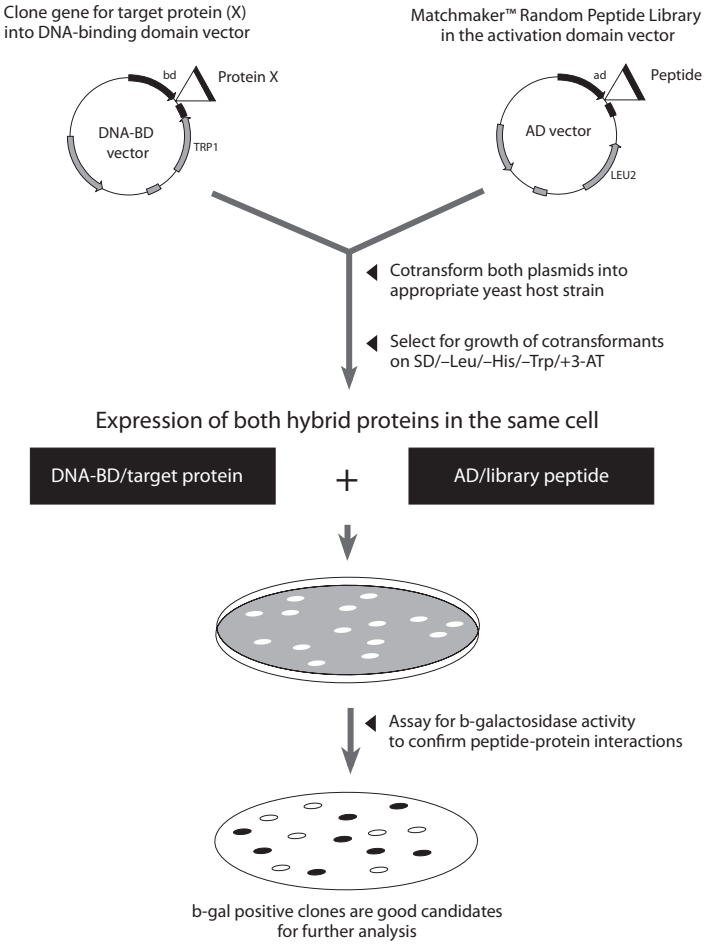

Figure 1. Screening the Matchmaker™ Random Peptide Library for sequences that interact with a target protein.

nucleus (Silver, 1984). Cotransformants are plated on SD/–His/–Leu/–Trp to select colonies in which a peptide interacts with a target protein. Thus, if the target protein interacts with a library peptide, then a functional GAL4 activator is reconstituted, and the expression of the HIS3 reporter gene is activated. To confirm the peptide-protein interaction, primary His^+ transformants are tested for expression of the second reporter gene lacZ using a β-galactosidase assay. All positives should then be rigorously tested to eliminate false positives.

I. Introduction *continued*

Before You Begin

For a successful two-hybrid assay, you must demonstrate that the target protein **does not autonomously activate the reporter genes** when fused to the GAL4 DNA-BD, for example, due to the presence of cryptic transcriptional activation sequences. If autonomous activation is observed, adding 3-aminotriazole (3-AT) to the growth medium may sufficiently suppress the background signal so that the target can be used. Alternatively, it may be possible to delete the activating regions from the target molecule before using it in a two-hybrid screening.

You should also demonstrate that when your target protein is expressed it is not toxic to yeast cells. If expression of your target protein does have toxic effects and if you have used a high-level expression vector (such as pAS2-1), you may wish to use a vector (such as pGBT9) that has a lower level of expression (Table II). To test this, perform a simple comparison of the growth curves of cells transformed with the DNA-BD vector alone and cells transformed with your DNA-BD/target plasmid. Other than possible toxicity or autonomous activation information, there is no evidence to suggest that certain types of proteins make particularly good or poor two-hybrid targets, except that certain proteins containing transmembrane regions work only after the transmembrane region is removed.

Before embarking on a two-hybrid experiment, one should be aware of the occurrence of false positives, and the potential for false negative results (Sections X.E & XI). Bona fide interactions may be missed if the hybrid proteins (1) are not stably expressed in the yeast nucleus; (2) are not localized to the yeast nucleus; (3) are not expressed at a high enough level; or (4) the GAL4 domains interfere with the ability of the test peptides and proteins to interact (van Aelst *et al.*,1993). Another potential cause of false negatives, at least in the case of some mammalian proteins, is that the host organism (yeast) may not provide the proper post-translational modifications required for native folding or interactions.

TABLE II. COMPARISON OF MATCHMAKER™ DNA-BD VECTORS

DNA-BD vectors	Description	Size	Unique Cloning Sites	Protein Expression
pGBT9	GAL4 $_{(1–147)}$ DNA-BD, TRP1, amp^r	5.4 kb	*Bam*H I, *Eco*R I, *Pst* I, Sal I, *Sma* I	Low
pAS2-1	GAL4 $_{(1–147)}$ DNA-BD, TRP1, amp^r, CYH^{s}2	8.4 kb	*Bam*H I, *Eco*R I, *Nco* I, *Nde* I, *Pst* I, *Sal* I, *Sfi* I, *Sma* I, *Xma* I	High

I. Introduction *continued*

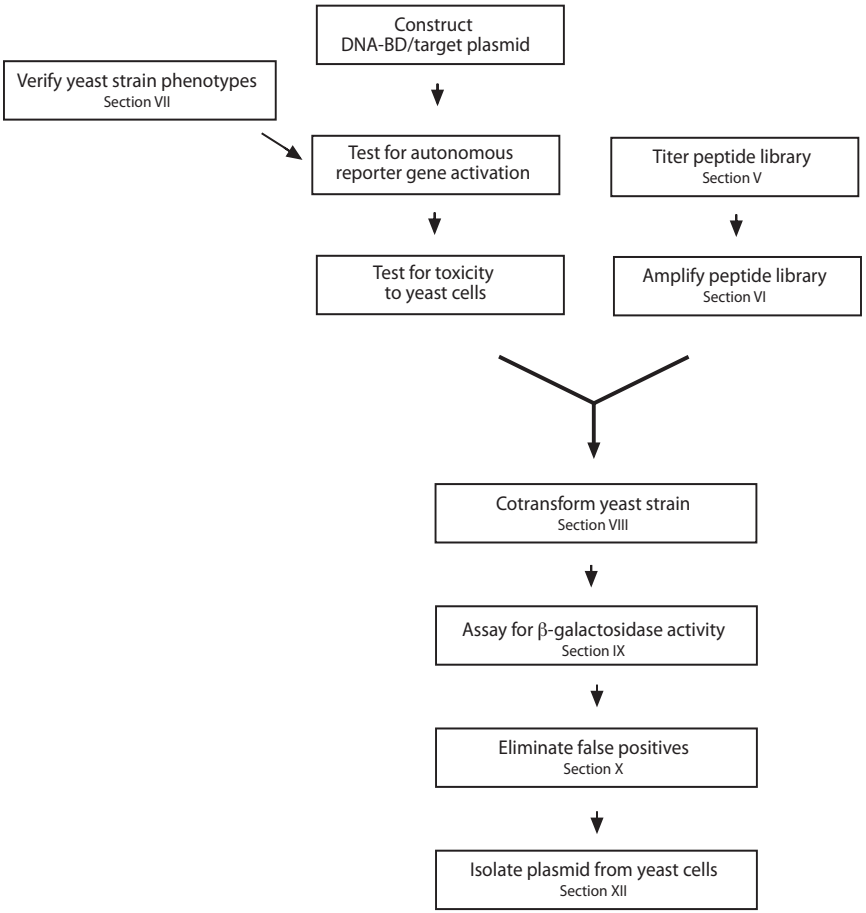

Figure 2. Guide to the Matchmaker™ Random Peptide Library protocols.

II. List of Components

Note: Store all components at –70°C.

- 2 x 1.0 ml **Plasmid Library Culture**

  Supplied as 1.0-ml liquid bacterial cultures in E. coli DH10B (in LB broth + 25% glycerol).

  Upon receipt, aliquot into working portions (~100 µl). Store at –70°C. Avoid multiple freeze/thaw cycles.

Control Plasmids

- 20 µl **pGBT9-Rb** (0.1 µg/µl)

  7.3-kb positive control DNA-binding domain hybrid; encodes the DNA-BD/retinoblastoma protein in pGBT9.

- 20 µl **pGAD GH-P1** (0.1 µg/µl)

  7.9-kb positive control activation-domain hybrid; encodes an AD/P1 peptide protein in pGAD GH, which interacts with the retinoblastoma protein.

Yeast Strains

- 0.5 ml Saccharomyces cerevisiae **Y190** reporter host strain; used when screening fusion libraries for proteins that interact with a target protein. (Genotype in Table III; Section VII.) Supplied as saturated culture in YPD medium/25% glycerol.
- 0.5 ml Saccharomyces cerevisiae **CG-1945** reporter host strain; used when screening fusion libraries for proteins that interact with a target protein. (Genotype in Table III; Section VII.) Supplied as saturated culture in YPD medium/25% glycerol.

