GAL4 Two-Hybrid Phagemid Vector Kits

INSTRUCTION MANUAL

Catalog #211351 (GAL4 Two-Hybrid Phagemid Vector Kit),
#235700 (pAD-GAL4-2.1 Phagemid Vector Kit), and
#235702 (pBD-GAL4 Cam Phagemid Vector Kit)
Revision A

For In Vitro Use Only
211351-12

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GAL4 Two-Hybrid Phagemid Vector Kits

CONTENTS

Note *The complete sequences for the pAD-GAL4-2.1 phagemid vector and the pBD-GAL4 Cam phagemid vector are available for downloading to your computer. The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vector sequences are available from the GenBank® database (Accession #AF033313 and #U46126, respectively).*

GAL4 Two-Hybrid Phagemid Vector Kits

MATERIALS PROVIDED

Product	Catalog #	Materials provided
GAL4 Two-Hybrid Phagemid Vector Kit	211351	Vectors pAD-GAL4-2.1 phagemid vector pBD-GAL4 Cam phagemid vector Control plasmids pGAL4 control plasmid pBD-WT control plasmid pAD-WT control plasmid pBD-MUT control plasmid pAD-MUT control plasmid pLamin C control plasmid Bacterial host strain XL1-Blue MRF´ strain[a] Yeast host strain YRG-2 strain
pAD-GAL4-2.1 Phagemid Vector Kit	235700	Vector pAD-GAL4-2.1 phagemid vector Bacterial host strain XL1-Blue MRF´ strain Yeast host strain YRG-2 strain
pBD-GAL4 Cam Phagemid Vector Kit	235702	Vector pBD-GAL4 Cam phagemid vector Bacterial host strain XL1-Blue MRF´ strain Yeast host strain YRG-2 strain

[a] Also available separately.

STORAGE CONDITIONS

Vectors: –20°C
Control Plasmids: –20°C
Bacterial Glycerol Stock: –80°C
Yeast Glycerol Stock: –80°C

Revision A

Vectors

- The pAD-GAL4-2.1 phagemid vector undigested (20 µg)
- The pBD-GAL4 Cam phagemid vector undigested (20 µg)

Store the pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors at –20°C.

Control Plasmids

- The pGAL4, pBD-WT, pAD-WT, pBD-MUT, pAD-MUT, and pLamin C control plasmids for the detection of protein–protein interactions in yeast [20 µg each at a concentration of 1 µg/µl in TE buffer (see *Preparation of Media and Reagents*)].

Store at –20°C.

Host Strains

For host strain storage conditions, see *Preparation of Host Strains*.

Bacterial Host Strain

XL1-Blue MRF´ Strain Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]

Yeast Host Strain

YRG-2 Strain Mata *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538* LYS2::UAS$_{GAL1}$-TATA $_{GAL1}$-HIS3 URA3::UAS$_{GAL4\ 17mers(x3)}$-TATA$_{CYC1}$-*lacZ*

Note *The* LYS2 *gene in this strain is nonfunctional.*

ADDITIONAL MATERIALS REQUIRED

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (phenol, chloroform, and sodium hydroxide) or physical (high-voltage electrophoresis systems). The researcher is advised to take proper precautions and care with these hazards and to follow the safety recommendations from each respective manufacturer.

Reagents and Solutions

Phenol–chloroform [1:1 (v/v)] and chloroform

Note *Do not use the low-pH phenol from the Stratagene RNA Isolation Kit for any phenol–chloroform extractions within the yeast plasmid isolation protocol. The low-pH phenol is specific for RNA isolation and may cause the DNA to remain in the organic phase following extraction.*

Ethanol (EtOH) [70% and 100% (v/v)]
Sterile distilled water (dH_2O)
Liquid nitrogen
Acid-washed glass beads (425–600 µm)
Dimethyl sulfoxide (DMSO)

Nucleic Acids

Salmon sperm DNA

ADDITIONAL MATERIALS REQUIRED (CONTINUED)

Equipment

Microcentrifuge and microcentrifuge tubes
Wide-bore pipet tips
Incubators (30° and 37°C) and water baths (30° and 42°C)
Vacuum evaporator
Whatman® No. 1 qualitative filter paper, Grade 1 {Fisher Scientific, Pittsburgh, Pennsylvania [Catalog #09-805C (7 cm diameter) and #09-805F (12.5 cm diameter)]}
VWRbrand™ qualitative filter papers, Grade No. 413 {VWR Scientific, Westchester, Pennsylvania [Catalog #28310-026 (7.5 cm diameter)] and #28310-106 (12.5 cm diameter)]}

NOTICE TO PURCHASER

Practice of the two-hybrid system is covered by U.S. Patent Nos. 5,283,173; 5,468,614 and 5,667,973 assigned to The Research Foundation of State University of New York. Purchase of any two-hybrid reagents does not imply or convey a license to practice the two-hybrid system covered by these patents. Commercial entities in the U.S.A. practicing the above technologies must obtain a license from The Research Foundation of State University of New York. Non-profit institutions may obtain a complimentary license for research not sponsored by industry. Please contact Dr. John Roberts, Associate Director, The Research Foundation of SUNY at Stony Brook, W5530 Melville Memorial Library, Stony Brook, NY 11794-3368; phone 631 632 4163; fax 631 632 1505 for license information.

INTRODUCTION

Protein–protein interactions occur in many biological processes including replication, transcription, secretion, signal transduction, and metabolism. A fundamental question in the study of any protein is to identify proteins that interact with a given protein in vivo. Intense research efforts are focused on the identification of these proteins.

The GAL4 two-hybrid phagemid vector system* (Figure 1), a eukaryotic system to detect protein–protein interactions in vivo, provides a method for the rapid identification of genes encoding proteins that interact with a given protein (i.e., a bait protein).[1,2] The system is based on the ability to separate eukaryotic transcriptional activators into two separate domains, the DNA-binding domain (BD) and the transcriptional activation domain (AD).[3] In the GAL4 two-hybrid phagemid vector system, proteins that interact with the bait protein are identified by generating hybrids of the yeast GAL4 BD and the bait protein (*X*) and the GAL4 AD and a library of proteins (*Y*). Neither hybrid protein is capable of initiating specific transcription of reporter genes in yeast in the absence of a specific interaction with the other hybrid protein (Figure 2A). When the hybrid protein *X* is expressed in yeast, the GAL4 BD binds *X* to specific DNA sequences in the yeast chromosome defined by the GAL1 or GAL4 upstream activating sequences (UAS_{GAL1} or UAS_{GAL4}, respectively), which regulate the expression of a reporter gene. Binding of *X* to the UAS is not sufficient to initiate transcription of the reporter gene. When *Y* is expressed in yeast, the AD interacts with other components of the transcription machinery required to initiate transcription of the reporter gene. However, *Y* alone is not localized to the reporter gene UAS and therefore does not activate transcription of the reporter gene. When a specific interaction between *X* and *Y* localizes both the GAL4 BD and GAL4 AD to the reporter gene UAS, transcriptional activation of the reporter gene occurs (Figure 2B). The reporter genes in the GAL4 two-hybrid phagemid vector system are β-galactosidase (*lacZ*) and histidine (*HIS3*).

* U.S. Patent Nos. 5,283,173 and 5,468,614.

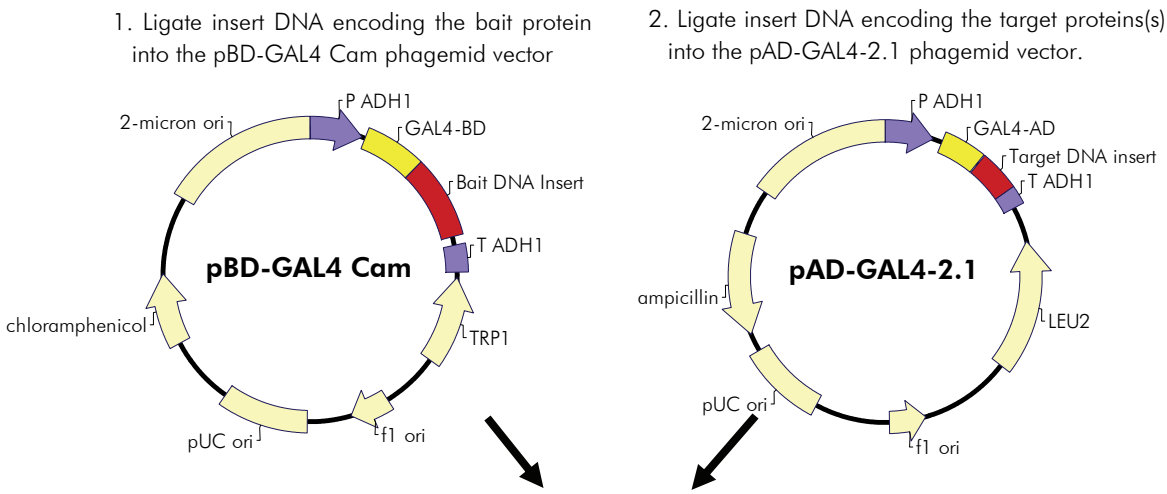


FIGURE 1 The GAL4 two-hybrid phagemid vector system. Inserts for the GAL4 AD vector may be prepared from DNA encoding a single target protein or a library of target proteins by conversion of mRNA to double-stranded cDNA or by restriction digestion of genomic DNA for expression of the GAL4 AD hybrid protein (target proteins or *Y*). DNA inserts are ligated into the pAD-GAL4-2.1 phagemid vector to generate a single clone or a primary library. The primary library is amplified to generate an amplified library. DNA encoding the bait protein is inserted into the pBD-GAL4 Cam phagemid vector for expression of the GAL4 BD hybrid protein (bait protein or *X*). The bait and target plasmids are transformed and coexpressed in the yeast host, YRG-2 strain. Colonies that contain DNA encoding target proteins, which interact with the bait protein, are identified by transcription of the *HIS3* and *lacZ* reporter genes in the yeast host strain. The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors contain the pUC origin for replication in *E. coli*, an f1 origin for production of single-stranded DNA (ssDNA) in *E. coli*, and the 2μ origin for replication in yeast. The pAD-GAL4-2.1 phagemid vector contains the ampicillin-resistance gene [β-lactamase (*bla*)] for selection with ampicillin in *E. coli*. The pBD-GAL4 Cam phagemid vector contains the chloramphenicol-resistance gene [chloramphenicol acetyltransferase] and promoter for selection with chloramphenicol in *E. coli*. For selection in yeast, the pAD-GAL4-2.1 phagemid vector contains the *LEU2* gene and the pBD-GAL4 Cam phagemid vector contains the *TRP1* gene. Hybrid proteins are expressed in yeast from the *ADH1* promoter (P *ADH1*) and terminated by the *ADH1* terminator (T *ADH1*).

