HybriZAP-2.1 Two-Hybrid Predigested Vector Kit

INSTRUCTION MANUAL

Catalog #235601

Revision A.01

For In Vitro Use Only

235601-12

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HybriZAP-2.1 Two-Hybrid Predigested Vector Kit

CONTENTS

HYBRIZAP-2.1 TWO-HYBRID PREDIGESTED VECTOR KIT

MATERIALS PROVIDED

Materials Provided	Quantity
HybriZAP-2.1 vector predigested with *Eco*R I and *Xho* I, SAP treated (1 µg/µl)	10 µg
pBR322 test insert (4.4 kb, 0.25 µg/µl)	2.5 µg
pBD-GAL4 Cam phagemid vector, undigested (1 µg/µl)	20 µg
pGAL4 control plasmid (1 µg/µl)	20 µg
pBD-WT control plasmid (1 µg/µl)	20 µg
pAD-WT control plasmid (1 µg/µl)	20 µg
pBD-MUT control plasmid (1 µg/µl)	20 µg
pAD-MUT control plasmid (1 µg/µl)	20 µg
pLamin C control plasmid (1 µg/µl)	20 µg
XL1-Blue MRF´ strain	glycerol stock
XLOLR strain	glycerol stock
ExAssist interference-resistant helper phage (~1× 10^{10} pfu/ml)	1 ml
YRG-2 yeast host strain	glycerol stock

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* www.stratagene.com *and from the GenBank® database (Accession #AF033313 and #U46126, respectively).*

STORAGE CONDITIONS

Vectors: –20°C. (On arrival, store the HybriZAP-2.1 vector arms at –20°C. After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month. Store the pBD-GAL4 Cam phagemid vector at –20°C.)
Test Insert: –20°C
Control Plasmids: –20°C
Helper Phage: –80°C. Retiter prior to each use if passed through more than one freeze–thaw cycle.
Bacterial Glycerol Stocks: –80°C
Yeast Glycerol Stock: –80°C

Revision A.01

ADDITIONAL MATERIALS REQUIRED

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.*

Gigapack III Gold packaging extract
Salmon sperm DNA

Equipment and Supplies

Acid-washed glass beads (425–600 µm)
Wide-bore pipet tips
Water baths (4°, 12°, 30°, 37°, and 70°C)
Vacuum evaporator
Incubator (30° and 37°C)
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
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)]}

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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 HybriZAP-2.1 two-hybrid 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 HybriZAP-2.1 two-hybrid 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 HybriZAP-2.1 two-hybrid vector system are β-galactosidase (*lacZ*) and histidine (*HIS3*).

* U.S. Patent Nos. 5,283,173; 5,468,614; 5,128,256; and 5,286,636.

Overview of HybriZAP-2.1 Two-Hybrid Library Screens

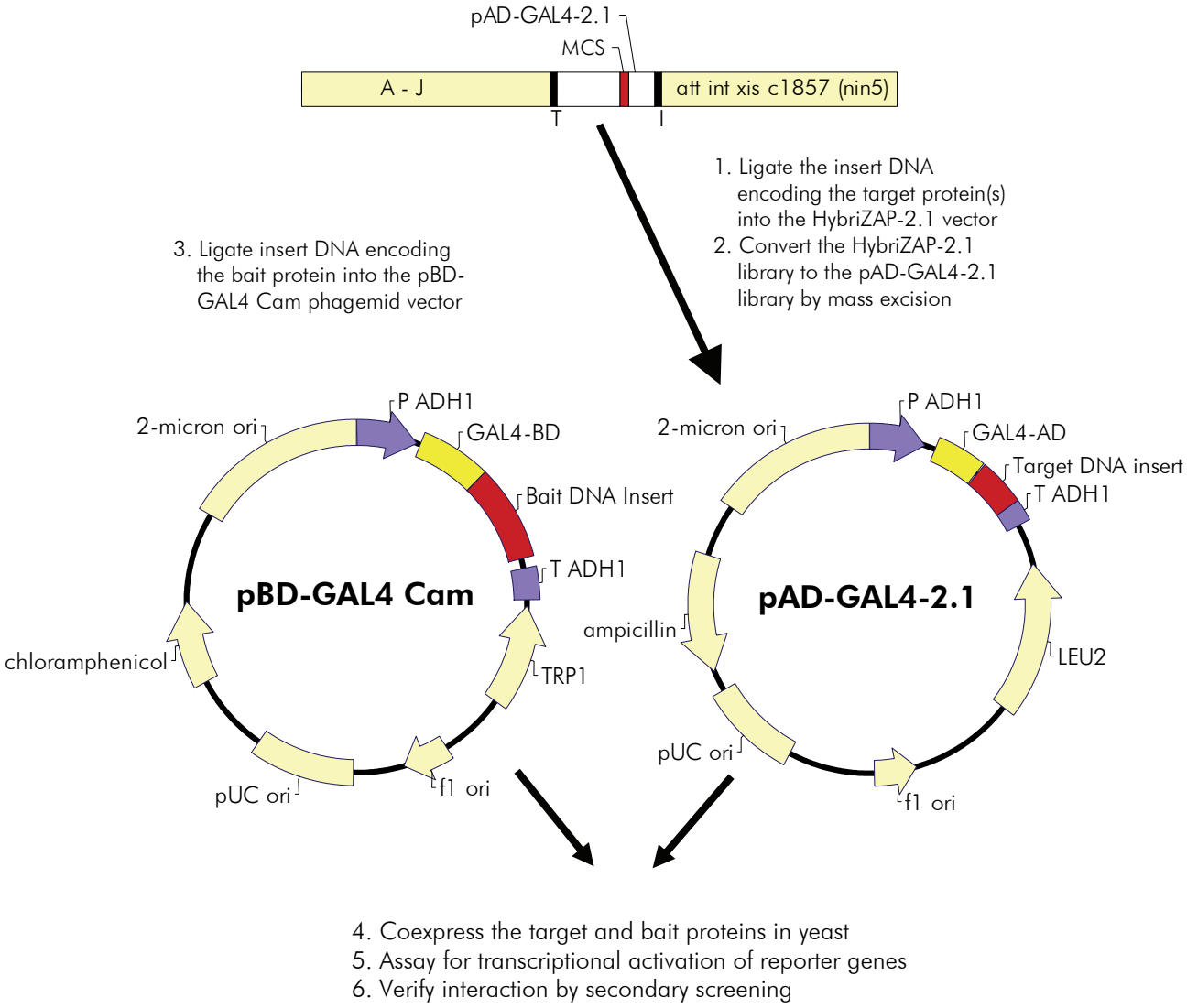


FIGURE 1 The HybriZAP-2.1 two-hybrid vector system. DNA inserts are ligated into the HybriZAP-2.1 vector to generate the primary lambda library. This primary lambda library is amplified and converted by *in vivo* mass excision to a pAD-GAL4-2.1 library. DNA that expresses a library of the GAL4 AD hybrid proteins (target proteins or *Y*) is isolated from *E. coli*. 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 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*).