III. Additional Materials Required

The following materials are required but not supplied.

A. Bacterial Growth and Maintenance

- **LB broth**

  10 g/L Bacto-tryptone
  5 g/L Bacto-yeast extract
  5 g/L NaCl

  Adjust pH to 7.0 with 5 N NaOH. Autoclave. Store at room temperature.

- **LB/amp plates**

  Prepare LB broth, then add agar (15 g/L), autoclave, and cool to 50°C. Add ampicillin to 50 µg/ml. Pour plates and store at 4°C.

B. Yeast Growth and Maintenance

Store yeast strain stocks in 15–30% glycerol at –70°C. To recover a strain, scrape a small amount of cells from the frozen stock with a loop or wooden stick and streak them onto YPD plates. Propagate yeast strains on YPD agar plates by incubating cells at 30°C for 1–3 days until colonies appear. YPD liquid medium is used to grow yeast strains for transformation.

Note: If you cannot recover the strain by scraping the frozen stock, the cells may have settled to the bottom of the tube before the stock was frozen. If this happens, thaw the frozen culture on ice and vortex it before restreaking.

Clontech carries a full line of yeast media (YPD and SD) and Dropout Supplements:

YPD Medium	Cat No. 630409
YPD Agar Medium	Cat No. 630410
Minimal SD Base	Cat No. 630411
Minimal SD Agar Base	Cat No. 630412
–Trp DO Supplement	Cat No. 630413
–Leu DO Supplement	Cat No. 630414
–His DO Supplement	Cat No. 630415
–Ura DO Supplement	Cat No. 630416
–Leu/–Trp DO Supplement	Cat No. 630417
–Leu/–His DO Supplement	Cat No. 630418
–Leu/–Trp/–His DO Supplement	Cat No. 630419

Otherwise, use the following recipes to make your own medias.

- **YPD medium**

  20 g/L Difco peptone
  10 g/L Yeast extract
  20 g/L Agar (for plates only)

  Add H_2O to 950 ml. Adjust pH to 5.8, autoclave, and cool to ~ 55°. Add dextrose (glucose) to 2% (50 ml of a sterile, 40% stock solution per L).

III. Additional Materials Required *continued*

- **SD medium**

  Synthetic dropout (SD) is a minimal medium used in yeast transformations to select for specific phenotypes. It includes a yeast nitrogen base, a carbon source, and a stock of "dropout" solution that contains essential nutrients, such as amino acids and nucleotides. One or more essential nutrients are often omitted to select for transformants carrying the corresponding nutritional gene.

  6.7 g/L Yeast nitrogen base without amino acids

  20 g/L Agar (for solid medium only)

  Add 850 ml of H_2O. Add 100 ml of the appropriate sterile 10X dropout solution. Adjust pH to 5.8, autoclave, and cool to ~55°C. Then add dextrose (glucose) to 2% (50 ml of a sterile 40% dextrose stock solution). For 3-AT-containing medium, add 1 M 3-AT to the appropriate concentration after the medium has cooled and before pouring plates.

- **10X Dropout solution**

  10X dropout solutions contain all but one or more of the following components; which components are omitted depends on the selection medium desired. To prepare SD/–Trp/–Leu, for example, use a 10X dropout solution lacking Trp and Leu. 10X Dropout solutions can be autoclaved and stored at 4°C for up to 1 year.

L-Isoleucine	300 mg/L
L-Valine	1,500 mg/L
L-Adenine hemisulfate salt	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl monohydrate	200 mg/L
L-Leucine	1,000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2,000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L

Note: Serine, aspartic acid, and glutamic acid are left out of the solutions because they make the media too acidic. The yeast can synthesize these amino acids endogenously.

III. Additional Materials Required *continued*

- **1 M 3-amino-1,2,4-triazole** (3-AT; Sigma Cat No. A-8056), dissolve 3-AT in H_2O and filter-sterilize.
  Notes:
  - 3-AT, a competitive inhibitor of the yeast HIS3 protein (His3p), is used to inhibit low levels of His3p expressed in a leaky manner in some reporter strains (Fields, 1993; Durfee *et al.*, 1993).
  - 3-AT is heat-labile; therefore, allow autoclaved medium to cool to approximately 55°C before adding 3-AT. The concentration of 3-AT used in the medium depends on the yeast strain.
- **40% dextrose**, autoclaved or filter-sterilized (avoid prolonged or repeated autoclaving)

C. Yeast Transformation

Purchase these yeast transformation reagents from Clontech (Yeastmaker™ Yeast Transformation System 2, Cat No. 630439), or prepare them as directed.

- **10 mg/ml Carrier DNA**
  Sonicated, herring testes carrier DNA in solution can be purchased separately (Cat No. 630440), or can be prepared using a standard method (Sambrook *et al.*, 1989). Just prior to use, denature the carrier DNA by placing it in a boiling water bath for 20 min and immediately cooling it on ice. **Use only high-quality carrier DNA; nicked calf thymus DNA is not recommended.**
- **1X PEG/LiAc solution** (polyethylene glycol/lithium acetate) Prepare fresh just prior to use.

	Final Conc.	To prepare 10 ml of solution
PEG 4000	40%	8 ml of 50% PEG
TE buffer	1X	1 ml of 10X TE
LiAc	1X	1 ml of 10X LiAc

- **1X TE/LiAc solution**; prepare just before use from 10X solutions.
- **50% PEG 4000** (Polyethylene glycol, avg. MW=3,350; Sigma Cat No. P-3640). Filter-sterilize or autoclave. Avoid repeated autoclaving.
- **DMSO** (Dimethyl sulfoxide; Sigma Cat No. D-8779)
- **10X TE buffer**: 0.1 M Tris-HCl, 10 mM EDTA, adjust pH to 7.5, and autoclave.
- **10X LiAc**: 1 M Lithium acetate (Sigma Cat No. L-6883), adjust pH to 7.5 with dilute acetic acid, and autoclave.

D. β-galactosidase Filter Assays

- **Z buffer**

$Na_2HPO_4 \bullet 7H_2O$	16.1	g/L
$NaH_2PO_4 \bullet H_2O$	5.5	g/L
KCl	0.75	g/L
$MgSO_4 \bullet 7H_2O$	0.246	g/L

Adjust pH to 7.0 and autoclave. Prepare fresh as needed.

III. Additional Materials Required *continued*

- **X-gal stock solution**

  Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL; Cat No. 8060-1) in N,N-dimethylformamide (DMF) at 20 mg/ml. Store in the dark at –20°C.

- **Z buffer/X-gal solution** Prepare fresh as needed.

  100 ml Z buffer
  0.27 ml β-mercaptoethanol (Sigma Cat No. M-6250)
  1.67 ml X-gal stock solution

- **Whatman No. 5 or VWR grade 413 paper filters**

  75-mm diameter filters (e.g., VWR Cat No. 28310-026) for use with 100-mm diameter petri plates. 125-mm diameter filters (e.g., VWR Cat No. 28310-106) for use with 150-mm plates. (Or, special order 85-mm and 135-mm filters directly from Whatman.)

E. Liquid β-galactosidase Assays with ONPG as Substrate

- **Z buffer** (see preceding section)
- **Z buffer with β-mercaptoethanol**
  To 100 ml of Z buffer, add 0.27 ml of β-mercaptoethanol.
- **ONPG (o-nitrophenyl β-D galactopyranoside;** Sigma Cat No. N-1127)
  Prepare fresh before each use. 4 mg/ml in Z buffer, mix well.
  Note: This takes 1–2 hr to dissolve.
- **1 M Na_2CO_3**
- **Liquid nitrogen**

F. Liquid β-galactosidase Assay with CPRG as Substrate

- **CPRG (chlorophenol red-β-D-galactopyranoside;** BMC Cat No. 884 308)
- **Buffer 1**

	To prepare 100 ml	
HEPES	2.38	g
NaCl	0.9	g
L-Aspartate [hemi-Mg salt; Sigma Cat No. A-9506]	0.065	g
BSA	1.0	g
Tween-20	50.0	µl

  Dissolve the above components in 75 ml of ddH_2O. Adjust pH to between 7.25 and 7.30, and then bring volume to 100 ml. Filter-sterilize. Store at 4°C for up to 3 months.

- **Buffer 2** (20 ml)

  Dissolve 27.1 mg of CPRG in 20 ml of Buffer 1 (final concentration of CPRG is 2.23 mM). Filter-sterilize. Store at 4°C in the dark for up to 3 months.