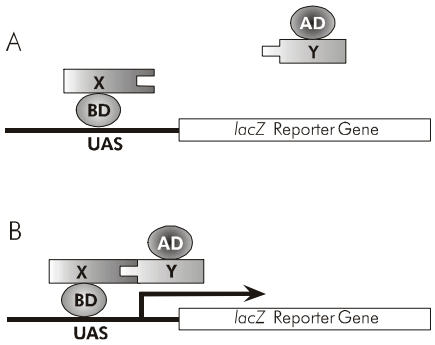


FIGURE 2 Detection of interacting proteins by transcription of the *lacZ* reporter gene. The GAL4 UAS and the *lacZ* reporter gene are integrated into the yeast chromosome. (A) The GAL4 BD hybrid protein (BD and the bait protein *X*) binds to the GAL4 UAS present upstream of the *lacZ* reporter gene. The GAL4 AD hybrid protein (AD and the target protein *Y*) binds transcription factors in the nucleus but does not localize to the GAL4 UAS. (B) If the bait (*X*) and target (*Y*) proteins interact, the GAL4 AD and the GAL4 BD are brought close to each other and act together with the bound transcription factors to initiate transcription of the *lacZ* reporter gene.

VECTORS

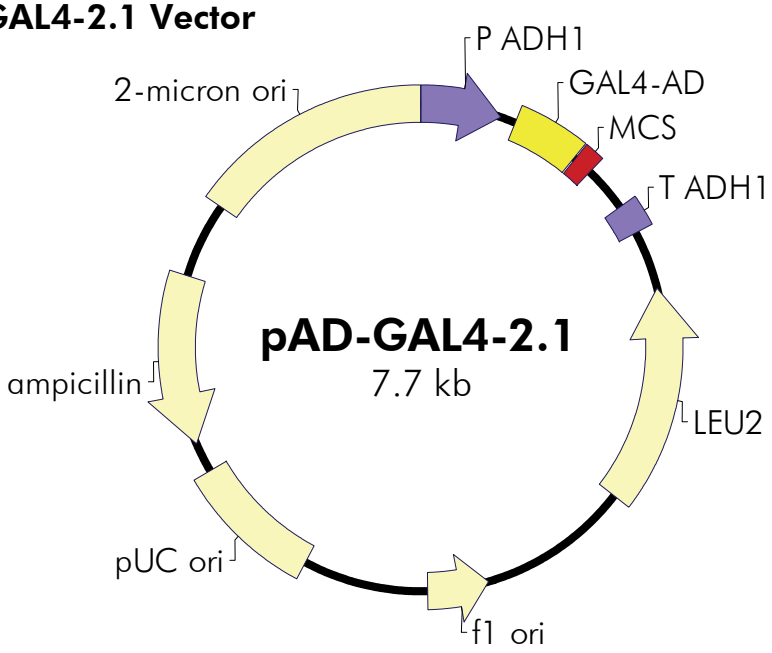
The pAD-GAL4-2.1 phagemid vector contains a multiple cloning site (MCS) with *Bam*H I, *Nhe* I, *Eco*R I, *Xho* I, *Sal* I, *Xba* I, *Pst* I, and *Bgl* II restriction sites. The pBD-GAL-4 Cam phagemid vector contains an MCS with *Eco*R I, *Srf* I, *Sma* I, *Xho* I, *Sal* I, *Xba* I, and *Pst* I restriction sites (Figures 3 and 4 and Table I). The unique *Eco*R I and *Xho* I cloning sites in the pAD-GAL4-2.1 vector make this vector compatible with the Stratagene cDNA Synthesis Kit for the preparation of unidirectional cDNA libraries. The unique *Eco*R I and *Sal* I cloning sites are used for the preparation of cDNA libraries in the pBD-GAL4 Cam phagemid vector because the *Xho* I site in the MCS is not unique. The unique *Bam*H I, *Nhe* I, and *Eco*R I sites at the 5´ end and the *Xho* I, *Sal* I, *Xba* I, and *Bgl* II sites at the 3´ end of the DNA insert facilitate the transfer of DNA encoding the target protein into commonly used protein expression/purification vectors. Genomic DNA digested with *Mbo* I, *Bam*H I, or *Sau*3A I and partially filled-in can be inserted into a partially filled-in *Xho* I site in the pAD-GAL4-2.1 phagemid vector. The *Xba* I site in the pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors is not unique and contains the UAG amber suppressor in the same translational reading frame as the GAL4 domain. DNA should therefore be inserted such that the *Xba* I site is not between the GAL4 domain and the DNA insert.

The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors contain the pUC origin for replication and an f1 origin for production of single-stranded DNA (ssDNA) in *E. coli*. Single-stranded DNA can be used for DNA sequencing or site-directed mutagenesis. The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors contain ampicillin-resistance gene [β-lactamase (*bla*)] and chloramphenicol acetyltransferase genes, respectively, for selection with ampicillin and chloramphenicol in *E. coli*. The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors contain the 2μ origin for replication. For selection in yeast, the pAD-GAL4-2.1 phagemid vector contains the *LEU2* gene and the pBD-GAL4 Cam phagemid vector contains the *TRP1* gene. The hybrid protein is expressed by the alcohol dehydrogenase 1 (*ADH1*) promoter (P *ADH1*) and is terminated by the *ADH1* terminator (T *ADH1*).

TABLE I
Unique Restriction Sites in the MCS

Restriction site in MCS	pAD-GAL4-2.1 phagemid vector	pBD-GAL4 Cam phagemid vector
*Bam*H I	Yes	No site
Nhe I	Yes	No site
*Eco*R I	Yes	Yes
Xho I	Yes	No
Sal I	Yes	Yes
Xba I	Yes	No
Pst I	Yes	Yes
Srf I	No site	Yes
Sma I	No site	No
Bgl II	Yes	No site

The pAD-GAL4-2.1 Vector



pAD-GAL4-2.1 Multiple Cloning Site Region

(sequence shown 812–958)

end of GAL4 activation domain

BamH I Nhe I EcoR I

5′ CCA AAC CCA AAA AAA GAG ATC GAA TTA GGA TCC TCT GCT AGC AGA GAA TTC AAT...

Xho I Sal I Xba I **T7 promoter**

...TCT CTA ATG CTT CTC GAG AGT ATT AGT CGA CTC TAG AGC CCT ATA GTG AGT CGT ATT...

Pst I Bgl II

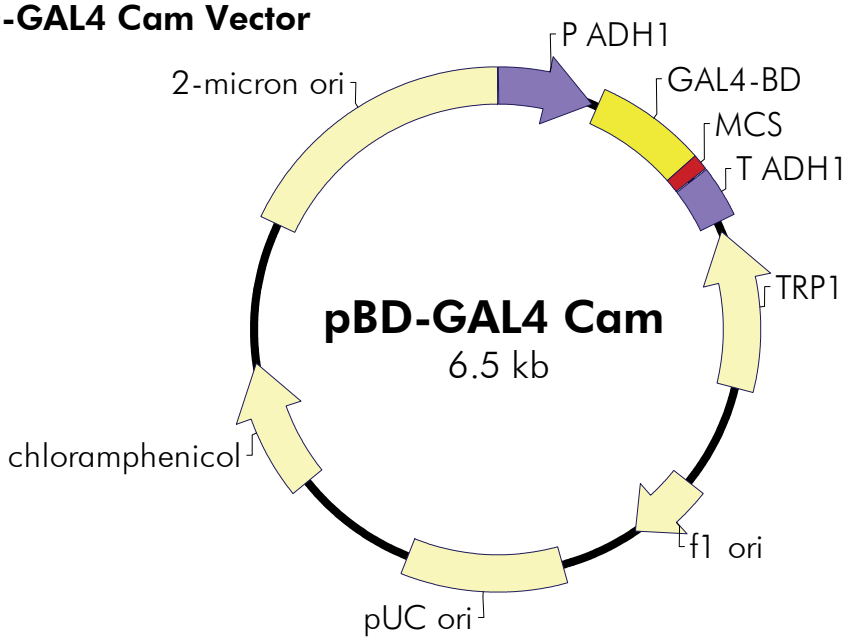
...ACT GCA GAG ATC TAT GAA TCG TAG ATA CTG AAA AAC 3′

STOP STOP STOP

Feature	Nucleotide position
yeast *ADH1* promoter	4–408
GAL4 activation domain (114 amino acids)	488–829
multiple cloning site	839–935
yeast *ADH1* terminator	1168–1318
yeast *LEU2* selection marker ORF	1615–2709
f1 origin of ss-DNA replication	3483–3789
pUC origin of replication	4427–5094
ampicillin resistance (*bla*) ORF	5245–6102
2μ yeast origin of replication	6489–7653

FIGURE 3 Circular map features of the excised pAD-GAL4-2.1 phagemid vector. The *Xba* I site contains the UAG amber suppressor in the same translational reading frame as the GAL4 domain. DNA should therefore be inserted such that the *Xba* I site is not between the GAL4 domain and the DNA insert. The complete sequence and list of restriction sites can be found at *www.stratagene.com*.