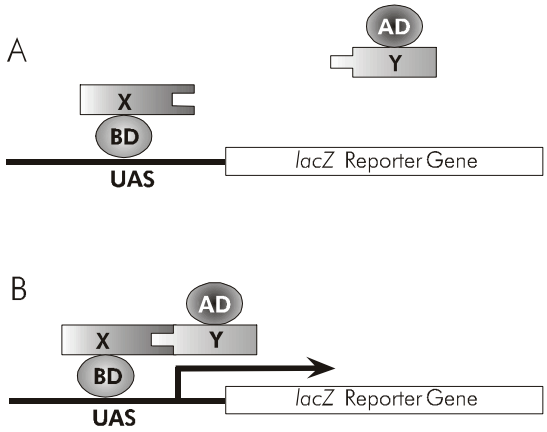


FIGURE 2 Detection of interacting proteins by transcription of the *lacZ* reporter gene. The GAL4 UAS and the *lacZ* reporter gene are integrated into the yeast chromosome. (A) The GAL4 BD hybrid protein (BD and the bait protein *X*) binds to the GAL4 UAS present upstream of the *lacZ* reporter gene. 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 HybriZAP-2.1 vector will accommodate DNA inserts from 0 to 6 kb in length. *In vivo* mass excision allows conversion of the HybriZAP-2.1 lambda library to a pAD-GAL4-2.1 phagemid library by the same excision mechanism found in the Lambda ZAP vectors.[4, 5, 6]

The HybriZAP-2.1 lambda vector and the pAD-GAL4-2.1 phagemid vector contain 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 3–5 and Table I). The unique *Eco*R I and *Xho* I cloning sites in the HybriZAP-2.1 lambda vector and the pAD-GAL4-2.1 vector make these vectors 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. The *Xba* I site in the HybriZAP-2.1 lambda vector and pAD-GAL4-2.1 and pBD-GAL4 Cam phagemid vectors 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. In the HybriZAP-2.1 lambda vector and pBD-GAL4 Cam phagemid vector, the *Xba* I site is not unique.

TABLE I

Unique Restriction Sites in the MCS

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

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 in yeast cells. 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. In both vectors, the hybrid protein is expressed by the alcohol dehydrogenase 1 (*ADH1*) promoter (P *ADH1*) and is terminated by the *ADH1* terminator (T *ADH1*).

HybriZAP-2.1 Vector Map

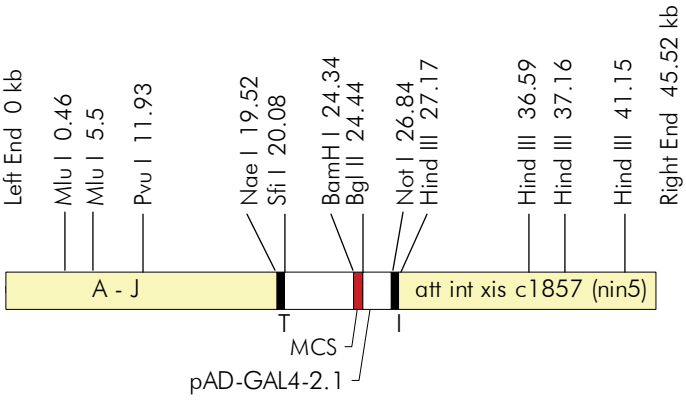
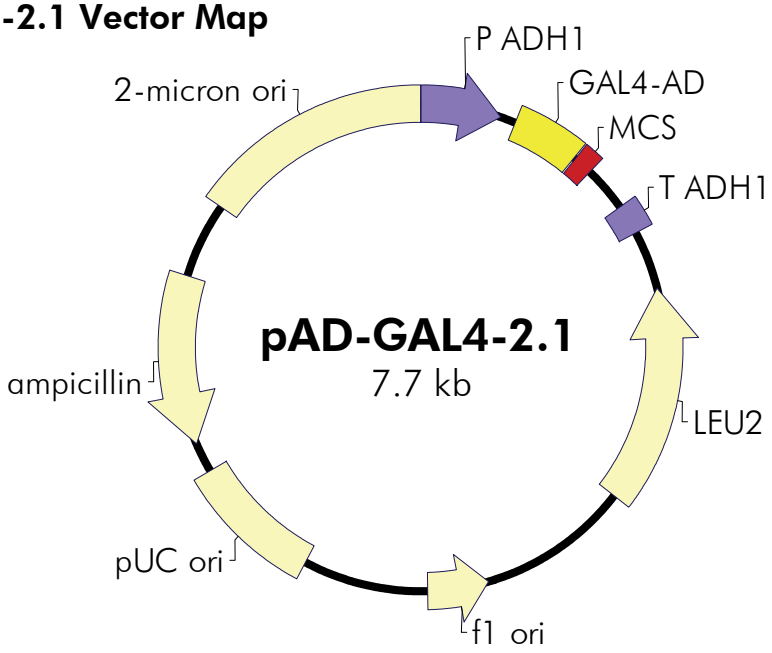


FIGURE 3 Restriction map of the HybriZAP-2.1 vector. The HybriZAP-2.1 vector contains lambda genes *A* through *J* in the left arm and *att*, *int*, *xis*, and cI857 in the right arm. The f1 initiator (I) and terminator (T) allow efficient *in vivo* excision of the pAD-GAL4-2.1 phagemid vector from the HybriZAP-2.1 vector.

pAD-GAL4-2.1 Vector Map



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

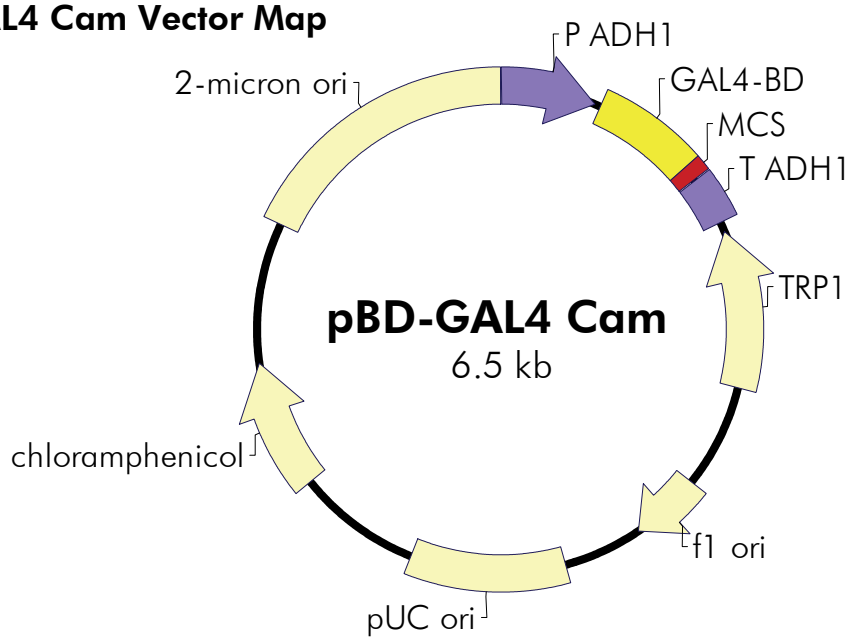
...ACT GCA GAG ATC TAT GAA TCG TAG ATA CTG AAA AAC 3′

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 ssDNA replication	3483–3789
pUC origin of replication	4427–5094
ampicillin resistance (*bla*) ORF	5245–6102
2μ yeast origin of replication	6489–7653

FIGURE 4 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*.

pBD-GAL4 Cam Vector Map



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

In the MCS of the pBD-GAL4 Cam phagemid vector, there is a non-unique *Xba* I site upstream of the T7 promoter. This *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*.