- **3 mM $ZnCl_2$** Filter-sterilize to preserve for ~3 months.
- **Liquid nitrogen**

III. Additional Materials Required *continued*

G. Preparation of Plasmid from Yeast

- **Yeast lysis solution**

2	%	Triton X-100
1	%	SDS
100	mM	NaCl
10	mM	Tris [pH 8.0]
1.0	mM	EDTA

- **Phenol: chloroform: isoamyl alcohol (25:24:1)**

  Prepare with neutralized (pH 7.0) phenol; see Sambrook *et al.* (1989) for information on equilibrating phenol.
- **Acid-washed glass beads** (425–600 µm; Sigma Cat No. G-8772)
- **95%** and **70% EtOH**
- **3 M NaOAc**

H. Transformation of E. coli with a LEU2-based Recombinant Plasmid (e.g., pGAD GH or its derivatives)

- **LB/amp plates** (see Section III.A)
- **LB broth** (see Section III.A)
- **M9 minimal medium** (Sambrook *et al.*, 1989)
- **leuB E. coli host strain** (e.g., HB101)

  HB101 has a defect in the leuB gene, which can be complemented by LEU2 from yeast. Thus, HB101 is useful for selection of the AD/library plasmid, which carries yeast LEU2. To grow HB101 transformants on minimal medium, add 4 ml of a 10 mg/ml solution of proline, 1 ml of 50 mg/ml ampicillin, and 1 ml of 1 M thiamine-HCl to M9 medium after autoclaving and cooling to 50°C. For plates, add 20 g/L agar before autoclaving.
- **50 mg/ml Ampicillin**, store at 4°C no longer than 1 month.
- **10 mg/ml Proline**, filter-sterilize
- **1 M Thiamine-HCl**, filter-sterilize

IV. Library Information

A. Library Preparation

Synthetic $(NNK)_{16}$ oligonucleotides having an open reading frame of 16 amino acids followed by a stop codon were directionally inserted into the BamH I and EcoR I sites of pGAD GH (Yang *et al.*, 1995). (N is an equal mixture of all four nucleotides, and K is an equal mixture of G and T.) The stop codon is in frame, and ensures that only one oligonucleotide sequence will be expressed as a GAL4 AD/peptide fusion. The library was reamplified by Clontech.

B. Number of Independent Clones

This is defined as the number of independent colonies estimated before amplification. The Matchmaker Random Peptide Library contains 10^7 independent clones.

C. Quality Control

The following quality control is performed with each Matchmaker Random Peptide Library.

1. Titer of the peptide plasmid library is confirmed to be >1 x 10^8 cfu/ml.
2. 50 clones were selected at random and their inserts sequenced using an automated sequencer. Sequencing showed that there was no bias towards one particular random sequence (i.e., all 50 clones contained a different sequence).
3. Six of 10 clones contain >1 $(NNK)_{16}$. All 10 clones confirmed the presence of the stop codon after 16 amino acids. The stop codon ensures that concatemers will not be expressed.
4. The control plasmids, pGBT9-Rb and pGAD GH-P1, were cotransformed into the yeast strain Y190 and tested for peptide-protein interaction. Following a colony lift β-galactosidase filter assay, colonies turned blue.

V. Library Titering

A. Library Titering Precautions

The titer, or number of colony- or plaque-forming units (cfu or pfu) per ml of culture, was determined when the library was constructed. Titers are usually stable at –70°C for at least one year.

- Diluted libraries are always less stable than undiluted libraries.
- Once 10^{-3} and 10^{-6} dilutions of the library are made, use them within the next hour, before drastic reductions in titer can occur.
- A 2–5-fold range in titer calculations is reasonable, especially if more than one person is doing the titering.
- Always use the recommended concentration of antibiotic in the medium to ensure plasmid stability.
- Use proper sterile technique when aliquoting and handling libraries.
- Design appropriate controls and include them during peptide plasmid library growth to test for cross-contamination.

B. Plasmid Library Titering Protocol

1. Prewarm LB/amp plates at 37°C (or 30°C) for 1–2 hr.
2. Thaw an aliquot of the Library and place on ice.
3. Remove 1 µl of the Library, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution A (1:10^3).
4. Remove 1 µl from Dilution A, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution B (1:10^6).
   Note: The diluted Library is unstable and should be plated within 1 hr.
5. Add 1 µl from Dilution A to 50 µl of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto a prewarmed LB agar plate.
6. Remove 50-µl and 100-µl aliquots from Dilution B and spread onto separate LB/amp plates.
7. Leave plates at room temperature for 15–20 min to allow the inoculum to soak into the agar.
8. Invert the plates and incubate at 37°C (or 30°C) overnight.
9. Count the number of colonies to determine the titer (cfu/ml). Calculate the titer according to the following formulas:
   - colony # Dilution A x 10^3 x 10^3 = cfu/ml
   - (colony # Dilution B/plating volume) x 10^3 x 10^3 x 10^3 = cfu/ml

VI. Amplification of Plasmid Libraries

You must amplify the premade Matchmaker Random Peptide Library to obtain enough plasmid for yeast transformations.

1. Note the titer of the Peptide Library (in cfu/ml) from Section V.B.
2. The Library is provided as E. coli transformants and should be plated directly on selective medium (e.g., LB/amp plates) at a high density so that the resulting colonies are nearly confluent. Approximately 100–150 150-mm plates will be necessary.
   a. Plate enough colonies, at 20,000–50,000 colonies/150-mm plate, to obtain at least 2–3X the number of independent clones in the library.
   b. Incubate plates at 37°C overnight.
      Note: Growing the transformants on solid instead of liquid medium minimizes uneven amplification of the individual clones.
3. Scrape the colonies into 1–2 L of LB/amp broth.
4. Incubate at 37°C for 2–4 hr with shaking.
5. Proceed to your protocol for large-scale isolation of highly purified plasmid. Any standard large- or mega-scale method that yields highly purified plasmid may be used for plasmid preparation. (See Sambrook *et al.* ,1989 for CsCl gradient purification, if necessary).

VII. Yeast Cell Stock Maintenance and Phenotype Verification

A. Yeast Reporter Strains

For additional information on yeast, we recommend Guthrie and Fink (1991) Guide to Yeast Genetics and Molecular Biology (Cat No. V2010-1).

1. Auxotrophic phenotypes

- Both yeast strains are deficient for TRP and LEU (i.e., they are Trp^-, Leu^-) and cannot grow on minimal medium lacking those nutrients unless functional TRP1 and LEU2 genes are introduced.
- Y190 is very leaky for HIS3 expression and, in the absence of 3-AT, grows normally on SD/–His; 25 mM 3-AT is usually required in the medium to suppress background growth of Y190 and transformant strains derived from Y190. In some cases, transforming Y190 with your DNA-BD/target protein can shift the sensitivity to 3-AT; therefore, you should titrate the concentration of 3-AT with each DNA-BD/target protein construct. To titrate 3-AT, plate Y190 transformed with your DNA-BD/target protein plasmid on SD/–Trp/–His plates with 3-AT concentrations from 25 to 60 mM (i.e., 25 mM, 35 mM, 45 mM, 55 mM). Use the lowest concentration of 3-AT which, after one week, produces only small (<1 mm) colonies.
- CG-1945 is slightly leaky for HIS3 expression and, in the absence of 3-AT, grows slowly (maximum colony size ~1 mm); 5 mM 3-AT is usually sufficient to suppress background growth of CG-1945 and transformant strains derived from CG-1945. In some cases, transforming CG-1945 with your DNA-BD/target protein

TABLE III. YEAST HOST STRAIN GENOTYPES

Strain	Genotype	Reporters	Transformation Markers	Reference
CG-1945	*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538,cyh^{r}2, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3 : : GAL4$_{17\text{-}mers(x3)}$-CyC1$_{TATA}$-lacZ*	*HIS3, lacZ*	*trp1,leu2, cyh^{r}2*	Feilotter *et al.*, 1994
Y190	*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2 : : GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3 : : GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	*HIS3, lacZ*	*trp1, leu2, cyh^{r}2*	Harper *et al.*, 1993

- CG-1945 is a derivative of HF7c (Feilotter *et al.*, 1994). The *LYS2* gene is nonfunctional.
- The *GAL*1 upstream activating sequence (UAS) and the three tandem copies of the *GAL*4 17-mer consensus sequence ($GAL4_{17\text{-}mers[x3]}$) are responsive to the *GAL*4 transcriptional activator.
- The *trp*1, *leu*2, *his*3, *gal*4, and *gal*80 mutations are all deletions.

VII. Yeast Cell Stock Maintenance and Phenotype *continued*

can shift the sensitivity to 3-AT; therefore, you should titrate the concentration of 3-AT with each DNA-BD/target protein construct. To titrate 3-AT, plate CG-1945 transformed with your DNA-BD/target protein plasmid on SD/–Trp/–His plates with 3-AT concentrations from 0 to 15 mM (i.e., 0 mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM, 12.5 mM, 15 mM). Use the lowest concentration of 3-AT which, after one week, produces only small (<1 mm) colonies.

2. Colony phenotype
   - Both strains carry the *ade2-101* mutation, which confers a pink or red color that turns darker as the colony ages (on minimal media, this can take up to 8 days). Certain spontaneous mitochondrial mutations will result in smaller white colonies (Holm, 1993); avoid such colonies.
   - When transformed with pAS2-1 (or any other pAS2-derived plasmid), CG-1945 and Y190 grow more slowly and form noticeably smaller colonies (on average) than the untransformed strain.
3. Additional notes
   - For unknown reasons, strain CG-1945 often forms clumps in liquid culture. Performance will not be affected if the clumps are dispersed by vigorous vortexing prior to use of cells.
   - Because of differences in the strength of the lacZ promoter constructs, Y190 exhibits a higher level of β-galactosidase activity than CG-1945 in a positive two-hybrid assay. Therefore, liquid cultures of Y190 should be used in quantitative β-galactosidase assays.
   - Both strains can be used in a cycloheximide counterselection (see the Matchmaker Two-Hybrid System 2 User Manual).