The pBD-GAL4 Cam Vector



pBD-GAL4 Cam Multiple Cloning Site Region

(sequence shown 854–992)

end of GAL4 binding domain

EcoR I Srf I Sal I

```
5´ CAA AGA CAG TTG ACT GTA TCG CCG GAA TTC GCC CGG GCC TCG AGC CCG GGT CGA...
```

T7 promoter

```
...CTC TAG AGC CCT ATA GTG AGT CGT ATT ACT GCA GCC AAG CTA ATT CCG GGC GAA...
```

```
...TTT CTT ATG ATT TAT GAT TTT TAT TAT TAA A 3´
```

STOP STOP STOP

Feature	Nucleotide position
yeast *ADH1* promoter	4–408
GAL4 DNA-binding domain (148 amino acids)	434–877
multiple cloning site	878–941
yeast *ADH1* terminator	948–1154
yeast *TRP1* selection marker ORF	1197–1871
f1 origin of ss-DNA replication	2322–2628
pUC origin of replication	2970–3637
chloramphenicol resistance ORF	4174–4725
2μ yeast origin of replication	5330–6489

FIGURE 4 Circular map and features of the pBD-GAL4 Cam phagemid vector. In the MCS of the pBD-GAL4 Cam phagemid vector, the *Eco*R I, *Srf* I, *Sal* I, and *Pst* I sites are unique; however, the *Xho* I, *Sma* I and *Xba* I sites are not. The *Xba* I site contains the UAG amber suppressor in the same translational reading frame as the GAL4 domain. DNA should therefore be inserted such that the *Xba* I site is not between the GAL4 domain and the DNA insert. The complete sequence and list of restriction sites can be found at *www.stratagene.com*.

Host Strains

The table in the appendix compares the qualities and features of *E. coli* and yeast host strains (see *Appendix: General Comparison of* Escherichia coli *versus Yeast Host Strains*).

Bacterial Host Strains

The RecA$^-$ *E. coli* host strain XL1-Blue MRF´ is supplied with the GAL4 Two-Hybrid Phagemid Vector Kits.[4] The episome is selectively maintained by the presence of the Tn*10* tetracycline-resistance gene on the F´ episome in the XL1-Blue MRF´ strain. The XL1-Blue MRF´ strain is used for amplification and excisions.

Note *The* mcrA, mcrCB, *and* mrr *mutations prevent restriction of methylated DNA, making XL1-Blue MRF´ compatible with cloning both genomic DNA and cDNA constructed using the Stratagene cDNA Synthesis Kit.*

TABLE II

Yeast Host Strain

Strain	Genotype	Reporter genes	Transformation markers
YRG-2[a]	Mat**a** *ura352 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538* LYS2::UAS_{GAL1}-$TATA_{GAL1}$-HIS3 URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-*lacZ*	*lacZ, HIS3*	*leu2, trp1*

[a] The *LYS2* gene in this strain is nonfunctional.

Yeast Host Strain

The GAL4 Two-Hybrid Phagemid Vector Kits contain the YRG-2 strain, a yeast strain with two reporter genes for the detection of in vivo protein–protein interactions (Table II). The YRG-2 strain is a derivative of the HF7c strain[5] and was selected for its ability to generate high-efficiency competent cells.[6] The YRG-2 strain carries a mutation which ensures that the endogenous *GAL4* gene is not expressed. In addition, *GAL80*, whose product inhibits function of the GAL4 gene product, is mutated. The YRG-2 strain also carries the auxotrophic markers, leucine (*leu2*) and tryptophan (*trp1*), for selection of yeast which have been transformed with the AD and BD phagemids, respectively, and the auxotrophic marker, histidine (*his3*), for selection of yeast which has been transformed with interacting proteins. For generalized protocols and techniques used to analyze the genetics and molecular biology of yeast, see Reference 7.

The YRG-2 strain contains a dual selection system with *lacZ* and *HIS3* reporter gene constructs. The promoters that govern expression of the reporter genes are optimized to reduce the number of detectable false positives; however, the expression level of the *lacZ* gene may not be suitable for quantitating an interaction. The *lacZ* reporter gene construct consists of three copies of the GAL4 17-mer consensus sequence (GAL4 DNA-binding sites) and the TATA portion of the iso-1-cytochrome *c* (CYC1) promoter (p_{CYC1}) which are fused to the *lacZ* reporter gene and regulate its expression. The *lacZ* reporter gene construct, including the *LYS2* yeast gene,* has been integrated into the nonfunctional *lys* locus. The *HIS3* reporter gene construct consists of the UAS_{GAL1} and the TATA portion of the GAL1 promoter (p_{GAL1}) which are fused to the *HIS3* reporter gene and regulate its expression. The *HIS3* reporter gene construct, including the *URA3* yeast gene, has been integrated into the nonfunctional *ura* locus. Expression of the functional *URA3* yeast gene allows the YRG-2 strain to grow in the absence of uracil. The UAS_{GAL1} contains four GAL4 DNA-binding sites. The GAL4 BD hybrid protein binds to the UAS_{GAL1} and the GAL4 17-mers present upstream of the reporter genes. If *X* and *Y* proteins interact, the AD and the BD are brought in close proximity to each other and act together to initiate transcription of the reporter genes (see Figure 2B).

* The *LYS2* gene in this strain is nonfunctional.

PREPARATION OF HOST STRAINS

Bacterial Host Strain

The bacterial host strain has been sent as a bacterial glycerol stock. For the appropriate media, please refer to the following table:

Host strain	Agar plates for bacterial streak	Medium for bacterial glycerol stock
XL1-Blue MRF′ strain	LB–tetracycline[a,b]	LB–tetracycline[a,b]

[a] 12.5 µg/ml.

[b] See *Preparation of Media and Reagents*.

On arrival, prepare the following from the bacterial glycerol stock using the appropriate media as indicated in the previous table:

Note *The host strain may thaw during shipment. The vial should be stored immediately at –20° or –80°C, but the strain remains viable longer if stored at –80°C. It is also best to avoid repeated thawing of the host strain in order to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.

2. Streak the splinters onto an LB agar plate (see *Preparation of Media and Reagents*) containing the appropriate antibiotic, if one is necessary.

3. Incubate the plate overnight at 37°C.

4. Seal the plate with Parafilm® laboratory film and store the plate at 4°C for up to 1 week.

5. Restreak the colonies onto a fresh plate every week.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of appropriate liquid medium containing antibiotic with one or two colonies from the plate. Grow the cells to late log phase (OD_{600} = 0.8–1.0).

2. Add 4.5 ml of a sterile glycerol–liquid medium solution (5 ml of glycerol + 5 ml of appropriate medium) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/ tube).

This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Yeast Host Strain

The yeast host strain has been sent as a yeast glycerol stock. For the appropriate media, please refer to the following table:

Host strain	Agar plate for yeast streak	Medium for yeast glycerol stock
YRG-2 strain	YPAD[a,b]	YPAD[a,b]

[a] See *Preparation of Media and Reagents*.

[b] Adenine sulfate is added to the medium to reduce the reversion rate of the ade2-101 mutation thereby reducing the amount of reddish pigment in the yeast colonies.

On arrival, prepare the following from the yeast glycerol stock using the appropriate media as indicated in the previous table:

Note *The yeast host strain should be stored immediately at –80°C. It is also best to avoid repeated thawing of the yeast host strain in order to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.

2. Streak the splinters onto a YPAD agar plate.

3. Incubate the plate at 30°C until colonies appear (~2–3 days).

4. Seal the plate with Parafilm laboratory film and store the plate at 4°C for up to 1 week.

5. Restreak the colonies onto a fresh plate every week.

Preparation of a –80°C Yeast Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of YPAD broth with one colony from the plate. Grow the cells to late log phase (OD_{600} = 0.8–1.0).

2. Add 4.5 ml of a sterile glycerol–liquid medium solution (5 ml of glycerol + 5 ml of appropriate medium) to the yeast culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/ tube).

This preparation may be stored at –80°C for more than 2 years.

Yeast Host Strain Phenotype

The phenotype of the yeast host strain should be verified as outlined below prior to performing the screening assays for the GAL4 two-hybrid phagemid vector system.

1. Prepare a fresh plate of the yeast host strain on a YPAD agar plate from the yeast glycerol stock as outlined in steps 1–3 of *Yeast Host Strain*. Incubate the plate at 30°C for 2–3 days.

2. Prepare SD agar plates using the appropriate 10× dropout solution (see *Synthetic Minimal Medium* in the *Two-Hybrid Vector System Media and Reagents* subsection of *Preparation of Media and Reagents*) to test the yeast host strain for the following nutritional requirements: tryptophan (Trp), leucine (Leu), histidine (His), and uracil (Ura). Streak the yeast host strain onto the agar plates with the appropriate 10× dropout solution and incubate the plates at 30°C for 2–3 days.

   The yeast host strain should grow only on the SD agar plates without Ura. The yeast host strain may grow slightly on the SD agar plates without His due to leaky expression of the *HIS3* gene. The yeast host strain should not grow on the SD agar plates without Trp or Leu. Although the p_{GAL1}, which governs expression of the *HIS3* gene, is slightly leaky, the addition of the histidine antimetabolite, 3-aminotriazole, to restore histidine auxotrophy is not necessary. 3-Aminotriazole slows the growth rate of the yeast cells and has not been shown to be effective at reducing background growth.

3. After the phenotype has been verified, use the tested colony to inoculate medium for the preparation of competent yeast cells.