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 ssDNA replication	2322–2628
pUC origin of replication	2970–3637
chloramphenicol resistance ORF	4174–4725
2µ yeast origin of replication	5330–6489

FIGURE 5 Circular map and features of the pBD-GAL4 Cam phagemid vector.

BACTERIAL HOST STRAINS

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

Bacterial Strain Genotypes

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})]

XLOLR Strain Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})] Su^{-} (nonsuppressing) λ^{R} (lambda resistant)

Note *Use the XLOLR strain for plating excised phagemids and the XL1-Blue MRF´ strain for all other manipulations.*

XL1-Blue MRF´ Bacterial Strain Description

The RecA^{-} *E. coli* host strain XL1-Blue MRF´ is supplied with the HybriZAP-2.1 two-hybrid vector system.[7] 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. It is the ideal strain for amplification and excisions.

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

The strains used for the Lambda gt11 vector (i.e., Y1088, Y1089, and Y1090) are not suitable for use with the HybriZAP-2.1 vector because these strains contain the plasmid pMC9, a pBR322 derivative, which contains many of the same sequences as those found in the phagemid portion of the HybriZAP-2.1 vector.

The F´ episome present in the XL1-Blue MRF´ strain contains the genes for expression of the bacterial F´ pili required for filamentous (i.e., f1 or M13) phage infection. The conversion of the HybriZAP-2.1 vector to the pAD-GAL4-2.1 phagemid vector requires superinfection with a filamentous helper phage. (This efficient *in vivo* excision process is outlined in *In Vivo Excision of the pAD-GAL4-2.1 Phagemid Vector from the HybriZAP-2.1 Vector*.)

Recommended Media

Host strain	Agar plates and liquid medium for bacterial streak and glycerol stock	Liquid medium for bacterial cultures prior to phage attachment	Agar plates and top agar for plaque formation	Agar plates for excision protocol
XL1-Blue MRF′ strain	LB-tetracycline[a]	LB broth with supplements[a-c]	NZY[a]	—
XLOLR strain	LB-tetracycline[a]	LB broth with supplements[a-c]	—	LB-ampicillin[a]

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

[b] LB with 0.2% (w/v) maltose and 10 mM $MgSO_4$.

[c] Maltose and magnesium supplements are required for optimal lambda phage receptor expression on the surface of the XL1-Blue MRF′ host cell. These media supplements are not required for helper phage infection, but are included in both protocols for simplified media preparation.

Establishing an Agar Plate Bacterial Stock

The bacterial host strains have been sent as bacterial glycerol stocks. On arrival, prepare the following from the bacterial glycerol stock using the appropriate media as indicated in the previous table:

Note *The host strains may thaw during shipment. The vials should be stored immediately at –20° or –80°C, but most strains remain viable longer if stored at –80°C. It is also best to avoid repeated thawing of the host strains 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 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 (prepared by mixing 5 ml of glycerol + 5 ml of appropriate medium) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile microcentrifuge 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

Yeast Strain Genotype and Phenotypic Verification

TABLE II

Strain	Genotype	Reporter genes	Transformation markers
YRG-2[a]	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*	*lacZ, HIS3*	*leu2, trp1*

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

Table II gives the genotype of the YRG-2 yeast host strain.

The phenotype of the yeast host strain should be verified as outlined below prior to performing the HybriZAP-2.1 two-hybrid vector system assays.

1. Prepare a fresh plate of the yeast host strain on a YPAD agar plate (see *Preparation of Media and Reagents*) from the yeast glycerol stock as outlined below:

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

   b. Streak the splinters onto a YPAD agar plate.

   c. 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 containing 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.

Yeast Strain Description

The HybriZAP-2.1 two-hybrid vector system includes the YRG-2 strain, a yeast strain with two reporter genes (see Table II) for the detection of protein–protein interactions *in vivo*. The YRG-2 strain is a derivative of the HF7c strain[8] and was selected for its ability to generate high-efficiency competent cells.[9] 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 carries the auxotrophic markers leucine (*leu2*) and tryptophan (*trp1*), for selection of yeast which have been transformed with the AD or BD plasmids, respectively. YRG-2 also carries the auxotrophic marker histidine (*his3*), for selection of yeast which have been transformed with plasmids encoding interacting proteins that together activate transcription from the *HIS3* reporter. For generalized protocols and techniques used to analyze the genetics and molecular biology of yeast, see Reference 10.

The YRG-2 strain contains a dual two-hybrid assay system with *lacZ* and *HIS3* reporter gene constructs. 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}, which contains four GAL4 DNA-binding sites, 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 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 the Yeast Host Strain

The yeast host strain has been sent as a yeast glycerol stock. For the appropriate medium, 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 medium 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 (prepared by mixing 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.

HELPER PHAGE

The ExAssist interference-resistant helper phage with XLOLR strain is designed to efficiently excise the pAD-GAL4-2.1 phagemid vector from the HybriZAP-2.1 vector while preventing problems associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E. coli* strain (e.g., XLOLR cells). Only the excised phagemid can replicate in the host, removing the possibility of co-infection from the ExAssist helper phage. Because ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using ExAssist helper phage. XLOLR cells are also resistant to lambda infection, thereby ensuring that the library is not lysed by residual lambda phage.

Note *The ExAssist helper phage is recommended only for excision of the pAD-GAL4-2.1 phagemid vector from the HybriZAP-2.1 vector. It should not be used for single-stranded rescue in general, because this f1 helper phage possesses α-complementing β-galactosidase sequences which may interfere with sequencing or site-directed mutagenesis where oligonucleotide primers hybridize to β-galactosidase sequences [e.g., M13 (–20) primer].*

Storing the Helper Phage

The ExAssist helper phage is supplied in 7% dimethylsulfoxide (DMSO) and should be stored at –80°C. If the titer drops over time, prepare a fresh high-titer stock of the helper phage as outlined in *Amplifying the Helper Phage*. It is important to titer the ExAssist helper phage prior to each use. Expect titers of approximately 10^{10} pfu/ml.

Titering the Helper Phage

Titer the helper phage with XL1-Blue MRF´ cells:

1. Transfer a colony of XL1-Blue MRF´ cells into 10 ml of LB broth with supplements in a 50-ml conical tube. Incubate the conical tube with shaking at 37°C until growth reaches an OD_{600} of 1.0.

2. Dilute the phage (10^{-4}–10^{-7}) in SM buffer[§] and combine 1 µl of each dilution with 200 µl of the XL1-Blue MRF´ cells (OD_{600} = 1.0).

3. Incubate the helper phage and the XL1-Blue MRF´ cells for 15 minutes at 37°C to allow the phage to attach to the cells.

4. Add 3 ml of NZY top agar,[§] melted and cooled to ~48°C, and then pour immediately onto prewarmed NZY agar plates.[§]

   Note *ExAssist plaques will have a cloudier appearance than lambda phage plaques.*

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

5. Incubate the plates overnight at 37°C.

6. To determine the titer [in plaque-forming units per milliliter (pfu/ml)], use the following formula:

$$\left[\frac{\text{Number of plaques (pfu)} \times \text{dilution factor}}{\text{Volume plated (µl)}}\right] \times 1000 \text{ µl / ml}$$

   where the volume plated (in microliters) refers to the volume of the helper phage solution added to the cells.