B. Yeast Strain Maintenance

1. Yeast strains are provided as stocks in YPD medium with 25% glycerol and can be stored indefinitely at –70°C. For storage >1 year, the temperature must be maintained below –55°C.

TABLE IV. GUIDELINES FOR CHOOSING A YEAST REPORTER STRAIN

Strain	Advantages	Disadvantages
Y190	• Higher lacZ reporter expression • Cited in literature	• Leaky *HIS3* expression may result in a higher percent of false positives, and the use of 3-AT in the selection medium may obscure some weak two-hybrid interactions
CG-1945	• *HIS3* expression much less leaky than in Y190, so CG-1945 provides more sensitive *HIS3* screening	• Relatively weak *lacZ* reporter expression may result in some LacZ false negatives

VII. Yeast Cell Stock Maintenance and Phenotype *continued*

2. To recover frozen strains and prepare working stock plates:
   a. Streak a small portion of the frozen stock onto a YPD (or SD) agar plate. Transformed yeast strains are best stored on appropriate SD dropout medium to keep selective pressure on the plasmid.
   b. Incubate the plate at 30°C until yeast colonies reach ~2 mm in diameter (this takes 3–5 days).
   c. Seal plates with Parafilm and store at 4°C for up to two months. After two months, streak a fresh plate from the frozen stock.
   d. If you cannot recover the strain, some of the cells may have settled; in this case, thaw the culture on ice, vortex, and restreak. The stock tube may be refrozen.
3. To prepare stock cultures of new yeast transformants:
   a. Use a sterile inoculation loop to scrape an isolated colony from the agar plate.
   b. Thoroughly resuspend the cells in 200–500 µl of YPD medium (or the appropriate SD dropout medium). Add sterile 50% glycerol to a final concentration of 25%.
   c. Tightly close the cap. Shake the vial before freezing at –70°C.

C. Phenotype Verification

Before starting any transformations, verify the phenotypes of the yeast strains provided.

1. Make a fresh working stock plate of each strain you plan to use. Propagate additional cultures only from isolated colonies on this plate.
2. Test the strains you plan to use for the following nutritional requirements: tryptophan (Trp), leucine (Leu), histidine (His), and uracil (Ura).
   a. Using a sterile loop or toothpick, streak 3–4 colonies from the working stock onto separate, appropriately prepared SD plates.
   b. Incubate plates at 30°C for 4–6 days; yeast strains grow more slowly on SD selection medium than on YPD.
   c. Compare your results with Table V and use in transformations only if the strain's phenotype still conforms to that expected.
3. Use colonies from the verified working stock plate to inoculate liquid cultures for preparing competent cells.
4. Seal the verified working stock plate with Parafilm and store at 4°C.

VII. Yeast Cell Stock Maintenance and Phenotype *continued*

TABLE V. GROWTH OF THE YEAST REPORTER STRAINS ON VARIOUS TYPES OF MEDIA

Strain	SD/–Trp	SD/–Leu	SD/–His	SD/–Ura	YPD
CG-1945	–	–	–[a]	+	+
Y190	–	–	–[b]	+	+

[a] In the presence of 5 mM 3-AT
[b] In the presence of 25 mM 3-AT

VIII. Yeast Transformation Protocols

A. General Considerations

When screening an AD library to find new peptides that interact with a target protein, the DNA-BD/target plasmid and the AD/library plasmids can be introduced into the yeast strain CG-1945 or Y190 either simultaneously or sequentially. See Section VII for a description of the yeast reporter strains and for guidelines in choosing one.

In sequential transformation, the DNA-BD/target plasmid is introduced first (small-scale transformation); selected transformants are then grown up and transformed with the AD/library (large-scale transformation). Simultaneous cotransformation is generally preferred because it is simpler to perform than sequential transformation—and because of the risk that expression of the DNA-BD/target protein may be toxic to the cells. If the DNA-BD/target protein is toxic, clones arising from spontaneous deletions in the DNA-BD/target plasmid will have a growth advantage and will accumulate at the expense of clones containing intact plasmids. However, if there is no selective disadvantage to cells expressing the first hybrid protein, sequential transformation can be used, and may be preferred because it uses significantly less plasmid DNA than simultaneous cotransformation (Table VII).

To find the desired interacting clone in an AD library, plan to screen at least 1.5–3X the number of independent clones in the library. We provide protocols for three library screening options:

- library-scale, simultaneous cotransformation
- large-scale simultaneous cotransformations
- large-scale, sequential transformation

Tables VI and VII compare the three library screening options.

VIII. Yeast Transformation Protocols *continued*

TABLE VI. GUIDE TO YEAST TRANSFORMATION PROTOCOLS

Small-scale

- Yields 1.5 ml of competent cells for 15 small-scale transformations using 0.1 µg of each plasmid
- Use to:
  - Verify that DNA-BD/target protein does not autonomously activate reporter genes
  - Look for toxicity effects of DNA-BD/ target protein
  - Transform with DNA-BD/target plasmid as first step of sequential transformation (only if DNA-BD/target protein is not toxic)
  - Practice for large-scale protocols

Large-scale

- Yields 1.5 ml of competent cells for 1 large-scale transformation using 10–50 µg of the limiting plasmid
- Use for:
  - Sequential transformation: transform with AD/library plasmids as second step
  - Simultaneous cotransformation: smaller version of the library scale simultaneous cotransformation. Must be repeated 10 times to screen a total of 1 x 10^6 independent clones

Library-scale

- Yields 8 ml of competent cells for 1 library-scale transformation using 100–500 µg of the limiting plasmid
- Use to:
  - Screen up to 1 x 10^6 independent clones in one simultaneous-cotransformation (Takes less time to screen an equal number of clones than performing several large-scale simultaneous cotransformations)

TABLE VII. COMPARISON OF LIBRARY TRANSFORMATION METHODS

Transformation Type	Amount of Limiting Plasmid	Trans-formation Efficiency	No. of Indep. Clones Screened*	No. of Plates (150-mm)
Library-scale Simultaneous	100–500 µg	10^3–10^4	1 x 10^6	50
Large-scale Simultaneous	10–50 µg	10^3–10^4	1 x 10^5	5
Large-scale Sequential	10–50 µg	10^3–10^4	1 x 10^6	50

***** Total approximate number of transformants expected on SD/–Leu/–Trp selection plates assuming, in each case: 1) the minimal amount of plasmid was used; 2) the transformed cells were resuspended in the volumes recommended in the protocol; 3) 200 µl of transformed cells were spread on each plate; and 4) the transformation efficiencies were optimal.

VIII. Yeast Transformation Protocols *continued*

B. Recommended Experiments

TABLE VIII: RECOMMENDED SET-UP FOR LIBRARY SCREENING

Trans-formation[a]	Plasmid(s)	Plate on SD Minimal Medium	LacZ Phenotype
Control 1	pGBT9-Rb	–Trp[b]	White
Control 2	pGBT9-Rb + pGAD GH-P1	–Leu/–Trp[c]	Blue
		–Leu/–Trp/–His/+3-AT[c]	Blue
Experimental	DNA-BD/ + AD/peptide target protein library	–Leu[d]	—
		–Trp[d]	—
		–Leu/–Trp[d]	—
		–Leu/–Trp/–His/+3-AT	(Blue)[e]

[a] For simultaneous cotransformation, all transformations are performed in CG-1945 or Y190; for the second transformation in the sequential procedure, use CG-1945 or Y190 for the controls and CG-1945 or Y190 previously transformed with your DNA-BD/target plasmid for the library transformation.
[b] Control for the individual plasmid selection marker and media
[c] Controls for interaction of two hybrid proteins and for activation of both reporter genes
[d] Control for transformation efficiency
[e] True interacting positives should turn blue on a β-galactosidase colony-lift assay. Many of the His^+ positives will not turn blue; these might be false positives.

C. Preparation of Yeast Competent Cells

The LiAc method for preparing yeast competent cells was developed by Ito *et al.* (1983) and modified by Schiestl & Gietz (1989), Hill *et al.* (1991), and Gietz *et al.* (1992).

- Prepare the appropriate selection media, and pour the required number of agar plates in advance.
- Inoculate liquid culture with a fresh (1–3-week-old) colony, 2–3 mm in diameter. If the colonies on the stock plate are smaller than 2–3 mm, then scrape several colonies into the medium. (Colonies should not be >4 weeks old.)
- If the overnight or 3-hr cultures are visibly clumped, disperse the clumps with vigorous vortexing before using them in the next step.
- When collecting cells by centrifugation, a swinging bucket rotor results in better recovery of the cell pellet.
- **For best results when screening a library, use competent cells immediately**. However, for small-scale (routine) transformations, the competent cells can be stored at room temperature for several hours without a significant reduction in competency.