CONTROL PLASMIDS

Description

The HybriZAP-2.1 two-hybrid vector system contains six control plasmids (see Table III and Figure 5). The pGAL4 control plasmid expresses the entire coding sequence of the wild-type GAL4 protein.[2] The pBD-WT control plasmid expresses the DNA-binding domain (BD) of GAL4 and amino acids (aa) 132–236 of wild-type lambda cI, fragment C, as a hybrid protein.[8,9] The pAD-WT control plasmid expresses the activation domain (AD) of GAL4 and aa 132–236 of wild-type lambda cI, fragment C, as a hybrid protein. The pAD-MUT control plasmid expresses the AD of GAL4 and aa 132–236 of E233K mutant lambda cI, fragment C, as a hybrid protein.[10,11] The pBD-MUT control plasmid expresses the BD of GAL4 and aa 132–236 of E233K mutant lambda cI, fragment C, as a hybrid protein. The lambda cI gene product (cI-wt) naturally forms homodimers. The cI-E233K mutation encodes a substitution in the gene product that interferes with the interaction between the homodimers, resulting in a weaker protein–protein interaction. The pLamin C control plasmid expresses the BD of GAL4 and aa 67–230 of human lamin C as a hybrid protein.[12]

Applications

These plasmids are used alone or in pairwise combination as positive and negative controls for the induction of the *HIS3* and *lacZ* genes (Tables IV and V). Induction of the *HIS3* gene enables the transformed host to grow on SD medium without His. Induction of the *lacZ* gene is detected by cleavage of a chromogenic substrate causing the transformed host to turn blue in color. The pGAL4 control plasmid can be used alone to verify that induction of the *lacZ* and *HIS3* genes has occurred and that the gene products are detectable in the assay used. The pLamin C control plasmid can be used in pairwise combination with the pAD-WT control plasmid and/or the pAD-MUT control plasmid to verify that the *lacZ* and *HIS*3 genes are not induced, as the proteins expressed by each of these pairs do not interact in vivo.

Two pair of control plasmids are used as positive controls to verify that induction of the *HIS3* and *lacZ* genes has occurred. The degree of color development of the transformed host depends on the strength of interaction of the expressed proteins. The pBD-WT and pAD-WT control plasmids express proteins that interact strongly (K_d = 20 nM) in vivo, and the transformed host turns blue in color. The pBD-MUT and pAD-MUT control plasmids express proteins that interact weakly (K_d = 200 nM) in vivo, and the transformed host turns light blue in color.

TABLE III

Description of the Control Plasmids

Control plasmid	Insert description[a]	Vector	Genotype	Function
pGAL4	Wild-type, full-length GAL4	pRS415	*LEU2*, Amp^r	Positive control
pBD-WT	Wild-type fragment C of lambda cI repressor (aa 132–236)	pBD-GAL4 Cam	*TRP1*, Cam^r	Interaction control
pAD-WT	Wild-type fragment C of lambda cI repressor (aa 132–236)	pAD-GAL4-2.1	*LEU2*, Amp^r	Interaction control
pBD-MUT	E233K mutant fragment of lambda cI repressor (aa 132–236)	pBD-GAL4 Cam	*TRP1*, Cam^r	Interaction control
pAD-MUT	E233K mutant fragment of lambda cI repressor (aa 132–236)	pAD-GAL4-2.1	*LEU2*, Amp^r	Interaction control
pLamin C	Human lamin C (aa 67–230)	pBD-GAL4	*TRP1*, Amp^r	Negative control

[a] *aa*, Amino acid.

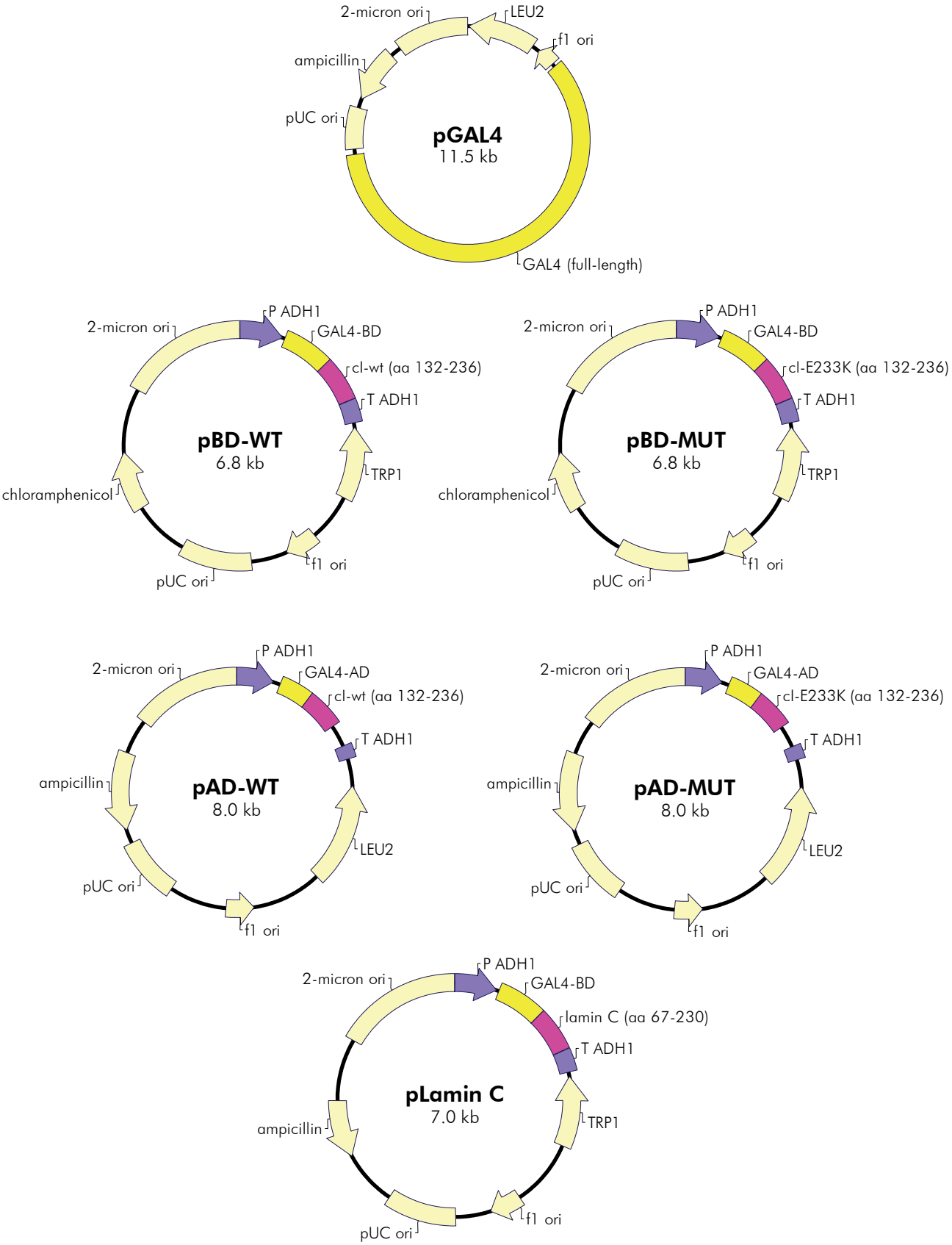


FIGURE 5 Circular maps of the control plasmids.

Expected Results

The expected results for transformation of the control plasmids alone or in pairwise combination into the YRG-2 strain when plated on selective media and assayed for expression of the *lacZ* gene are outlined in Tables IV and V.

TABLE IV

Expected Results for the pGAL4 Positive Control[a]

Control plasmid	Expected results
pGAL4	Growth, blue

[a] When transformed into YRG-2 competent cells, plated on SD agar plates without Leu and assayed for expression of the *lacZ* reporter gene.

TABLE V

Expected Results for Interaction Control Plasmids[a]

Control plasmids		Expected results			
BD fusion	**AD fusion**	**SD agar plates without Leu**	**SD agar plates without Trp**	**SD agar plates without Leu and Trp**	**SD agar plates without Leu, Trp, and His**
pBD-WT			Growth, white		
pBD-MUT			Growth, white		
	pAD-WT	Growth, white			
	pAD-MUT	Growth, white			
pLamin C			Growth, white		
pBD-WT	pAD-WT			Growth, blue	Growth, blue
pBD-MUT	pAD-MUT			Growth, light blue	Growth, light blue
pLamin C	pAD-WT			Growth, white	No growth
pLamin C	pAD-MUT			Growth, white	No growth

[a] When transformed into YRG-2 competent cells, plated on the SD media indicated, and assayed for expression of the *lacZ* reporter gene.

ACTIVATION DOMAIN AND DNA-BINDING DOMAIN VECTOR CONSTRUCTION

Background

The GAL4 two-hybrid phagemid vector system is particularly useful for the identification of protein domains or amino acids critical for the interaction between a bait and target protein. Specific mutations, insertions, or deletions that affect the encoded amino acid can be introduced into DNA encoding the target and/or bait protein, and the mutant target proteins can be assayed for the effect on the protein–protein interaction. Additionally, random mutations in the encoded target and/or bait protein can be introduced.

Note *We recommend using the HybriZAP-2.1 two-hybrid cDNA Gigapack cloning kit for the preparation of cDNA libraries and the HybriZAP-2.1 two-hybrid undigested vector kit for the preparation of genomic libraries.*

Target and Bait Protein Insert Preparation, Ligation, and Transformation

DNA that encodes the target and bait proteins is inserted into the pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors, respectively, and expressed as hybrid proteins. The hybrid proteins are then assayed for protein–protein interaction. DNA encoding the target and bait proteins may be prepared by either restriction digestion or PCR amplification and ligated into the same reading frame as the GAL4 AD of the pAD-GAL4-2.1 phagemid vector (see Figure 3) and the GAL4 BD of the pBD-GAL4 Cam phagemid vector (see Figure 4), respectively. In the MCS of the pAD-GAL4-2.1 phagemid vector, the *Eco*R I, *Srf* I, *Xho* I, *Sal* I, *Xba* I, *Pst* I, and *Bgl* II sites are unique. In the MCS of the pBD-GAL4 Cam phagemid vector, the *Eco*R I, *Srf* I, *Sma* I, *Sal* I, and *Pst* I sites are unique; **however, the *Xho* I, *Sma* I, and *Xba* I sites are not**. In addition, the *Xba* I site contains the UAG amber suppressor in the same translational reading frame as the GAL4 domain. DNA should therefore be inserted such that the *Xba* I site is not between the GAL4 domain and the DNA insert.