Amplifying the Helper Phage

1. Transfer a colony of XL1-Blue MRF´ cells from a fresh LB-tetracycline plate into 10 ml of LB broth with supplements in a 50-ml conical tube.

2. Incubate the conical tube with shaking at 37°C until growth reaches an OD_{600} of 0.3.

   Note *An OD_{600} of 0.3 corresponds to 2.5 × 10^8 cells/ml.*

3. Add the helper phage at a multiplicity of infection (MOI) of 20:1 (phage-to-cells ratio).

4. Incubate the conical tube at 37°C for 15 minutes to allow the phage to attach to the cells.

5. Incubate the conical tube with shaking at 37°C for 8 hours.

6. Heat the conical tube at 65°C for 15 minutes.

7. Spin down the cell debris and transfer the supernatant to a fresh conical tube.

8. The titer of the supernatant should be between 7.5 × 10^{10} and 1.0 × 10^{12} pfu/ml for ExAssist helper phage.

9. Add dimethylsulfoxide (DMSO) to a final concentration of 7% (v/v) and store at –80°C.

For a helper phage titering protocol, please see *Titering the Helper Phage*.

PACKAGING EXTRACTS

Packaging extracts are used to package recombinant lambda phage with high efficiency, which increases the size of gene libraries.

Gigapack III packaging extracts have been developed to increase the efficiency and representation of libraries constructed from highly methylated DNA. The packaging extracts are restriction minus ($HsdR^-$ $McrA^-$ $McrBC^-$ $McrF^-$ Mrr^-) to optimize packaging efficiency and library representation. When used in conjunction with restriction-deficient plating cultures, Gigapack III packaging extracts should improve the quality of DNA libraries constructed from methylated DNA.[11-14]

Lambda vectors are linear and contain *cos* sites at each end of the vector. A *cos* site can ligate to another *cos* site either intra- or intermolecularly to form concatameric or circular DNA molecules, respectively. Optimal packaging efficiencies are obtained with lambda DNAs that are concatameric. Ligations should be carried out at DNA concentrations of ≥0.2 µg/µl, which favors concatameric and not circular DNA molecules. DNA to be packaged should be relatively free from contaminants such as polyethylene glycol (PEG), which is contained in some ligase buffers and inhibits packaging. The volume of DNA added to each extract should be <5 µl.

DNA that has been digested with restriction enzymes and religated will be packaged less efficiently (by a factor of 10–100) than uncut lambda DNA. For example, uncut wild-type lambda DNA will be packaged with efficiencies exceeding 2×10^9 plaques/µg of vector when using Gigapack III packaging extracts. However, predigested arms, when ligated to a test insert, will yield ~1×10^7 recombinant plaques/µg of vector.

CONTROL PLASMIDS

Description

The HybriZAP-2.1 two-hybrid vector system contains six control plasmids (see Table III and Figure 6). 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.[15, 16] 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.[17, 18] 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.[19]

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.

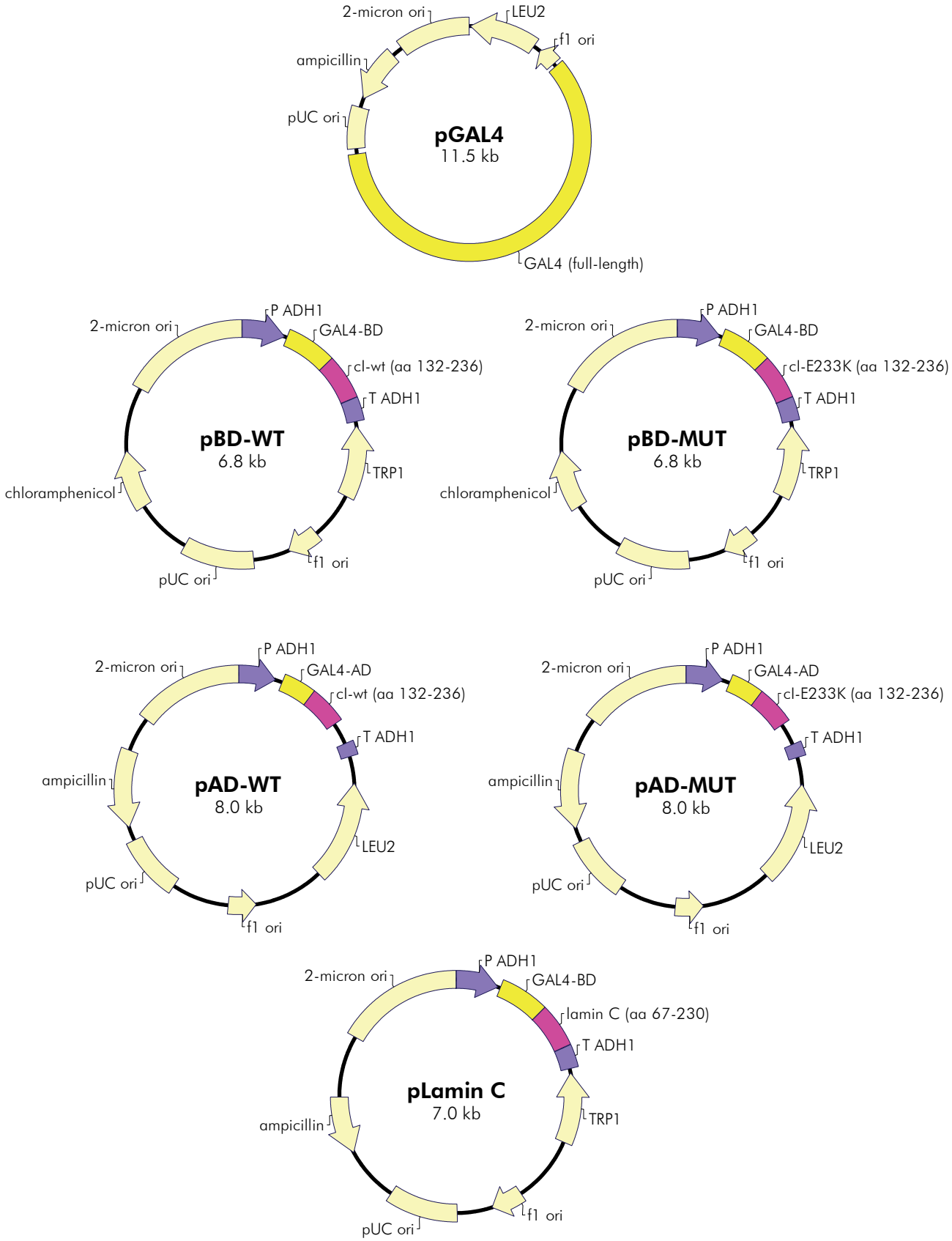


FIGURE 6 Circular maps of the control plasmids.