VIII. Yeast Transformation Protocols *continued*

	Transformation Scale		
	Small	Large	Library
1. Inoculate several colonies, 2–3 mm in diameter, into 0.5 ml of YPD*.			
2. Vortex vigorously to disperse any clumps.			
3. Transfer this into a flask containing YPD*:	50 ml	50 ml	150 ml
4. Incubate at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD_{600}>1.5).			
5. Transfer enough overnight culture to produce an OD_{600} = 0.2–0.3 into YPD:	300 ml	300 ml	1 L
6. Incubate at 30°C for 3 hr with shaking at 230–270 rpm.			
7. Centrifuge the cells at 1,000 x g for 5 min at room temperature (20–21°C).			
8. Discard the supernatant and vortex to resuspend the cell pellet in H_2O:	25–50 ml	25–50 ml	500 ml
9. Centrifuge the cells at 1,000 x g for 5 min at room temperature.			
10. Decant the supernatant.			
11. Resuspend the cell pellet in freshly prepared, sterile 1X TE/LiAc:	1.5 ml	1.5 ml	8 ml

* Use SD/–Trp instead of YPD for overnight culture when performing the second transformation in a sequential transformation protocol.

VIII. Yeast Transformation Protocols *continued*

D. Transformation of Yeast Competent Cells

		Transformation Scale		
		Small	Large	Library
1.	Prepare PEG/LiAc solution:	10 ml	10 ml	100 ml
2.	Add the following to each tube and mix: DNA-BD vector construct[a]:	0.1 µg	20–100 µg	0.2–1.0 mg
	AD vector construct[a]:	0.1 µg	10–50 µg	0.1–0.5 mg
	herring testes carrier DNA:	0.1 mg	2 mg	20 mg
3.	Add indicated amount of yeast competent cells to each tube and mix well:	0.1 ml	1 ml	8 ml
4.	Add sterile PEG/LiAc solution: to each tube and vortex to mix	0.6 ml	6 ml	60 ml
5.	Incubate at 30°C for 30 min with shaking (200 rpm).			
6.	Add DMSO to 10%: and mix gently by inversion.	70 µl	700 µl	7.0 ml
7.	Heat shock in a 42°C water bath for 15 min. Swirl occasionally to mix (large- and library-scale only).			
8.	Chill cells on ice.			
9.	Pellet cells by centrifugation for: (swinging bucket rotor best) at:	5 sec 14 K rpm	5 min 1,000 x g	5 min 1,000 x g
10.	Remove the supernatant.			
11.	Resuspend cells in 1X TE buffer:	0.5 ml	1.0 ml or 10.0 ml[b]	10 ml

[a] A molar ratio of 2:1 (DNA-BD vector:AD vector) is recommended for optimal transformation efficiency for large- and library-scale transformations. When performing a sequential transformation experiment, you will add either the DNA-BD vector construct or the AD vector construct, but not both.

[b] 1.0 ml for simultaneous cotransformation; 10.0 ml for sequential transformation.

VIII. Yeast Transformation Protocols *continued*

E. Plating Transformation Mixtures

1. For small-scale transformations and all control transformations:
   - Plate 100 µl on each 100-mm plate.
2. For large- and library-scale transformations:
   - Spread 100 µl of a 1:1,000, 1:100, and 1:10 dilution on SD/–Trp/–Leu plates for transformation efficiency controls (100-mm plates).
   - Spread 1 µl (diluted in 100 µl of H_2O) on SD/–Trp, and SD/–Leu plates to check transformation efficiency of each plasmid (100-mm plates).
   - Spread the remaining transformation suspension on SD/–Trp/–Leu/–His/+3-AT plates (200 µl per 150-mm plate).

   Notes:
   - Use 5 mM 3-AT for CG-1945, and 25 mM 3-AT for Y190, unless your DNA-BD/target protein titered otherwise (Section VII.A).
   - If using CG-1945 and low-expression vectors such as pGBT9 and pGAD424, 3-AT should be eliminated from the selection plates.
3. Incubate plates, colony side down, at **30°C** until colonies appear.
4. If you are screening an AD/library, calculate the transformation efficiency and estimate the number of clones screened as described in Section VIII.F.
5. Chose positive AD/library clones for further analysis.

   Notes:
   - After 2–3 days, some His^+ colonies will be visible on the library screening (SD/–Trp/–Leu/–His/+3-AT) plates, but plates should be incubated for 5–10 days to allow slower growing colonies (i.e., weak positives) to appear. Ignore the small, pale colonies that may appear after 2 days but never grow to >2 mm in diameter. True His^+ colonies are robust and can grow to >2 mm in diameter.
   - Not all of the transformants surviving this selection will be true two-hybrid positives. The most common class of false positives can be eliminated by screening for expression of the second reporter gene (lacZ; Section IX). Other types of false positives can be eliminated as described in Section X).
6. Streak out His^+ colonies on fresh SD/–Trp/–Leu/–His/+3-AT master plates and grow for 2–4 days at 30°C until colonies are at least 1 mm in diameter. At this point, you can perform a β-galactosidase filter assay (Section IX.A) on the fresh colonies using a sterile filter. After lifting the colonies for the β-galactosidase assay, place the master plates at 30°C for 1–2 days to allow the colonies to regrow. Then seal the master plates with Parafilm and store at 4°C for up to 3–4 weeks.

VIII. Yeast Transformation Protocols *continued*

F. Calculate Cotransformation Efficiency and Clones Screened

1. To calculate the cotransformation efficiency, count the colonies (cfu) growing on the SD/–Leu/–Trp dilution plate that has 30-300 cfu.

   $$\frac{\text{cfu X total suspension vol. (µl)}}{\text{Vol. plated (µl) X dilution factor X amt. DNA used (µg)*}} = \text{cfu/µg DNA}$$

   * In a cotransformation, this is the amount of limiting plasmid, i.e., the lesser of the two plasmids, not the sum of them.

2. To estimate the number of clones screened:

   cfu/µg X amt. DNA used (µg) = # of clones screened

   Sample calculation:

   - 100 colonies grew on the 1:100 dilution transformation efficiency control plate (dilution factor = 0.01);
   - resuspension volume was 10 ml;
   - amount of library plasmid used = 100 µg

     $$\frac{100 \text{ cfu}}{100 \text{ µl X } 0.01} \text{ X } \frac{10 \text{ ml X } 10^3 \text{ µl/ml}}{100 \text{ µg}} = 1 \text{ X } 10^4 \text{ cfu/µg}$$

   - In this example, 1 X 10^6 clones were screened.

   Note: If, in your library transformation, you screened <10^6 clones, you may wish to repeat the transformation using more DNA. Calculate the amount of DNA to use in the repeat transformation as follows:

   $$\frac{10^6 \text{ clones}}{\text{(observed \# of clones/ amt. DNA used)}} = \text{µg DNA needed}$$

IX. β-galactosidase Assays

Use a colony lift β-galactosidase filter assay (not replica plating) for blue/white screening, and use liquid culture β-galactosidase assay, with either ONPG or CPRG as substrate, for quantitative data. The CPRG assay is more sensitive than ONPG, yet the substrate is more expensive, and the procedure may be tricky to perform. β-galactosidase units are not directly comparable for the latter two assays because of substrate differences; therefore, use the appropriate formula provided.

A. Colony Lift β-galactosidase Filter Assay

Best results will be obtained using fresh colonies 1–3 mm in diameter. To assay only a few colonies, transfer them to filters placed on selection medium. Incubate plate at 30°C for 1–2 days, and then lift out the filters and assay the colonies for β-galactosidase activity (Steps 4–6 below).

1. Prepare Z buffer/X-gal solution.
2. Presoak one sterile Whatman No. 5 or VWR grade 410 filter for each plate of transformants to be assayed in this solution as follows:
   a. Add 1.75 ml of Z buffer/X-gal solution to a clean 100-mm plate. (Use 3.5 ml for 150-mm plate.)
   b. Layer a 75-mm filter onto the liquid to soak it up.
      Notes:
      • You can use nitrocellulose filters, but they often crack when frozen.
      • For 150-mm plates, use 3.5 ml of Z buffer/X-gal solution and 125-mm filters.
3. Place a clean, dry filter over the surface of the agar plate containing transformants.
4. Poke holes through the filter into the agar in three or more asymmetric locations to orient the filter to the agar.
5. Freeze/thaw to permeabilize the cells as follows:
   a. As soon as the filter has wetted from the agar, carefully lift it off the agar plate with forceps, and transfer it with colonies facing up into a pool of liquid nitrogen.
   b. Using forceps, completely submerge the filters for 10 sec or until uniformly frozen.
      Note: Always handle liquid nitrogen wearing thick gloves and goggles.
   c. Remove filter and thaw it at room temperature.
6. Carefully place the filter, colony side up, on the presoaked filter (from step 2 above). Do not trap air bubbles under or between filters.