We suggest dephosphorylation of the digested pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors with CIAP (according to the manufacturer's instructions) prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by selective precipitation using ammonium acetate, eliminating the small fragment that appears between the two restriction enzyme sites.

After purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) such that the concentration of the vector DNA is the same as the concentration of the insert DNA (~0.1 µg/µl).

For ligation, the ideal ratio of insert-to-vector DNA is variable; however, a reasonable starting point is 1:1 (insert-to-vector molar ratio), measured in available picomole ends. This is calculated as follows:

$$\text{Picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

Suggested Ligation Reactions

Ligation Reaction Components	Experimental		Control		
	1[a]	2[a]	3[b]	4[c]	5[d]
Prepared vector (0.1 µg/µl)	1.0 µl	1.0 µl	1.0 µl	1.0 µl	0 µl
Prepared insert (0.1 µg/µl)	*X* µl	*X* µl × 2	0 µl	0 µl	1.0 µl
10 mM rATP (pH 7.0)	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
10× ligase buffer	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.5 µl	0.5 µl	0 µl	0.5 µl
Double-distilled water (to 10 µl)	*Y* µl	*Y* µl	*X* µl	*X* µl	*X* µl

[a] Experimental samples 1 and 2 vary the insert-to-vector ratio.

[b] Control sample 3 tests to ensure the effectiveness of the digestion and CIAP treatment of the vector.

[c] Control sample 4 tests to ensure the vector was cleaved completely or if residual uncut vector remains.

[d] Control sample 5 tests to ensure the insert alone is not contaminated with the vector DNA.

1. Ligate overnight at 4°C. When using blunt ends, ligate overnight at 12–14°C.
2. Transform 1–5 µl of the ligation mix into the appropriate competent bacteria. Plate on selective media. (See Table VI for expected results.)

TABLE VI

Expected Results

Sample	Amount of transformation plated	Expected colony number	Efficiency (cfu/µg of DNA)
Sample 1 (experimental)	≤200 µl	will vary[a]	will vary
Sample 2 (experimental)	≤200 µl	will vary[a]	will vary
Sample 3 (control)	≤200 µl	low number[b]	—
Sample 4 (control)	≤200 µl	no colonies[c]	—
Sample 5 (control)	≤200 µl	no colonies[d]	—

[a] These plates represent recombinants.

[b] This plate should have low numbers of colonies if the digestion and CIAP treatment were effective.

[c] This plate should have no colonies if the digest was complete.

[d] This plate should have no colonies if the insert did not contain vector DNA.

Select isolated colonies for miniprep analysis to identify transformed colonies containing the pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors with the DNA insert. The nucleotide sequence of the DNA insert should be determined to verify that the DNA insert will be expressed as a fusion protein with the GAL4 AD and the GAL4 BD and that the DNA insert does not contain mutations. The oligonucleotide primers in the table below can be used to identify recombinants and to determine the nucleotide sequence of the DNA insert.

Vector	Primer	Binds to nucleotide (nt)	Nucleotide sequence (5′ to 3′)
pAD-GAL4-2.1	5′ AD	745–765	AGGGATGTTTAATACCACTAC
	3′ AD	962–982	GCACAGTTGAAGTGAACTTGC
pBD-GAL4 Cam	5′ BD	816–836	GTGCGACATCATCATCGGAAG
	3′ BD	1021–1041	CCTAAGAGTCACTTTAAAATT

Expression of the bait and target proteins may be verified by Western blot analysis with an antibody that immunoreacts with either the protein expressed from the DNA insert, the GAL4 BD, or the GAL4 AD. However, if the antibody used fails to detect expression of the protein, it may not indicate that the protein is not expressed. The ability of the antibody to detect the protein is dependent on several factors including the affinity of the antibody for the protein and the expression level of the protein.

The expression of a low number of bait proteins may be advantageous. Only the number of bait proteins required to bind to the UAS_{GAL4} or UAS_{GAL1} in the yeast chromosome is needed. Overexpression of a toxic bait and/or target protein can inhibit cell growth and even be lethal. Overexpression of the bait protein can also result in a phenomenon known as "squelching." When squelching occurs, excess unbound bait proteins bind to the target proteins thereby preventing the target proteins from interacting with the bait proteins, which are bound to the UAS. Consequently, transcription of the reporter genes is not activated and interacting proteins are not detected.

Yeast Transformation and Assay for Expression of Reporter Genes

The pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors containing DNA encoding the target and bait proteins (target and bait plasmids) must be transformed separately into the yeast host and assayed for expression of the *lacZ* and *HIS3* reporter genes (described in *Yeast Transformation* and *Screening*). If the target or bait plasmid is capable of inducing expression of the *lacZ* and *HIS3* reporter genes in the absence of the other phagemid vector containing an insert, the target or bait plasmid is unsuitable for detecting protein–protein interactions in the GAL4 two-hybrid phagemid vector system. Expression of the reporter genes by the target protein may occur if the target protein is capable of binding to the UAS_{GAL4} and UAS_{GAL1}. Expression of the reporter genes by the bait protein may occur if the bait protein is a transcriptional activator or contains a region of amino acids which are highly acidic and are capable of binding transcription factors in the yeast host. Deletion of portions of the target or bait protein may eliminate expression of the reporter genes but may also eliminate portions of the protein required for interaction.

YEAST TRANSFORMATION

Note *A number of specialized media and reagents are required for the protocols in this section and in the* Screening *and* Verification of Interaction *sections that follow. Please consult the* Two-Hybrid Vector System Media and Reagents *subsection of* Preparation of Media and Reagents *for detailed recipes and instructions for preparation of the appropriate media and reagents.*

TABLE VII

Selective Media for Yeast Transformations

Yeast transformations	Selective media		
	SD medium	SD agar	
		Transformation	Interaction
Control plasmids			
pGAL4	Without Leu	Without Leu	—
pBD-WT	Without Trp	Without Trp	—
pAD-WT	Without Leu	Without Leu	—
pBD-MUT	Without Trp	Without Trp	—
pAD-MUT	Without Leu	Without Leu	—
pLamin C	Without Trp	Without Trp	—
pBD-WT and pAD-WT	Without Leu and Trp	Without Leu and Trp	Without Leu, Trp, and His
pBD-MUT and pAD-MUT	Without Leu and Trp	Without Leu and Trp	Without Leu, Trp, and His
pLamin C and pAD-WT	Without Leu and Trp	Without Leu and Trp	Without Leu, Trp, and His
pLamin C and pAD-MUT	Without Leu and Trp	Without Leu and Trp	Without Leu, Trp, and His
Bait plasmid	Without Trp	Without Trp	—
Bait and target plasmids	Without Leu and Trp	Without Leu and Trp	Without Leu, Trp, and His

Transform the control plasmids into the YRG-2 strain prior to the initial transformation of the bait and target plasmids and concurrently with all subsequent transformations of the bait and target plasmids. The control plasmids are used separately or in pairwise combination in the transformation of the YRG-2 yeast strain as outlined in Table VII and in the *Yeast Transformation Protocol*.

Yeast are cotransformed with the bait and target plasmids by sequential transformation. First, yeast are transformed with the bait plasmid and assayed for expression of reporter genes as described in the *Yeast Transformation Protocol* and *Screening* sections. Second, yeast competent cells containing the bait plasmid are prepared and transformed with the target plasmid(s).

Alternatively, yeast are transformed with the bait and target plasmids in a cotransformation with YRG-2 yeast competent cells. Cotransformation is especially useful when the bait plasmid is toxic to the yeast cells thereby increasing the difficulty of preparing competent cells containing the bait plasmid and generates results 5 days faster than sequential transformation.[6] Toxicity of the bait protein can be determined by comparing growth curves of YRG-2 yeast competent cells containing the bait plasmid and YRG-2 yeast competent cells containing the pBD-WT or pBD-MUT bait plasmid when grown in selective media.

Yeast Transformation Protocol

Note *Sterile technique must be used throughout the* Yeast Transformation Protocol*.*

Use wide-bore pipet tips when pipetting yeast competent cells to reduce the shear forces associated with standard pipet tips.

Competent cells should be used immediately after preparation.

Preparation of Yeast Competent Cells

1. Prepare a yeast culture as follows:

   a. Inoculate 1 ml of YPAD broth in a 1.5-ml microcentrifuge tube with two to four YRG-2 yeast colonies that are 2–3 mm in diameter and no more than 1 week old. Vortex the culture vigorously until no cell clumps are visible.

   b. In a 250-ml flask, add the 1 ml of the yeast culture to 50 ml of YPAD broth.

   c. Incubate the diluted culture for 18–24 hours at 30°C with shaking at 225–250 rpm.

   d. Check the OD_{600}. If the OD_{600} is ≥1.2, continue with step 2. If the OD_{600} is <1.2, return the flask to the incubator for 1–2 hours and then check the OD_{600} again. If OD_{600} is <1.2 after 24 hours, restart culture with new colonies.