Expected Results for Control Plasmid Assays

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 VECTOR CONSTRUCTION

Background

The HybriZAP-2.1 two-hybrid vector system is particularly useful for the identification of novel (target) proteins from a cDNA or genomic library, which interact with a bait protein, and for the subsequent determination of protein domains or amino acids critical for the interaction. Specific mutations, insertions, or deletions that affect the encoded amino acid can be introduced into DNA encoding the target protein, and the mutant target proteins can be assayed for the protein–protein interaction with the bait protein.

DNA inserts to be ligated into the HybriZAP-2.1 vector may be prepared from either mRNA or genomic DNA. If the inserts are to be prepared from mRNA, the Stratagene cDNA Synthesis Kit is highly recommended. The cDNA Synthesis Kit provides the reagents required to convert mRNA to cDNA inserts prior to unidirectional insertion into the HybriZAP-2.1 vector. DNA inserts must have *Eco*R I and *Xho* I sites at the 5´ and 3´ ends, respectively, for unidirectional insertion into the predigested HybriZAP-2.1 vector arms. Sites that generate compatible ends can also be used. The protocol for inserting prepared DNA may be found in *Ligating DNA into the HybriZAP-2.1 Vector Arms*.

Ligating DNA into the HybriZAP-2.1 Vector Arms

We strongly recommend quantitating the prepared DNA before ligation (see *Appendix II: Ethidium Bromide Plate Assay—Quantitation of DNA*). Best results are usually obtained by ligating 100 ng of DNA/1 µg of vector. Store the remaining DNA at –20°C.

Note *Polyethylene glycol, which is contained in some ligase buffers, may inhibit packaging.*

1. Set up a test ligation as follows to ligate the test insert into the HybriZAP-2.1 vector:

   1.0 µl of the HybriZAP-2.1 vector (1 µg)
   0.8 µl of test insert (0.2 µg)
   0.5 µl of 10× ligase buffer (see *Preparation of Media and Reagents*)
   0.5 µl of 10 mM rATP
   1.7 µl of water

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

2. To prepare the sample ligation, add the following components:

   X µl of resuspended DNA (~100 ng)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   1.0 µl of the HybriZAP-2.1 vector (1 µg/µl)
   X µl of water for a final volume of 4.5 µl

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

   Note *In all ligations, the final glycerol content should be less than 5% (v/v).* ***Do not exceed 5% (v/v) glycerol!***

3. Incubate the reaction tubes overnight at 12°C or for up to 2 days at 4°C. The ligated DNA–vector will form very large concatamers. Breakage of these strands via over-manipulation or multiple freeze–thaw cycles will decrease the efficiency.

After ligation is complete, package 1 µl of the sample ligation as outlined in *Packaging Reaction* using a packaging extract according to the manufacturer's instructions.

Packaging Reaction

Preparation of Host Bacteria

Notes *The XL1-Blue MRF' host bacteria are used in this protocol to titer the packaged lambda phage.*

XL1-Blue MRF´ cells are RecA⁻ and consequently grow slowly.

1. Streak the XL1-Blue MRF' bacterial glycerol stock onto LB-tetracycline agar plates. Incubate the plates overnight at 37°C.

2. Inoculate 50 ml of LB broth with supplements with a single colony.

[ll] The HybriZAP-2.1 vector arms can accommodate inserts ranging from 0 to 6 kb. If there are no contaminants in the DNA inhibiting ligation, expect >2 × 10^6 recombinant plaques when using a high-efficiency packaging extract such as Gigapack III Gold packaging extract.

3. Grow at 37°C, with shaking for 4–6 hours (do not grow past an OD_{600} of 1.0). Alternatively, grow overnight at 30°C, with shaking at 200 rpm (the lower temperature keeps the bacteria from overgrowing, which reduces the number of nonviable cells). (Phage can adhere to nonviable cells resulting in a decreased titer.)

4. Spin the cells at 500 × *g* for 10 minutes and discard the supernatant.

5. *Gently* resuspend the cells in half the original volume with sterile 10 mM $MgSO_4$.

   Note *For later use, store the cells at 4°C overnight in 10 mM $MgSO_4$.*

Packaging Guidelines

6. Package 1–4 µl containing 0.1–0.5 µg of ligated DNA with a packaging extract as outlined in the manufacturer's instructions.

Titer Determination

7. Dilute the XL1-Blue MRF' cells from step 5 to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

   Note *The bacteria should be used immediately following dilution.*

8. To determine the titer of the packaged ligation product, mix the following components:

   1 µl of the final packaged reaction
   200 µl of XL1-Blue MRF´ cells at an OD_{600} of 0.5

   and

   1 µl of a 1:10 dilution of packaged reaction
   200 µl of XL1-Blue MRF´ cells at an OD_{600} of 0.5

   Note *In order to obtain accurate titers, use freshly prepared XL1-Blue MRF´ cells. The XL1-Blue MRF´ strain is $RecA^-$ $McrA^-$ and $McrCB^-$ Mrr^- and does not restrict methylated DNA. Use of any other cell line may result in dramatically reduced titer.*

9. Incubate the phage and the bacteria at 37°C for 15 minutes to allow the phage to attach to the cells. (Best results are obtained with gentle shaking.)

10. Add 2–3 ml of NZY top agar (held at 48°C).

11. Plate immediately onto NZY agar plates and allow the plates to set undisturbed for 10 minutes. Place the plates upside down in a 37°C incubator.

12. Plaques should be visible after 6–8 hours. The number of background plaques should be $<1 \times 10^5$ pfu/µg of arms, while the number of recombinant plaques should be 10- to 100-fold above the background. (See *Verification of Insert Percentage and Size*). If the results of the test insert ligation and the sample ligation give the expected results, package the remaining 4 µl of the sample ligation in four separate packaging reactions.

    Note *Primary libraries can be unstable; therefore, amplification of the libraries within 1–2 weeks is recommended.*

Verification of Insert Percentage and Size

Individual lambda clones can be examined to determine the percentage of vectors with inserts and the average insert size. Clones may be analyzed either by PCR directly from the plaque (see *Appendix III: Polymerase Chain Reaction Amplification of Lambda DNA from Individual Plaques*) or by conversion to the phagemid format by *in vivo* excision (see *In Vivo Excision of the pAD-GAL4-2.1 Phagemid Vector from the HybriZAP-2.1 Vector*) followed by phagemid DNA isolation and restriction digestion.

Amplification of the HybriZAP-2.1 Library

It is usually desirable to amplify libraries prepared in lambda vectors to make a large, stable quantity of a high-titer stock of the library. However, more than one round of amplification is not recommended, since slower growing clones may be significantly underrepresented.

Amplification Protocol

The following protocol is recommended for amplifying the HybriZAP-2.1 library. In this procedure, each aliquot of bacteriophage containing 5×10^4 pfu is combined with 600 µl of cells and plated on one 150-mm NZY plate. To amplify the library to 1×10^6 plaques, use a total of 20 aliquots (each aliquot contains 5×10^4 pfu/150-mm plate).

Day 1

1. Prepare the host strains as outlined in steps 1–5 of *Preparation of Host Bacteria* in *Packaging Reaction*.

    Note *In order to obtain the highest amplification efficiency, use freshly prepared XL1-Blue MRF´ cells (see* Preparation of Bacterial Host Strains*).*

Day 2

2. Dilute the cells to an OD_{600} of 0.5 in 10 mM $MgSO_4$.

3. Combine aliquots of the packaged mixture or library suspension containing ~5 × 10^4 pfu of bacteriophage with 600 µl aliquots of host cells at an OD600 of 0.5 in BD Falcon polypropylene tubes. To obtain 10^6 plaques, use a total of 20 aliquots.