IX. β-galactosidase Assays *continued*

7. Incubate the filters at 30°C or room temperature and check periodically for the appearance of blue colonies.
   Notes:
   - The time it takes colonies producing β-galactosidase to turn blue varies, especially with Y190. It will typically take from 30 min to 8 hr in a library screening. Prolonged incubation (>8 hr) tends to give false positives.
   - Yeast transformed with pCL1 (the wild-type GAL4 control) will turn blue within 20–30 min. Y187 or Y190 cotransformed with pVA3-1 and pTD1-1 should give a positive blue signal within 60 min; CG-1945 cotransformed with the same controls may take an additional 30 min to develop. If the controls do not behave as expected, check the reagents and repeat the assay.
8. Identify the β-galactosidase-producing colonies by aligning the filter to the agar plate using the orienting marks.
9. Pick the corresponding positive colonies from the original plates, and transfer them to fresh medium.
   Note: If all of a colony was lifted onto the membrane, pick it from the filter, or incubate the plate for 1–2 days to regrow the colony.

B. Liquid Culture β-galactosidase Assay with ONPG as Substrate

- To reduce variability, assay five separate transformants, and with each, perform the assay in triplicate.
- Due to promoter strength differences, it may be possible to quantitate an interaction in Y190, but not in CG-1945.

1. Inoculate a yeast colony into 5 ml of SD/–His/–Leu/–Trp/+3-AT.
2. Grow overnight at 30°C with shaking (250 rpm).
3. Dissolve ONPG at 4 mg/ml in Z buffer with shaking for 1–2 hr.
4. Inoculate 2 ml of overnight culture into 8 ml of YPD liquid medium.
5. Grow culture at 30°C for 3–5 hr with shaking (250 rpm) until the OD_{600} of 1 ml = 0.5–0.8 (mid-log phase).
6. Vortex for 0.5–1 min to disperse the culture. Record exact OD_{600}.
7. Put 1.5 ml of culture into each of three 1.5-ml tubes. Centrifuge at 14,000 rpm for 30 sec.
8. Carefully remove supernatants. Wash and resuspend each pellet in 1.5 ml of Z buffer/tube.
9. Spin cells again, and resuspend in 300 µl of Z buffer, thereby concentrating cells 5X.
   Note: It may be necessary to try several dilutions of cells to remain within the linear range of the assay.
10. Pipet 100 µl of cell suspension into a fresh microcentrifuge tube.
11. Place tubes in liquid nitrogen until the cells are frozen.

IX. β-galactosidase Assays *continued*

12. Thaw in a 37°C water bath for 0.5–1 min.
13. Set up a blank tube with 100 µl of Z buffer.
14. Add 0.7 ml of Z buffer + β-mercaptoethanol to reaction and blank tubes (do not add prior to freezing).
15. Start timer. Immediately add 0.16 ml of ONPG in Z buffer to reaction and blank tubes.
16. Incubate tubes at 30°C.
17. After yellow color develops, add 0.4 ml of 1 M Na_2CO_3 to the reaction and blank tubes. The time needed will vary, weak interactions may take 24 hr.
18. Record elapsed time in min.
19. Spin reaction tubes for 10 min at 14,000 rpm to pellet cell debris.
20. Calibrate spectrophotometer against the blank at A_{420}.
21. Carefully remove supernatant and read OD_{420} of the samples relative to the blank.

    Note: The cellular debris, if disturbed, will strongly interfere with the accuracy of this test. The OD should be between 0.02–1.0.
22. Calculate β-galactosidase units, where 1 unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of ONPG to o-nitrophenol and D-galactose per min (Miller, 1972), as follows:

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

    where: t = elapsed time (in min) of incubation

    V = 0.1 ml X concentration factor

    OD_{600} = A_{600} of 1 ml of culture

    Notes:
    - X-gal is ~10^6 times more sensitive than ONPG. Transient or weak two-hybrid interactions may not be quantifiable by ONPG.
    - Cell recovery differences after the wash in Step 9 produce sample variation which you may correct by rereading the OD_{600} after resuspending them (Step 10).

C. Liquid Culture β-galactosidase Assay with CPRG as Substrate

Use CPRG and β-galactosidase units (Miller, 1972) to analyze the strength of interaction between two proteins.

1. Prepare culture as described for ONPG assay (Steps X.B.1–7).
2. Decant the media by pouring (cells will stay pelleted).
3. Resuspend cells in 1.0 ml of Buffer 1.
4. Centrifuge at 14,000 rpm for 30 sec to pellet the cells.
5. Decant the medium by pouring, and resuspend the cells in residual liquid (volume should be ~100 µl of Buffer 1).
6. Place tubes in a liquid nitrogen bath until frozen (≤1 min).

IX. β-galactosidase Assays *continued*

7. Place frozen tubes at 37°C for 1 min to thaw.
8. Repeat steps 6 and 7 once.
9. Suspend sample in 900 µl of Buffer 2 by vortexing (~95% total volume). Mixing the sample is critical to the assay.
10. Record the starting time.
11. Centrifuge at 14,000 rpm for 1 min to pellet cell debris.
12. Set up a blank containing 1 ml of Buffer 2 and 0.5 ml of 3.0 mM $ZnCl_2$.
13. Place supernatant in a cuvette and look for color change.
14. When the color is between yellow/grey and red, the absorption at 578 nm is between 0.25 and 1.8, and a linear relationship between absorption and activity per time exists; therefore, immediately add 0.5 ml of 3.0 mM $ZnCl_2$ to the sample to stop color development (final concentration of 1.0 mM).
15. Zero spectrophotometer on the blank and measure the sample absorbance at 578 nm.
16. Calculate β-galactosidase units as follows:

    Units = (1,000 X OD_{578})/ (elapsed min X 1.5 ml culture X OD_{600})

X. Elimination of False Positives

False positives are yeast transformant colonies that are His^+ and $LacZ^+$, but harbor plasmids that do not encode hybrid proteins that directly interact. Such colonies arise in the two-hybrid system for a number of reasons. (See Bartel, 1993b for more information.) Sometimes either the AD/library peptide plasmids alone will activate reporter gene transcription, or the plasmids appear to require the DNA-BD/target for their activity. You should have already tested the DNA-BD/target protein for autonomous activation function (Section I) before using it in a library screening experiment. If the DNA-BD/target protein has activation function, it should not have been used, or it should have been modified appropriately before use.

Yeast strains CG-1945 and Y190 carry two different reporter genes (HIS3 and lacZ) under the control of different promoters. This automatically eliminates many false positives, particularly those that do not bind to the DNA-BD/target protein but instead interact with promoter sequences flanking the GAL4 binding site or to proteins bound to the flanking sequences. Nevertheless, putative true positives should be tested further to determine that their activity is specific for your DNA-BD/target protein. The following procedures are designed to quickly eliminate most false positives (Bartel *et al.*, 1993a).

A. Restreak positive clones.

Several different AD/library peptide plasmids may be present in each β-galactosidase positive colony. Restreaking each colony on SD/–Trp/–Leu selection medium will, in many cases, allow multiple AD/library plasmids to segregate. Reassay completely isolated colonies to verify the $LacZ^+$ phenotype.

B. Eliminate DNA-BD/target plasmid

Generate $Leu^+ Trp^-$ yeast segregants with only the AD/library plasmid. Eliminate the DNA-BD/target plasmid, using either of the following methods:

1. Segregate plasmids in yeast by removing the Trp selection.
   a. Culture individual Leu^+, Trp^+, $LacZ^+$ transformants separately in 3 ml of SD/–Leu liquid for 1–2 days. Under these conditions, the AD/library plasmid carrying the LEU2 gene will be maintained, but the DNA-BD/target plasmid carrying TRP1 will be randomly lost from some transformants (estimated frequency ~10–20%).
   b. Dilute a sample of this culture and plate it on SD/–Leu medium.
   c. Incubate plates at 30°C for 2–3 days for colonies to appear.
   d. Using sterile toothpicks, transfer in an orderly grid, 20–30 single colonies to two SD plates, one –Leu /–Trp and the other –Leu only.
   e. Colonies that grow on +Trp but not on –Trp medium are called Trp auxotrophs. Presumably, they have lost the DNA-BD/target plasmid with TRP1, yet still have the AD/library plasmid with LEU2.