2. Add the 50-ml yeast culture to 300 ml of YPAD broth in a 1- or 2-liter flask.

3. Incubate the culture for 3 hours at 30°C with shaking at 225–250 rpm.

4. Harvest the cells by centrifugation at 1000 × g for 5 minutes at room temperature.

5. Discard the supernatant and resuspend the cells in 50 ml of deionized water.

6. Centrifuge the cells at 1000 × g for 5 minutes at room temperature.

7. Discard the supernatant and resuspend the cells in 1.5 ml of freshly prepared TE–LiAc solution. [§]

Transformation of Yeast Competent Cells

Note *Each transformation requires one sterile 1.5-ml microcentrifuge tube.*

1. Prepare the carrier DNA (salmon sperm DNA at 20 mg/ml) by boiling the salmon sperm DNA for 20 minutes. Chill the salmon sperm DNA on ice.

2. Using wide-bore pipet tips, aliquot 100 µl of competent yeast cells per microcentrifuge tube.

3. Add 100 µg of carrier DNA to each tube.

4. Add 100 ng of the desired plasmid to each tube; for pairwise transformations, add 200 ng of each plasmid for a total of 400 ng of plasmid DNA in each tube.

5. Add 600 µl of TE–LiAc–PEG solution[§] to each tube and mix the contents by vortexing.

6. Incubate the samples at 30°C for 30 minutes with shaking at 200 rpm.

7. Add 70 µl of DMSO to each tube and mix the contents gently.

8. Heat-shock the samples for 15 minutes in a 42°C water bath.

9. Place the tubes on ice for 10 minutes.

10. Centrifuge the samples at 3000 rpm for 10 seconds to pellet the cells.

11. Using standard pipet tips, carefully remove all of the supernatant from the tubes. If necessary after removing the supernatant, spin the tubes in a microcentrifuge for a few seconds, and using a pipet, remove any residual supernatant.

12. Add 0.5 ml of 1× TE buffer to each tube and vortex the tube to resuspend cells. If pipetting is required to resuspend cells, use of a wide-bore pipet tip is recommended to reduce the shearing stress on the yeast cells.

13. Using wide-bore pipet tips, plate the transformed cells on the appropriate SD-selective plates. For single transformations, plate 150 µl of the transformed cells on each 100-mm plate. For cotransformations, plate 125 µl of the transformed cells on each of two 100-mm plates.

[§] See *Preparation of Media and Reagents*.

14. Incubate the plates at 30°C for 2–4 days until colonies appear.

Proceed with the filter lift assay described in *Screening* to confirm the interactions outlined by the expected results in Tables VIII and IX.

TABLE VIII
Expected Results for the Yeast Transformation Controls

Yeast transformation	Expected results[a]		
	SD agar plates w/o Leu	SD agar plates w/o Trp	SD agar plates w/o Leu and Trp
pGAL4[b]	Growth, blue		
pBD-WT		Growth, white	
pAD-WT	Growth, white		
pBD-MUT		Growth, white	
pAD-MUT	Growth, white		
pLamin C		Growth, white	
pBD-WT and pAD-WT			Growth, blue
pBD-MUT and pAD-MUT			Growth, light blue
pLamin C and pAD-WT			Growth, white
pLamin C and pAD-MUT			Growth, white
Bait plasmid		Growth, white	

[a] When plated on the selective medium and assayed for expression of the *lacZ* reporter gene.

[b] The expected transformation efficiency of the pGAL4 control plasmid may be as much as 10-fold lower than the expected transformation efficiencies of the other control plasmids.

TABLE IX
Expected Results for Interactions Between Control Plasmids

Yeast transformation	Purpose of control	SD medium	Expected Result[a]
pBD-WT and pAD-WT	Positive interaction	SD agar plates w/o Leu, Trp, and His	Growth, blue
pBD-MUT and pAD-MUT	Positive interaction	SD agar plates w/o Leu, Trp, and His	Growth, light blue
pLamin C and pAD-WT	Negative interaction	SD agar plates w/o Leu, Trp, and His	No growth
pLamin C and pAD-MUT	Negative interaction	SD agar plates w/o Leu, Trp, and His	No growth
pGAL4	Positive control for *lacZ* expression	SD agar plates w/o Leu	Growth, blue
pBD-WT	Negative control for *lacZ* expression	SD agar plates w/o Trp	Growth, white

[a] When assayed for expression of the *lacZ* reporter gene.

If the Expected Results are Obtained

Prepare and transform yeast competent cells with control plasmids according to the protocols in *Preparation of Yeast Competent Cells* and *Transformation of Yeast Competent Cells*.

Prepare yeast competent cells containing the bait plasmid for transformation with target plasmid(s) according to the protocol in *Preparation of Yeast Competent Cells*. This protocol prepares enough competent cells for one transformation and can be adjusted for the number of transformations to be performed. Incorporate the following modifications into the protocol:

- In step 1a, inoculate 1 ml of SD medium lacking Trp with yeast colonies containing the bait plasmid.
- In step 1b, add 1 ml of the culture of yeast cells containing the bait plasmid to 50 ml of SD medium lacking Trp.

Transfect the target plasmid(s) into the prepared yeast competent cells containing the bait plasmid according to the protocol in *Transformation of Yeast Competent Cells*, incorporating the following modifications:

- In step 2, add 1 ml of yeast competent cells containing the bait plasmid to each 50-ml conical tube.
- In step 3, add 2 mg of carrier DNA to each tube.
- In step 4, add 40 µg of each target plasmid to be transfected to each tube.
- In step 5, add 6 ml of TE–LiAc–PEG solution to each tube and vortex the tubes to mix the contents.
- In step 7, add 700 µl of DMSO to each tube.
- In step 10, centrifuge the samples at 1000 × g for 5 minutes.
- In step 12, add 10 ml of 1× TE buffer to each tube.
- In step 13, spread 1, 10, and 100 µl of the transformed cells on SD agar plates lacking Leu and Trp. Spread 1 µl of the transformed cells on an SD agar plate lacking Leu and 1 µl on an SD and agar plate lacking Trp. Spread the remaining transformed cells on SD agar plates lacking His, Leu, and Trp at 250 µl of transformation/100-mm plate.

Confirmation of Protein–Protein Interaction

Colonies that grow on SD agar plates without His, Trp, and Leu are either due to the leaky expression of the *HIS3* reporter gene or to the specific interaction between the bait and target proteins resulting in expression of the *HIS3* gene. To distinguish between leaky expression and specifically interacting proteins, detection of the expression of the second reporter gene (*lacZ*) is determined by the filter lift assay described in *Screening*.

SCREENING

Filter Lift Assay

Notes *Do not try to bypass the filter lift assay by simply adding 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) directly to the plate. Addition of X-gal directly to the plate will inhibit yeast cell growth.*

Wear gloves and use sterile technique throughout the Filter Lift Assay. *Handle the qualitative filter papers* ***carefully*** *as the papers tend to tear easily when wet.*

Colonies are transferred to filter paper, permeabilized in liquid nitrogen, and assayed for expression of the *lacZ* reporter gene by the detection of β-galactosidase activity with a solution containing an X-gal substrate. Colonies producing β-galactosidase turn blue in color.

Note *Nitrocellulose paper can be substituted for filter paper. White colonies will eventually turn blue on filter paper, but colonies will maintain their blue or white color on nitrocellulose paper.*

1. Allow the transformants from step 14 of the *Transforming Yeast Competent Cells* to grow for 3–7 days or until the colonies are 1–2 mm in diameter.

2. Prepare the Z buffer with X-gal (see *Preparation of Media and Reagents*).

3. Add 2 ml of Z buffer with X-gal to the bottom of a 100-mm petri dish. Add a sterile qualitative filter paper to the dish (see *Equipment in Additional Materials Required*). Ensure that the filter paper is completely wet. Excess buffer should be poured off into a waste beaker.

   Note *If the transformations were plated on 150-mm plates, use 4.5 ml of Z buffer and 150-mm petri dishes.*

4. Label a separate piece of sterile filter paper. Hold the paper with forceps and starting from the edge of the paper, slowly place the filter on the plate. Ensure that the filter paper contacts all of the colonies on the plate; allow contact for approximately 1 minute. Mark the orientation on the plate and on the filter.

5. Using forceps and starting at one side of the plate, carefully lift the filter paper from the plate.

6. Holding the filter paper with forceps, dip the paper **colony side up** in liquid nitrogen for ten seconds. Remove the filter paper from the liquid nitrogen and allow it to thaw (colony side up). Repeat this step two or three times with each filter paper.

7. Carefully place the thawed filter paper **colony side up** onto the filter paper soaked in the Z buffer with X-gal (see step 3). Carefully remove any air bubbles trapped between the two pieces of filter paper.

8. Allow the plates containing the filter papers to incubate at room temperature for 3 hours. During the incubation, the colonies containing the pGAL4 control will turn blue. The pAD-WT and pBD-WT cotransformants will turn a similar shade of blue. The pAD-MUT and pBD-MUT cotransformants will turn light blue. No color change should be observed in the pAD-WT and the pLaminC cotransformants (see Table VIII).

   Note *Colonies containing the pGAL4 control plasmid will be a more intense blue color than colonies containing the positive control plasmids. The pGAL4 control plasmid expresses the complete GAL4 protein and activates transcription of the* lacZ *reporter gene more efficiently than the portions of the GAL4 protein that are reconstituted by the interacting cI-wt or cI-E233K protein. The most important factor in evaluating the color of the yeast colonies containing control plasmids is whether the blue color of the cI-wt or cI-E233K-containing colony can be distinguished from the color of the pAD-WT or pAD-MUT and pLamin C-containing colonies.*

9. Colonies with β-galactosidase activity can be isolated by aligning the filter paper and the plate. Colonies should be streaked again on a new plate with selective media to select for His^+ colonies. Repeating this assay to verify the presence of β-galactosidase activity in $LacZ^+$ colonies is recommended.

DNA representing target DNA inserts can be prepared directly from His^+–$LacZ^+$ yeast colonies.