   Note *Do not add more than 300 µl of phage/600 µl of cells.*

4. Incubate the tubes containing the phage and host cells for 15 minutes at 37°C.

5. Mix 6.5 ml of melted NZY top agar with each aliquot of infected bacteria and spread evenly onto a 2- to 3-day-old 150-mm NZY agar plate (make sure the NZY top agar is cooled to ~48°C before adding it to the aliquot).

6. Incubate the plates at 37°C for 6–8 hours. Do not allow the plaques to get larger than 1–2 mm.

7. Overlay the plates with ~8–10 ml of SM buffer. Store the plates at 4°C overnight (with *gentle* rocking if possible). This allows the phage to diffuse into the SM buffer.

Day 3

8. Recover the bacteriophage suspension from each plate and pool it into a sterile polypropylene container. Rinse the plates with an additional 2 ml of SM buffer and pool. Add chloroform to a 5% (v/v) final concentration. Mix well and incubate for 15 minutes at room temperature.

9. Remove the cell debris by centrifugation for 10 minutes at 500 × *g*.

10. Recover the supernatant and transfer it to a sterile polypropylene container. If the supernatant appears cloudy or has a high amount of cell debris, repeat steps 8 and 9. If the supernatant is clear, add chloroform to a 0.3% (v/v) final concentration and store at 4°C. Store aliquots of the amplified library in 7% (v/v) DMSO at –80°C.

11. Check the titer of the amplified library using host cells and serial dilutions of the library. (Assume ~10^8–10^{11} pfu/ml.)

IN VIVO EXCISION OF THE PAD-GAL4-2.1 PHAGEMID VECTOR FROM THE HYBRIZAP-2.1 VECTOR

Converting the HybriZAP-2.1 two-hybrid library to the phagemid form allows screening of the phagemid library in yeast cells by transformation of yeast cells with supercoiled phagemid DNA. The HybriZAP-2.1 vector has been designed to allow simple, efficient *in vivo* excision of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. [5, 6, 20]

This *in vivo* excision depends on engineered DNA sequences in the HybriZAP-2.1 vector and on the presence of a variety of proteins, including helper phage-derived proteins. The helper phage proteins recognize a region of DNA normally serving as the f1 bacteriophage "origin of replication" for positive-strand synthesis. This origin of replication can be divided into two overlying parts: (1) the site of initiation and (2) the site of termination for DNA synthesis.[21] These two regions have been subcloned separately into the HybriZAP-2.1 vector. The lambda phage is made accessible to the helper phage-derived proteins by simultaneously infecting a strain of *E. coli* with both the lambda vector and the helper phage.

Inside *E. coli*, the helper phage-derived proteins recognize the initiator DNA that is within the lambda vector. One of these proteins then nicks one of the two DNA strands. At the site of this nick, new DNA synthesis begins and duplicates whatever DNA exists in the lambda vector "downstream" (3´) of the nicking site. DNA synthesis of a new single strand of DNA continues through the cloned insert until a termination signal, positioned 3´ of the initiator signal, is encountered within the constructed lambda vector. The ssDNA molecule is circularized by the gene II product from the helper phage, forming a circular DNA molecule containing the DNA between the initiator and terminator. In the case of the HybriZAP-2.1 vector, this includes all sequences of the pAD-GAL4-2.1 phagemid vector and the insert, if one is present. This conversion is the "subcloning" step, since all sequences associated with normal lambda vectors are positioned outside of the initiator and terminator signals and are not contained within the circularized DNA. In addition, the circularizing of the DNA automatically recreates a functional f1 origin as found in f1 bacteriophage or phagemids.

Signals for "packaging" the newly created phagemid are linked to the f1 origin sequence. The signals permit the circularized ssDNA to be "packaged" into phagemid particles and secreted from the *E. coli*. Following secretion of the phagemid particle, the *E. coli* cells used for *in vivo* excision of the cloned DNA are killed and the lambda phage is lysed by heat treatment at 70°C. The phagemid is not affected by the heat treatment. *Escherichia coli* is infected with the phagemid and can be plated on selective media to form colonies. DNA from colonies can be used for analysis of insert DNA, including DNA sequencing, subcloning, and mapping. Colonies from the excised pAD-GAL4-2.1 phagemid vector can also be used for subsequent production of ssDNA suitable for dideoxy-sequencing and site-specific mutagenesis.

ExAssist Helper Phage and XLOLR Strain

The ExAssist helper phage, used with the XLOLR strain, is designed to efficiently excise the pAD-GAL4-2.1 phagemid vector from the HybriZAP-2.1 vector, while eliminating problems associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that prevents replication of the helper phage genome in a nonsuppressing *E. coli* strain such as XLOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using this helper phage.

Mass Excision Protocol

Note *The ratios of bacterial cells, HybriZAP-2.1 library phage particles and ExAssist helper phage strongly influence excision efficiency. For a library titering protocol, see* Titer Determination *in* Packaging Reaction.

If the ExAssist helper phage has been stored at 4°C for >1 month or passed through a freeze–thaw cycle, titer the helper phage with XL1-Blue MRF´ cells prior to use (see Titering the Helper Phage*).*

Day 1

1. Grow separate 50-ml overnight cultures of XL1-Blue MRF´ and XLOLR cells in LB broth with supplements at 30°C.

Day 2

2. Gently spin down the XL1-Blue MRF´ and XLOLR cells (1000 × *g*). Resuspend each of the cell pellets in 25 ml of 10 mM $MgSO_4$. Adjust the cell concentration to an OD_{600} of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$.

3. In a 50-ml conical tube, combine a portion of the amplified lambda bacteriophage library with XL1-Blue MRF´ cells at a MOI of 1:10 lambda phage-to-cell ratio. Excise 10- to 100-fold more lambda phage than the size of the primary library to ensure statistical representation of the excised clones. Add ExAssist helper phage at a 10:1 helper phage-to-cells ratio to ensure that every cell is co-infected with lambda phage and helper phage.

   For example, use

   10^7 pfu of the lambda phage (i.e., 10- to 100-fold above the primary library size)
   10^8 XL1-Blue MRF´ cells (1:10 lambda phage-to-cell ratio, noting that an OD_{600} of 1.0 corresponds to 8×10^8 cells/ml)
   10^9 pfu of ExAssist helper phage (10:1 helper phage-to-cells ratio)

   Note *Briefly spin the lambda phage stock to ensure that the chloroform is separated completely before removing the aliquot used in the excision reaction.*

4. Incubate the conical tube at 37°C for 15 minutes to allow the phage to attach to the cells.

5. Add 20 ml of LB broth with supplements and incubate the conical tube for 2.5–3 hours at 37°C with shaking.

   Notes *Incubation times for mass excision in excess of 3 hours may alter the clonal representation.*

   The turbidity of the media is not indicative of the success of the excision.

6. Heat the conical tube at 65–70°C for 20 minutes to lyse the lambda phage particles and the cells. Spin down the debris at 1000 × *g* for 10 minutes.