X. Elimination of False Positives *continued*

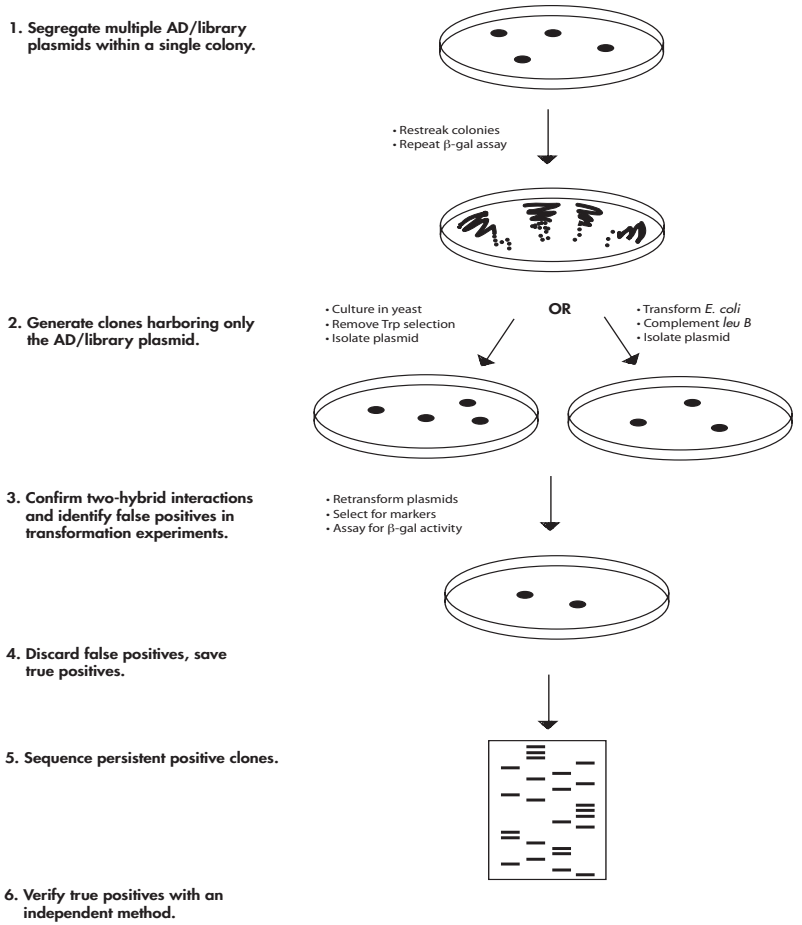

Figure 3. Procedures for eliminating false positives.

f. Assay the Trp auxotrophs for lacZ function. Save colonies that are **negative** for additional tests and discard **positive** colonies which most likely contain an AD/library plasmid encoding a transcriptional activator that recognizes the *GAL1* promoter.

g. Isolate plasmid DNA from Trp auxotrophs that are negative for β-galactosidase. These AD/library plasmids are candidates for encoding a genuine interacting protein.

2. Alternatively, isolate plasmids, transform them into *E. coli*, and select for the AD/library plasmid using its LEU2 to complement an *E. coli* leuB mutation (e.g., in HB101).

X. Elimination of False Positives *continued*

For transformation of E. coli with plasmids isolated from yeast and consequently likely to be contaminated with yeast genomic DNA, use electroporation for a high transformation efficiency (Sambrook *et al.*, 1989; and Dower, 1988). See Kaiser & Auer (1993) for an alternative, rapid method for the transfer of a shuttle plasmid from yeast to *E. coli.*

a. Culture 3–4 individual His^+, $LacZ^+$ transformant yeast colonies each in 5 ml of SD/–Leu liquid medium for 1 day only.
b. Isolate plasmid from these liquid yeast cultures (Section XII).
c. Dissolve the DNA in 10–20 µl of TE buffer.
d. Prepare electrocompetent leuB E. coli cells (i.e., HB101).
e. Add 1 µl of the plasmid to 40 µl of the leuB E. coli cells on ice.
f. Perform the electroporation.
g. Transfer cells to a sterile 1.5-ml microcentrifuge tube containing 1 ml of LB medium without antibiotics.
h. Incubate them at 37°C for 60 min with shaking at 250 rpm.
i. Centrifuge to pellet the cells at 2,500 rpm for 5 min.
j. Wash cells twice with M9 minimal medium.
k. Plate cells on M9 medium containing 50 µg/ml ampicillin, 40 µg/ml proline, and 1 mM thiamine-HCl.
Note: For better recovery of transformants, supplement medium with 10X dropout solution (–Leu) to 1X.
l. Isolate plasmids from Leu^+, Amp^r leuB E. coli transformants using a standard mini-prep procedure (Sambrook *et al.*, 1989).
Note: Do not use boiling lysis with *E. coli* HB101.
m. Verify plasmid genotype by restriction enzyme digestion.

C. Perform transformation experiments to eliminate false positives from these candidates.

1. Retransform the plasmid encoding the candidate interacting AD/library plasmid into yeast host strain (Y190 or CG-1945), in the combinations, and using the selection medium, shown in Table IX.
2. Assay transformants for β-galactosidase activity.
3. Refer to Table IX for the results expected when lacZ expression is truly dependent on interaction of the two hybrid proteins.
4. Discard false positives, and save true positives.

TABLE IX. TRANSFORMATION EXPERIMENTS TO ELIMINATE FALSE POSITIVES

Expt.	Purpose	Plasmid 1	Plasmid 2	SD Selection Medium	Expected Result
1	Confirm phenotype	none	AD/library	–Leu	—

X. Elimination of False Positives *continued*

2	Look for autonomous lacZ activation	DNA-BD/ no insert	AD/library	–Trp/–Leu/–His (+3-AT)	— [a]
3	Confirm specific interaction	DNA-BD/ target	AD/library	–Trp/–Leu/–His (+3-AT)	+ [b]
4	Identify artifactual interactions	DNA-BD/ control[d]	AD/library	–Trp/–Leu/–His (+3-AT)	— [c]

[a] False positives may result from a chance interaction between the candidate library protein and the GAL4 DNA-BD which activates lacZ expression.

[b] β-gal negatives could be due to the segregation of multiple AD/library plasmids present in one original colony. Subsequent plasmid segregation may result in both β-gal negative and positive phenotypes among the isolated colonies.

[c] False positives may be revealed when, for unknown reasons, the presence of a DNA-BD/ control protein activates the reporter gene. This could happen if the AD/library plasmid encodes a protein that does not bind to the DNA-BD/target protein but interacts with promoter sequences flanking the GAL4 binding site,or to proteins bound to the flanking sequence.

[d] Use a GAL4 DNA-BD/"any unrelated protein" fusion plasmid.

D. Sequence positive clones.

Unlike other Matchmaker libraries, the small insert sizes in the Matchmaker Random Peptide Library precludes rapid indentification of inserts by restriction enzyme digestion of PCR-amplified inserts. Therefore, to further characterize the random peptide inserts, it is necessary to sequence the inserts. After the inserts have been sequenced, the amino acid sequence of positive clones may be analyzed and compared to find patterns of positive interacting inserts.

1. Isolate plasmids from a 5-ml culture of each His^+ $LacZ^+$ positive clone (Section XIII).
2. Dissolve the plasmid in 10–20 µl of TE buffer.
3. Transform plasmid into E. coli.
   Note: The plasmid isolated from yeast is not clean enough to be used in sequencing.
4. Innoculate a 5-ml culture of LB/amp broth and incubate at 37°C overnight with shaking.
5. Isolate the plasmid using any standard miniprep procedure (Sambrook *et al.*, 1989).
6. Sequence the random peptide inserts. (The Matchmaker AD LD-Insert Screening Amplimer Set [Cat No. 9103-1] may be used.)
   Note: It may be necessary to sequence inserts using both standard and secondary structure destorying (i.e., dITP or 7-deaza-dGTP) sequencing techniques. Also, confirm that residue 17 is a stop codon, if it is not, there may be a sequencing error.

XI. Verification of Positive Interactions

Verify a positive result obtained in the two-hybrid assay by cotransforming the two hybrid vectors into the other yeast reporter strain (Y190 or CG-1945) which was not used for screening; thus, testing for protein interaction with a lacZ gene under control of a different reporter. Therefore, any positive result observed in both strains is likely to require binding of the GAL4 DNA-BD to the GAL4-responsive elements (Bartel *et al.*, 1993b).

A. [Optional] Additional Two-Hybrid Tests to Verify Positive Interactions

1. Switch cloning vectors by moving the library insert from the AD to the DNA-BD vector and vice versa, and then repeat the two-hybrid assay (Chien *et al.*, 1991; van Aelst *et al.*, 1993).
2. Test the library and target inserts using a different two-hybrid system, such as that based on the lexA transcriptional regulator (Zervos *et al.*, 1993; Vojtek *et al.*, 1993).
3. Create a frameshift mutation upstream of the library insert in the AD plasmid by cutting at the Mlu I site, filling in the overhangs, and then religating (Bendixen *et al.*, 1994). Cotransform the mutated AD/library plasmid and the DNA-BD/target plasmid into CG-1945 (or Y190), select for cotransformants, and assay for lacZ expression. The frameshift mutation should knock out the reporter gene expression, relative to a control experiment using the unmutated AD library plasmid.

B. Other Methods to Verify Positive Interactions

True positives are those AD/library clones exhibiting reporter gene expression only when the AD/library plasmid is cotransformed, or introduced by mating, with the plasmid encoding the DNA-BD/target protein. Candidate AD/library clones should be further verified by sequencing and by independent methods.