Following verification that the target and bait proteins interact in the GAL4 two-hybrid phagemid vector system, the amino acid sequence of the target or bait protein can be altered by mutation of the plasmid DNA encoding the target or bait protein of interest. Mutations can be introduced by a variety of methods. The Stratagene QuikChange site-directed mutagenesis kits provide a simple and rapid method for making specific point mutations or deletions/insertions in order to alter one or more specific amino acids. Stratagene XL1-Red competent cells can be used to introduce random mutations into plasmid DNA by transformation of the plasmid into a mutator strain with a spontaneous mutation rate that is approximately 5000-fold higher than the mutation rate of the wild-type parent strain.

Target or bait plasmids containing the desired mutations are then cotransformed with the appropriate partner into yeast and expression of the *HIS3* and *lacZ* reporter genes is assayed.

If desired, the pAD-GAL4-2.1 and pBD-GAL4 phagemid vectors can be isolated from transformed yeast as described in *Isolation of Plasmid DNA from Yeast*.

ISOLATION OF PLASMID DNA FROM YEAST

Plasmid DNA can be isolated from yeast in sufficient quality and quantity to transform *E. coli* either by using the Yeast DNA Isolation System or by following this quick and easy procedure.[13] This procedure yields a mixture of intact plasmid DNA and fragmented chromosomal DNA; therefore, the resultant plasmid DNA is not of sufficient purity for gel analysis.

1. Inoculate 2 ml of YPAD broth with an isolated His^+–$LacZ^+$ yeast colony. Incubate the culture at 30°C until the media is saturated (~2–3 days).

2. Transfer the yeast culture to a 1.5-ml microcentrifuge tube and spin at 14,000 × *g* for 10 seconds to pellet the yeast cells. Decant the supernatant.

3. Add 0.2 ml of yeast lysis solution[§] and resuspend the yeast cells by vortexing. Add 0.2 ml of phenol–chloroform–isoamyl alcohol[§] [25:24:1 (v/v/v)] and 0.3 g of acid-washed glass beads. Vortex the suspension for 2 minutes.

4. Spin the suspension at 14,000 × *g* for 5 minutes at room temperature. Transfer the top aqueous phase containing the DNA to a new microcentrifuge tube.

5. Precipitate the DNA with 1/10 volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of ethanol. Spin the suspension at 14,000 × *g* for 10 minutes. Decant the supernatant.

6. Wash the DNA pellet with 1 ml of 70% (v/v) ethanol and respin the pellet at 14,000 × *g* for 10 minutes. Decant the supernatant and dry the DNA pellet under a vacuum.

7. Resuspend the DNA pellet in 50 µl of TE buffer. Use 5–20 µl to transform the XL1-Blue MRF´ competent cells and select for the target or bait plasmid by plating on LB–ampicillin agar plates[§] or LB–chloramphenicol agar plates,[§] respectively.

8. Identify colonies that contain the target or bait plasmid by preparing miniprep DNA from isolated colonies from the LB–ampicillin or LB–chloramphenicol agar plates, respectively, and by restriction digest analysis.

[§] See *Preparation of Media and Reagents*.

APPENDIX: GENERAL COMPARISON OF *Escherichia coli* VERSUS YEAST HOST STRAINS

Quality/feature	Host strain	
	Escherichia coli	**Yeast**
Doubling time	20 minutes	>1 hour
Complex media (nonselective)	LB and NZY	YPAD
Chemically defined media (selective)	M9	SD
pH	7 (neutral)	5.8 (acidic)
Growth temperature	37°C	30°C
Antibiotic sensitivity	Sensitive to most antibiotics	Resistant to most antibiotics including ampicillin
Selection method for presence of plasmid	Add antibiotic to media	Remove amino acid from media
Colonial morphology	Small, flat colonies	Large, rounded colonies
Cell diameter	1 μm	3–5 μm
Odor	Musty, pungent	Bread dough

TROUBLESHOOTING

Two-Hybrid Vector System Screening

Observation	Suggestion
The target protein is not detected in Western blot analysis	Verify the nucleotide sequence of the GAL4 AD and the insert DNA to ensure that they are in the same frame
	If the antibody does not have a sufficiently high affinity for the target protein, the target protein may be expressed but may not be detectable. If the nucleotide sequence encoding the target protein is correct, continue with the two-hybrid screening
Transformation with the target plasmid alone results in His^+–$LacZ^+$ colonies	Subclone portions of the target protein (see *Yeast Transformation and Assay for Expression of Reporter Genes*) to ensure that the target protein alone does not activate transcription of the reporter genes
The bait protein is not detected in Western blot analysis	Verify the nucleotide sequence of the GAL4 BD and the insert DNA to ensure that they are in the same frame
	Ensure the insert DNA is expressed at levels sufficient to be detectable with the antibody used. To activate transcription of the reporter genes, only a sufficient number of bait proteins to bind to all of the GAL4 operators is required; therefore, a low level of bait protein may be adequate in the two-hybrid assay. If the nucleotide sequence encoding the bait protein is correct, continue with the two-hybrid screening
	If the antibody does not have a sufficiently high affinity for the bait protein, the bait protein may be expressed but may not be detectable. If the nucleotide sequence encoding the bait protein is correct, continue with the two-hybrid screening
Transformation with the bait plasmid alone results in His^+–$LacZ^+$ colonies	Subclone portions of the bait protein (see *Yeast Transformation and Assay for Expression of Reporter Genes*) to ensure that the bait protein alone does not activate transcription of the reporter genes
The control plasmids do not give the expected results	Verify that correct control plasmid pairs are used
	Prepare a fresh solution of Z buffer with X-gal
	Incubate the qualitative filter paper colony side up during color development to avoid smearing of the colonies
	Ensure that Z buffer and X-gal solutions are prepared correctly
	Avoid exceeding the recommended amount of Z buffer with X-gal; excessive amounts may cause smearing of colonies
	Verify the pH of the SD agar plates using a pH indicator strip
	Verify the phenotype of a yeast colony as described in *Yeast Host Strain Phenotype* and prepare new yeast competent cells using the same yeast colony
	Use sterile technique when preparing and transforming the yeast competent cells to avoid contamination with a different yeast strain or *E. coli*
	Ensure that the filter paper makes good contact with the yeast colonies
	Verify that the colony material has been transferred by visual inspection of the filter paper and the colonies on the plate
The transformants of the pBD-WT and pAD-WT pair of control plasmids or the pBD-MUT and pAD-MUT pair of control plasmids do not turn blue	If the colonies do not contain both control plasmids, blue color will not be observed. Verify that medium was made correctly to select for both control plasmids

(*table continues on the next page*)

(table continues from the previous page)

Observation	Suggestion
No His^+–$LacZ^+$ transformants are present	Verify the nucleotide sequence of the GAL4 AD and insert DNA and confirm expression of the hybrid protein In Western blot analysis
	Verify the nucleotide sequence of the GAL4 BD and insert DNA and confirm expression of the hybrid protein In Western blot analysis
	Verify the pH of the SD agar plates using a pH indicator strip
	Vary the fusion point of the GAL4 AD and the target protein or the GAL4 BD and the bait protein to highlight possible steric inhibition
	For good transfer of yeast colonies, ensure that the filter paper makes good contact with the yeast colonies
	Verify that the colony material has been transferred by visual inspection of the filter paper and the colonies on the plate
The colonies are small and are His^+–$LacZ^+$, but the colonies are also flat and do not grow larger with continued incubation	Visually compare the *E. coli* and yeast on the plate, noting that *E. coli* colonies are small and flat and yeast are large, round, and white. If further comparison is required, prepare a slide and view the *E. coli* and yeast under a microscope, noting that yeast are large and round and *E. coli* cells are very small and rod shaped

Plasmid Isolation from Yeast

Observation	Suggestion
Absence of Amp^r or Cam^r colonies when *E. coli* is transformed with DNA isolated from yeast	Transform *E. coli* with a greater volume of isolated DNA or reisolate plasmid DNA to ensure that the transformation is performed with a sufficient amount of plasmid DNA
	Continue incubation of the transformants to compensate for the slow growth rate of the Cam^r transformants
No discernible bands following restriction analysis of the recovered plasmid DNA	Transform *E. coli* with plasmid DNA isolated from yeast before restriction analysis, as the plasmid DNA isolated may be contaminated with yeast chromosomal DNA and is not suitable for restriction analysis

PREPARATION OF MEDIA AND REAGENTS

Standard Media and Reagents

Note *All media must be autoclaved prior to use.*

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM DTT

Note *rATP is added separately in the ligation reaction*

LB–Ampicillin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 10 ml of 10 mg/ml ampicillin (filter-sterilized)
Pour into petri dishes (~25 ml/100-mm plate)

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Adjust to pH 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

LB–Chloramphenicol Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 30 mg of filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

LB–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 12.5 mg of filter-sterilized tetracycline
Pour into petri dishes (~25 ml/100-mm plate)
Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods as tetracycline is light-sensitive

LB–Tetracycline Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 12.5 mg of filter-sterilized tetracycline
Store broth in a dark, cool place as tetracycline is light-sensitive

Super Broth (per Liter)[‖]

35 g of tryptone
20 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 with 5 M NaOH
Autoclave

[‖] LB broth is the medium of choice for overnight growth. However, when growing XL1-Blue MRF´ for in vivo excision, rescue, or minipreps, super broth may be used. Growing host cells overnight plating cultures at 30°C also increases plating efficiency.

Two-Hybrid Vector System Media and Reagents

Media for Growth and Maintenance of Yeast

YPAD Medium

YRG-2 cells are grown on YPAD medium. YPAD is a rich medium and does not select for yeast containing a plasmid. Yeast are streaked for isolation on YPAD agar plates and are incubated at 30°C for 1–2 days until colonies appear. Liquid YPAD broth is used for growing yeast for transformation. Adenine sulfate is added to the medium to reduce the reversion rate of the *ade2-101* mutation thereby reducing the amount of reddish pigment in the yeast colonies.