7. Transfer the supernatant into a fresh sterile conical tube. This stock contains the excised pAD-GAL4-2.1 phagemid packaged as filamentous phage particles. (This stock may be stored at 4°C for 1–2 months.)

8. To titer the excised phagemids, combine 1 µl of this supernatant with 200 µl of the XLOLR cells from step 2 in a 1.5-ml microcentrifuge tube.

9. Incubate the microcentrifuge tube at 37°C for 15 minutes.

10. Plate 100 µl of the cell mixture onto LB–ampicillin (100 µg/ml) agar plates and incubate the plates overnight at 37°C.

    Note *It may be necessary to further dilute the cell mixture to achieve single-colony isolation.*

Day 3

11. Determine the titer of excised phagemid (in cfu/ml) as follows:

$$\left[\frac{\text{Number of colonies (cfu)} \times \text{dilution factor}}{\text{Volume of phagemid plated } (\mu\text{l})}\right] \times 1000\ \mu\text{l / ml}$$

Mass Excision Results

Determine the excision efficiency as the ratio of the number of colony-forming units rescued to the number of input lambda phage. Because the excision efficiency is dependent on the ratio between the helper phage, the phage stock, and the cells, the excision efficiency may vary [5, 22]. If the number of excised phagemid recovered is lower than expected when performing mass excisions, repeat the excision with a higher number of lambda phage and with freshly prepared XL1-Blue MRF´ cells.

At this point, single rescued colonies may be selected for plasmid preps and DNA analysis.

Amplification of the Excised Phagemid Library

To generate the excised phagemid library, the supernatant containing the excised phagemid particles from step 7 of the *Mass Excision Protocol* is incubated with XLOLR host cells in the presence of ampicillin to produce a stable amplified phagemid library.

Day 1

1. Grow an overnight culture (50 ml) of XLOLR cells, in LB broth with supplements at 30°C.

Day 2 (Early)

2. Re-grow the cells to mid-log phase by adding 0.25 ml of the XLOLR cells to 50 ml of LB broth with supplements, in a 250-ml flask. Incubate the cells at 37°C, with shaking, until the culture reaches an OD_{600} of 0.3–0.4.

3. Gently spin down the XLOLR cells (1000 × *g*). Resuspend the cells in 10 mM $MgSO_4$ to an OD_{600} of 1.0 (8×10^8 cells/ml).

4. In a 2-liter flask, combine XLOLR cells with a portion of the excision supernatant (from step 7 of the *Mass Excision Protocol)* at a minimum cells-to-phagemid ratio of 10:1. (Assume an OD_{600} of 1.0 equals a cell concentration of 8×10^8 cells/ml.) Amplify a portion of the excision supernatant which represents *at least* 10-fold more clones than found in the primary lambda library. Incubate the phagemids and cells at 37°C for 15 minutes.

5. Add 500 ml of LB broth containing 100 µg/ml of ampicillin[§]. Incubate with shaking at 37°C until an OD_{600} of 0.3–0.4 is reached. Do not incubate the cells overnight.

6. Spin at 500 × *g* for 10 minutes to pellet the cells. Isolate the plasmid DNA from the pelleted cells using any suitable method such as alkaline lysis.

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

Single-Clone Excision Protocol

Day 1

1. Core the plaque of interest from the agar plate and transfer the plaque to a sterile microcentrifuge tube containing 500 µl of SM buffer and 20 µl of chloroform. Vortex the microcentrifuge tube to release the phage particles into the SM buffer. Incubate the microcentrifuge tube for 1–2 hours at room temperature or overnight at 4°C. This phage stock is stable for up to 6 months at 4°C.

2. Grow separate 50-ml overnight cultures of XL1-Blue MRF´ and XLOLR cells in LB broth with supplements at 30°C.

Day 2

3. Gently spin down the XL1-Blue MRF´ and XLOLR cells (1000 × *g*). Resuspend each of the cell pellets in 25 ml of 10 mM $MgSO_4$. Measure the OD_{600}, then adjust the cell concentration to an OD_{600} of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$.

4. Combine the following components in a BD Falcon polypropylene tube:

   200 µl of XL1-Blue MRF´ cells at an OD_{600} of 1.0
   250 µl of phage stock (containing $>1 \times 10^5$ phage particles)
   1 µl of the ExAssist helper phage ($>1 \times 10^6$ pfu/µl)

   Note *Briefly spin the lambda phage stock to ensure that the chloroform is separated completely before removing the aliquot used in the excision reaction.*

5. Incubate the BD Falcon polypropylene tube at 37°C for 15 minutes to allow the phage to attach to the cells.

6. Add 3 ml of LB broth with supplements and incubate the BD Falcon polypropylene tube for 2.5–3 hours at 37°C with shaking. Because clonal representation is not relevant, single-clone excision reactions can be safely performed overnight.

   Note *The turbidity of the media is not indicative of the success of the excision.*

7. Heat the BD Falcon polypropylene tube at 65–70°C for 20 minutes to lyse the lambda phage particles and the cells. Spin the tube at 1000 × *g* for 15 minutes to pellet the cell debris.

8. Transfer the supernatant into a sterile BD Falcon polypropylene tube. This stock contains the excised pAD-GAL4-2.1 phagemid packaged as filamentous phage particles. (This stock may be stored at 4°C for 1–2 months.)

9. To plate the excised phagemids, add 200 µl of freshly grown XLOLR cells from step 3 (OD_{600} = 1.0) to two 1.5-ml microcentrifuge tubes. Add 100 µl of the phage supernatant (from step 8) to one microcentrifuge tube and 10 µl of the phage supernatant to the other microcentrifuge tube.

10. Incubate the microcentrifuge tubes at 37°C for 15 minutes.

11. Plate 200 µl of the cell mixture from each microcentrifuge tube on LB–ampicillin (100 µg/ml) agar plates and incubate the plates overnight at 37°C.

Due to the high-efficiency of the excision process, it may be necessary to titrate the supernatant to achieve single-colony isolation.

Colonies appearing on the plate contain the pAD-GAL4-2.1 double-stranded phagemid with the cloned DNA insert. Helper phage will not grow, since helper phage is unable to replicate in the Su^- (nonsuppressing) XLOLR strain and does not contain ampicillin-resistance genes.

To maintain the pAD-GAL4-2.1 phagemid, streak the colony on a new LB–ampicillin agar plate. For long-term storage, prepare a bacterial glycerol stock and store at –80°C.

DNA-BINDING DOMAIN VECTOR CONSTRUCTION

Bait Protein Insert Preparation, Ligation, and Transformation

DNA encoding the bait protein is prepared for insertion into the pBD-GAL4 Cam phagemid vector either by restriction digestion or PCR amplification. DNA encoding the bait protein must be inserted so that the bait protein is expressed in the same reading frame as the GAL4 BD (Figure 5). 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**. 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.

Dephosphorylate the digested pBD-GAL4 Cam phagemid vector 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 that will allow the concentration of the vector DNA to be 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:

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

We suggest the following protocol, which includes three control ligations:

	Experimental		Control		
Ligation Reaction Components	**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 pBD-GAL4 Cam phagemid vector 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 BD and that the DNA insert does not contain mutations.