1. An example of an independent verification method is to transfer the positive AD/library plasmids to an expression vector, express the encoded protein at high levels, and purify the protein. The protein can be fixed to a chromatographic column which is then used to fractionate a cellular extract. Bound proteins are eluted, and the presence of the target protein in this fraction can often be detected by an immunoassay. (For examples of verification methods using coimmunoprecipitation, see Durfee *et al.*, 1993; and Zhang *et al.*, 1993.)
2. If possible, identify the functional relationship between the target protein and the interacting AD/library protein (such as described in Miyata *et al.*, 1994; Takayama *et al.*, 1995; and Yang, E. *et al.*, 1995).

XII. Plasmid Isolation from Yeast Cells

This rapid procedure is based on Hoffman & Winston (1987) and Kaiser & Auer (1993). The purified plasmid is suitable for E. coli transformation or for PCR screening, but not for restriction analysis or sequencing due to yeast genomic DNA contamination. For these applications, transform E. coli and isolate plasmid using a standard mini-prep procedure (Sambrook *et al.*, 1989).

We recommend that you grow the culture in SD/–Leu/–Trp/–His. Although the cells will grow slower (than in YPD or SD/Leu), the triple dropout medium will maintain selective pressure on the positive, interacting plasmid. (Growth in SD/–Leu would maintain selection for any AD/library plasmid. However, some colonies may contain two or more different AD/library plasmids, in which case the positive interacting plasmid may be lost when the cells are grown without active selection for the positive interaction [i.e., growth on medium lacking His]. This is particularly likely if the true positive AD/library plasmid expresses a fusion protein that is mildly toxic to the host cell.)

1. For each candidate clone, inoculate 5 ml of SD/–Leu/–Trp/–His liquid medium with a single yeast transformant colony.
   Note: If you used 3-AT during your initial screening, use the same concentration of 3-AT in the medium at this step.
2. Incubate at 30°C for at least 20 hr with shaking at 250 rpm (until the culture is saturated).
3. Pellet the cells by spinning at 1,000 x g for 5 min at room temperature.
4. Decant the supernatant. Vortex to resuspend the pellet in residual liquid, and transfer to a 1.5-ml microcentrifuge tube.
5. Add 0.2 ml of yeast lysis solution.
6. Add 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads. Vortex for 2 min.
7. Spin at 14,000 rpm for 5 min at room temperature.
8. Transfer the supernatant to a clean 1.5-ml tube.
9. Add 1/10 volume of 3M NaOAc, pH 5.2, and 2.5 volumes of ethanol to precipitate the DNA.
10. Wash the pellet with 70% ethanol and dry under vacuum.
11. Resuspend the DNA pellet in 20 µl of TE buffer.

XIII. Troubleshooting Guide

A. False Positives

1. During the initial library screening, you may see background growth: small colonies in which a low level of leaky HIS3 expression in the Y190 or CG-1945 host strains permits slow growth on SD/–His medium.

   Solution: Check to make sure that you have prepared the SD/–Leu/–Trp/–His/+3-AT selection media properly. Confirm that you have added 3-AT to the selection medium to a final concentration of no more than 5 mM for CG-1945 and at least 25 mM for Y190.

2. The DNA-BD/target protein could induce reporter gene expression without an AD/library protein if the target protein has a transcriptional activation domain. This is especially likely if the target protein is a transcription factor; however, other proteins that are not normally involved in transcription are sometimes capable of activating transcription (Ruden, 1992).

   Solution: It may be possible to remove the AD of the target protein by creating specific deletions within the gene and assaying the deletion constructs for those that no longer have activation function. However, such deletions may also eliminate a potential interaction domain.

3. False positives can also occur if an AD hybrid activates transcription inappropriately. Refer to Section XI or Bartel *et al.* (1993a) for discussion and methods to eliminate these false positives.

B. False Negatives

Failure to detect interaction between two proteins that normally interact in vivo will result in false negatives.

Solution 1: If the overall transformation efficiency was low, try obtaining more transformants by a "recovery" period after the heat shock: perform the simultaneous cotransformation as described (Section VIII), but insert the following steps after Step VIII.B.11:

1. Resuspend cells in 1.0 L of YPD medium.
2. Incubate cells for at 30°C 1 hr with shaking at 230 rpm.
3. Pellet cells by centrifuging at 1,000 x g for 10 min at room temperature.
4. Continue protocol from Step 11.

Solution 2: Too much 3-AT in the initial library transformation can kill recently shocked cells; therefore, use less 3-AT initially for screening, but increase the 3-AT concentration in the master plates to regain stringency.

XIII. Troubleshooting Guide *continued*

Solution 3: If high-level expression of one or both of the hybrid proteins is toxic to the cell, transformants will not grow. Sometimes truncation of one of the proteins will alleviate the toxicity and still allow the interaction to occur. Alternatively, vectors which provide a low level of expression may be used. Please call Technical Services for advice.

Solution 4: It may help to construct hybrids containing different domains of the target protein if one of the following is true: (1) the hybrid proteins are not stably expressed in the host cell; (2) the fused GAL4 domains occlude the site of interaction; (3) the hybrid protein folds improperly; or (4) the hybrid protein cannot be localized to the yeast nucleus (e.g., if the expressed protein contains a transmembrane region; see van Aelst *et al.*, 1993 for one example.)

Solution 5: For protein interactions requiring a ligand, it may be necessary to express the ligand or add it to the media (Lee *et al.*, 1994; Lee *et al.*, 1995; Chen & Evans, 1995; Horlein *et al.*, 1995)

However, for certain protein pairs, there may be no way to detect an interaction using this system.

XIV. References

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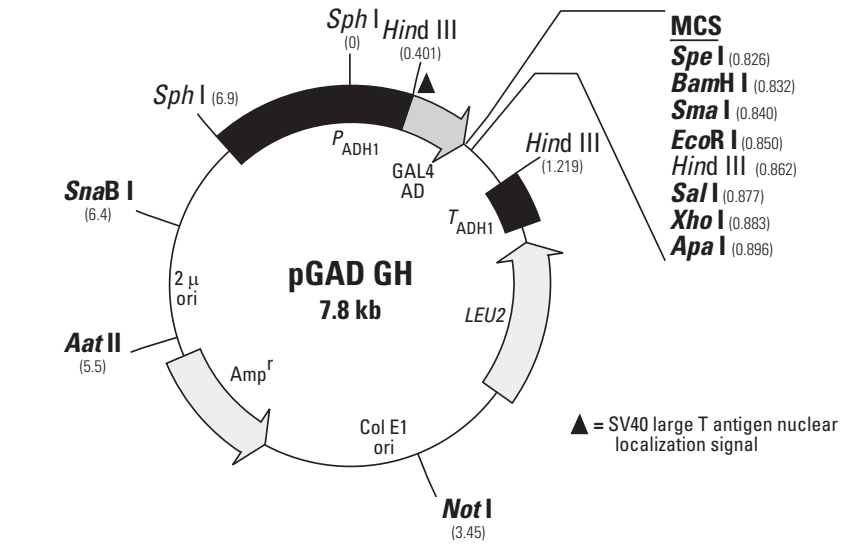
XV. Related Products

Product	Cat. No.
• Matchmaker™ Two-Hybrid System 3	630303
• Mammalian Matchmaker™ Two-Hybrid Assay Kit	630301
• Matchmaker™ Library Construction & Screening Kit	630445
• Matchmaker™ cDNA & Genomic Libraries	many
• Matchmaker™ AD LD-Insert Screening Amplimer Set	630433
• GAL4 AD Monoclonal Antibody	630402
• GAL4 DNA-BD Monoclonal Antibody	630403
• Yeast Media and Dropout Supplements	many
• Yeastmaker™ Yeast Transformation System 2	630439
• Yeastmaker™ Carrier DNA	630440

Appendix A. Vector Information



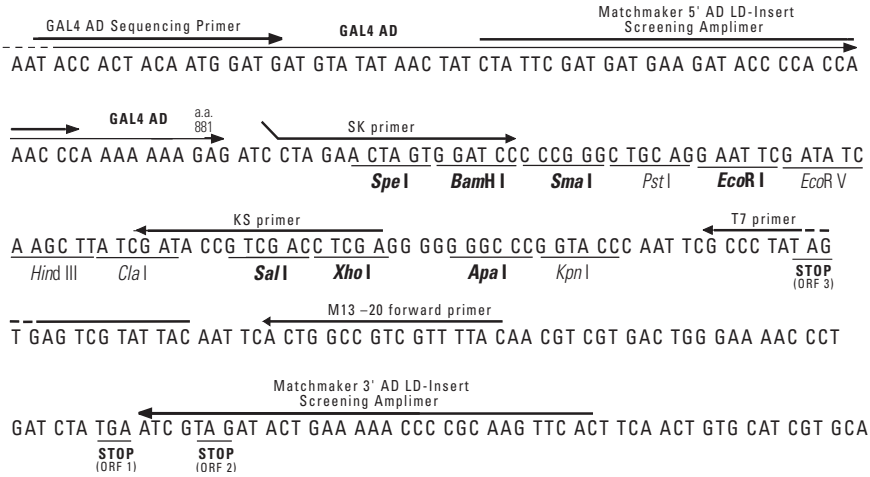


Figure 4. Map and MCS of pGAD GH Activation Domain Vector. (All unique sites are bold.)