YPAD Agar (per Liter)	YPAD Broth (per Liter)
20 g of Difco peptone 10 g of yeast extract 15–20 g of agar Add deionized H_2O to a final volume of 960 ml Adjust the pH to 5.8 Add 40 mg of adenine sulfate Autoclave Cool to 55°C Add glucose to 2% (v/v) by adding 40 ml of a 50% stock solution which has been filter sterilized or autoclaved separately	20 g of Difco® peptone 10 g of yeast extract Add deionized H_2O to a final volume of 960 ml Adjust the pH to 5.8 Add 40 mg of adenine sulfate Autoclave Cool to 55°C Add glucose to 2% (v/v) by adding 40 ml of a 50% stock solution which has been filter sterilized or autoclaved separately

Synthetic Minimal Medium

Synthetic minimal (SD) medium is used for selection of yeast containing a plasmid. SD medium contains a yeast nitrogen base, a carbon source [2% (w/v) glucose], and a dropout solution. The dropout solution contains specific amino acids and other nutrients required for growth of the yeast. The omission of Leu from SD medium selects for the pAD-GAL4-2.1 vector or the pAD-WT or pAD-MUT control plasmid, which contain the *LEU2* gene. The omission of Trp from SD medium selects for the pBD-GAL4 Cam vector or the pBD-WT, pBD-MUT, or pLamin C control plasmid, which contain the *TRP1* gene. The omission of both Leu and Trp from SD medium selects for both vectors or a pair of control plasmids. The omission of Leu, Trp, and His from SD medium selects for both phagemid vectors and for hybrid proteins that interact.

SD Agar (per Liter)	**SD Medium (per Liter)**
6.7 g of Difco yeast nitrogen base without amino acids (Difco Catalog #0919-15-3) 182.2 g of D-sorbitol 15–20 g of agar Add deionized H_2O to a final volume of 860 ml. Adjust pH to 5.8 Autoclave Cool to 55°C Add 100 ml of the appropriate 10× dropout solution (see *10× Dropout Solution*) and 40 ml of a 50% stock solution of glucose which has been filter sterilized or autoclaved separately Pour into 100- and 150-mm petri dishes	6.7 g of Difco yeast nitrogen base without amino acids (Difco Catalog #0919-15-3) 182.2 g of D-sorbitol Add deionized H_2O to a final volume of 860 ml Adjust pH to 5.8 Autoclave Add 100 ml of the appropriate 10× dropout solution (see *10× Dropout Solution*) and 40 ml of a 50% stock solution of glucose which has been filter sterilized or autoclaved separately

10× Dropout Solution

To prepare the appropriate 10× dropout solution for the desired SD medium, simply omit the appropriate component as indicated in the footnotes to the table that follows. All amino acids and nutrients can be autoclaved with the exception of tryptophan, threonine and aspartic acid, which should be filter sterilized. After sterilization, the 10× dropout solutions can be stored in 100-ml aliquots at 4°C for up to 1 year.

Component	Weight (mg/liter)	Sigma Catalog #
L-Isoleucine	300	I 2752
L -Valine	1500	V 0500
L -Adenine hemisulfate salt	200	A 9126
L -Arginine HCl	200	A 5131
L -Histidine HCl monohydrate[a]	200	H 8125
L -Leucine[b]	1000	L 8000
L -Lysine HCl	300	L 5626
L -Methionine	200	M 9625
L -Phenylalanine	500	P 2126
L -Threonine	2000	T 8625
L -Tryptophan[c]	200	T 0254
L -Tyrosine	300	T 3754
L -Uracil	200	U 0750
L -Glutamic acid	1000	G 1251
L -Aspartic acid	1000	A 9256
L -Serine	4000	S 4500

[a] Omit L-histidine HCl monohydrate for selection of interacting proteins.

[b] Omit L-leucine for selection of the pAD-GAL4-2.1 phagemid vector or the pAD-WT or pAD-MUT control plasmid.

[c] Add these amino acids only after autoclaving the 10× dropout solution.

[d] Omit L-tryptophan for selection of the pBD-GAL4 Cam phagemid vector or the pBD-WT, pBD-MUT, or pLamin C control plasmid.

Yeast Transformation Solutions

Stock Solutions

The following stock solutions are necessary in order to prepare the yeast transformation solutions outlined below:

10× Lithium Acetate (LiAc) 1 M LiAc (Sigma Catalog #L 6883) Adjust pH to 7.5 with dilute acetic acid Autoclave Store at room temperature	**50% (w/v) PEG 3350** 50 g of PEG (Sigma Catalog #P 3640, average molecular weight: 3350) dH_2O to 100 ml Filter sterilize or autoclave Store at room temperature
TE–LiAc–PEG Solution (1× TE buffer, 1× LiAc, 40% (w/v) PEG 3350) 1 ml of 10× TE buffer 1 ml of 10× LiAc 8 ml of 50% (w/v) PEG 3350	**10× TE buffer** 100 mM Tris-HCl (pH 7.5) 10 mM EDTA (pH 8.0) Autoclave Store at room temperature
TE–LiAc Solution (1× TE buffer and 1× LiAc) 1 ml of 10× TE buffer 1 ml of 10× LiAc 8 ml of sterile dH_2O	**1× TE Buffer** 1 ml of 10× TE buffer 9 ml of dH_2O

Salmon Sperm DNA

Sonicate or randomly shear the salmon sperm DNA. For higher efficiency, phenol–chloroform extract and resuspend in TE buffer at a concentration of 20 mg/ml. Store the aliquots at –20°C. Before use, boil the salmon sperm DNA for 5 minutes.

Solutions for the Filter Lift Assay

Z Buffer Stock Solution (per Liter)

16.1 g of $Na_2HPO_4 \cdot 7H_2O$
5.5 g of $NaH_2PO_4 \cdot H_2O$
0.75 g of KCl
0.246 g of $MgSO_4 \cdot 7H_2O$
Add dH_2O to a volume of 1 liter
Adjust the pH to 7.0
Autoclave or filter sterilize
Store at 4°C

Z Buffer with X-gal (100 ml)

Note *Prepare fresh each time*

98 ml of Z buffer
0.27 ml of β-mercaptoethanol
1.67 ml of X-gal stock solution

X-gal Stock Solution

Dissolve X-gal in *N,N*-dimethyl-formamide (DMF) at a concentration of 20 mg/ml
Store at –20°C

Solutions for Plasmid DNA Isolation from Yeast

Phenol–Chloroform–Isoamyl Alcohol (100 ml)

50 ml of neutralized phenol (neutralized with Tris-HCl as described in Reference 14)
48 ml of chloroform
2 ml of isoamyl alcohol
Store at 4°C

Yeast Lysis Solution

2% (v/v) Triton® X-100
1% (w/v) SDS
100 mM NaCl
10 mM Tris-HCl (pH 8.0)
1 mM EDTA
Store at room temperature

REFERENCES

1. Mullinax, R. L. and Sorge, J. A. (1995) *Strategies* 8(1):3-5.
2. Fields, S. and Song, O. (1989) *Nature* 340(6230):245-6.
3. Ma, J. and Ptashne, M. (1987) *Cell* 51(1):113-9.
4. Jerpseth, B., Greener, A., Short, J. M., Viola, J. and Kretz, P. L. (1992) *Strategies* 5(3):81–83.
5. Feilotter, H. E., Hannon, G. J., Ruddell, C. J. and Beach, D. (1994) *Nucleic Acids Res* 22(8):1502-3.
6. Callahan, M., Jerpseth, B., Mullinax, R. L. and Greener, A. (1995) *Strategies* 8(2):45–46.
7. Guthrie, C. and Fink, G. R. (Eds.). (1991). *Guide to Yeast Genetics and Molecular Biology*, Vol. 194. Academic Press, Inc., Boston.
8. Ptashne, M., Backman, K., Humayun, M. Z., Jeffrey, A., Maurer, R. *et al.* (1976) *Science* 194(4261):156-61.
9. Estojak, J., Brent, R. and Golemis, E. A. (1995) *Mol Cell Biol* 15(10):5820-9.
10. Cohen, S., Knoll, B. J., Little, J. W. and Mount, D. W. (1981) *Nature* 294(5837):182-4.
11. Gimble, F. S. and Sauer, R. T. (1989) *J Mol Biol* 206(1):29-39.
12. Bartel, P. L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993). Using the Two-hybrid System to Detect Protein-Protein Interactions. In *Cellular Interactions in Development: A Practical Approach*, D. A. Hartley (Ed.), pp. 153–179. Oxford Univ. Press, Oxford, England.
13. Hoffman, C. S. and Winston, F. (1987) *Gene* 57(2-3):267-72.
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. *et al.* (1987). *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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GAL4 Two-Hybrid Phagemid Vector Kits

Catalog #211351 (GAL4 Two-Hybrid Phagemid Vector Kit), #235700 (pAD-GAL4-2.1 Phagemid Vector Kit), and #235702 (pBD-GAL4 Cam Phagemid Vector Kit)

QUICK-REFERENCE PROTOCOL

- Ligate the bait DNA insert into the pBD-GAL4 Cam phagemid vector (bait plasmid)
- Transform into yeast and assay for reporter gene expression
- Ligate the target DNA insert into the pAD-GAL4-2.1 phagemid vector (target plasmid)
- Transform into yeast and assay for reporter gene expression
- Cotransform the target and bait plasmids into yeast
- Assay cotransformants for reporter gene expression
- Introduce desired point mutation, deletion, or insertion into DNA encoding the target and/or bait plasmid
- Cotransform mutants into yeast and assay for reporter gene expression
- Quantitate and compare expression of the *lacZ* reporter gene