Expression of the bait protein may be verified by Western blot analysis with an antibody that immunoreacts with either the protein expressed from the DNA insert or the GAL4 BD. However, if the antibody used fails to detect expression of the bait protein, it may not indicate that the bait protein is not expressed. The ability of the antibody to detect the bait protein is dependent on several factors including the affinity of the antibody for the bait protein and the expression level of the bait 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 protein can inhibit cell growth and even be lethal. Over-expression 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 pBD-GAL4 Cam phagemid vector containing DNA encoding the bait protein (bait plasmid) must be transformed into the yeast host and assayed for expression of the *lacZ* and *HIS3* reporter genes (described in *Yeast Transformation* and *Screening*). If the bait plasmid is capable of inducing expression of the *lacZ* and *HIS3* reporter genes in the absence of the pAD-GAL4-2.1 phagemid vector containing an insert, the bait plasmid is unsuitable for detecting protein–protein interactions in the HybriZAP-2.1 two-hybrid vector system. Expression of the reporter genes by the bait plasmid 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 bait protein may eliminate expression of the reporter genes but may also eliminate portions of the protein required for interaction.

YEAST TRANSFORMATION

Notes *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 agar	
	SD medium	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, YRG-2 yeast competent cells may be transformed with the bait and target plasmids in a cotransformation procedure. 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.[9] 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

Notes *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.

[§] See *Preparation of Media and Reagents*

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.

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.
- In step 2, add the 50 ml of the yeast cells containing the bait plasmid to 300 ml of SD medium lacking Trp.
- In step 3, grow the yeast cells in selective medium at 30°C with shaking at 225–250 rpm until the OD_{600} reaches approximately 0.5.

Transform 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 transformed 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.

VERIFICATION OF INTERACTION

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.[23] 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 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*.

TABLE X

Verification of the Transformation of the Bait and Target Plasmids

AD vector	BD vector	Selective medium	Expected result[a]
Target vector	—	SD agar plate without Leu	Growth, white
Target vector	pBD-WT	SD agar plate without Leu and Trp	Growth, white
Target vector	pBD-MUT	SD agar plate without Leu and Trp	Growth, white
Target vector	pLamin C	SD agar plate without Leu and Trp	Growth, white
Target vector	pBD-GAL4 Cam	SD agar plate without Leu and Trp	Growth, white
Target vector	Bait vector	SD agar plate without Leu and Trp	Growth, blue

[a] When transformed into YRG-2, plated on the selective medium and assayed for expression of the *lacZ* reporter gene.

TABLE XI

Verification of the Specificity of the Interaction between the Bait and Target Proteins

AD vector	BD vector	Selective medium	Expected result[a]
Target vector	pBD-WT	SD agar plate without Leu, Trp, and His	No growth
Target vector	pBD-MUT	SD agar plate without Leu, Trp, and His	No growth
Target vector	pLamin C	SD agar plate without Leu, Trp, and His	No growth
Target vector	pBD-GAL4 Cam	SD agar plate without Leu, Trp, and His	No growth
Target vector	Bait vector	SD agar plate without Leu, Trp, and His	Growth, blue

[a] When transformed into YRG-2, plated on the selective medium and assayed for expression of the *lacZ* reporter gene.

Verification of Specificity of Protein–Protein Interactions

To verify the specificity of the interaction between the bait and target proteins, transform yeast and plate on selective media as indicated in Tables X and XI. Assay the transformants for expression of the *HIS3* and *lacZ* reporter genes and compare the results of the assay to the expected results.

1. Prepare and transform the yeast competent cells by sequential transformation as described in the *Yeast Transformation Protocol*.

2. Streak the transformants that grow on the SD agar plates without Leu and Trp onto SD agar plates without Leu, Trp, and His. Incubate the plates for 3–7 days at 30°C.

3. Determine expression of the *lacZ* gene of the cotransformants from step 2 by the filter lift assay described in *Screening*.

If transformants do not give the expected results, see *Troubleshooting*. For additional discussion regarding false positives, see references 19, 24 and 25.

To identify the protein encoded by the target DNA, the nucleotide sequence of the target DNA can be determined and compared to protein and nucleotide sequence databases to identify related or homologous proteins. In addition, the target DNA can be used as a hybridization probe to screen the lambda library for full-length target DNA clones and for clones with high homology to the target DNA.

The DNA insert encoding the target protein can be transferred from the pAD-GAL4-2.1 vector to a protein expression/purification vector by digesting the vector with *Bam*H I, *Nhe* I, or *Eco*R I restriction enzymes at the 5´ end of the DNA insert and with *Xho* I, *Sal* I, *Xba* I, or *Bgl* II restriction enzymes at the 3´ end of the DNA insert. Prokaryotic expression vectors having compatible restriction sites include the pCAL-n and pCAL-n-EK vectors and the pGEX-4T-1, pGEX-4T-2, pGEX-4T-3, and pGEX-5X-1 vectors (available from Amersham Biosciences, Piscataway, New Jersey). A eukaryotic expression vector having compatible restriction sites is the pESP-2 vector. The pCAL-n and pCAL-n-EK vectors express the target protein as a fusion protein with the calmodulin peptide and the pGEX-4T-1, pGEX-4T-2, pGEX-4T-3, pGEX-5X-1, and pESP-2 vectors express the target protein as a fusion protein with glutathione-*s*-transferase (GST). These fusion proteins can then be used in *in vitro* immunoprecipitation assays with the bait protein.

Discussion regarding further verification of protein–protein interactions can be found in numerous publications.[19, 26]

APPENDIX I: 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

APPENDIX II: ETHIDIUM BROMIDE PLATE ASSAY—QUANTITATION OF DNA

An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

Preparation of Ethidium Bromide Plates

Note *Prepare the EtBr plates under a fume hood.*

Prepare 100 ml of 0.8% (w/v) agarose in TAE buffer. Cool the molten agarose to 50°C and then add 10 µl of EtBr stock solution (10 mg/ml). The EtBr stock solution is prepared in dH_2O and is stored in the dark at 4°C. Swirl to mix the EtBr stock solution and pour the solution into 100-mm petri dishes using ~10 ml/plate. Allow the plates to harden and incubate the plates at 37°C to dry, if necessary. These plates may be stored in the dark at 4°C for up to 1 month.

Preparation of Standards

Using a DNA sample of known concentration, make seven serial dilutions in 100 mM EDTA to cover the range from 200 to 10 ng/µl. These standards may be stored at –20°C for 3 months.

Plate Assay for Determination of DNA Concentration

Using a marker, label the petri dish to indicate where the sample and the standards (200, 150, 100, 75, 50, 25, and 10 ng/µl) will be spotted.

Thaw the standards and carefully spot 0.5 µl of each standard onto the surface of a prepared EtBr plate. Be careful not to dig into the surface of the plate. Let capillary action pull the small volume from the pipet tip to the plate surface and do not allow a bubble to form. Change pipet tips between each standard.

After spotting all of the standards, immediately spot 0.5 µl of the cDNA sample onto the plate adjacent to the line of standards. Allow all spots to absorb into the plate for 10–15 minutes at room temperature. Invert the plate and photograph it using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates.

Standards and unknowns must be spotted within 10 minutes of each other.

APPENDIX III: POLYMERASE CHAIN REACTION AMPLIFICATION OF LAMBDA DNA FROM INDIVIDUAL PLAQUES

The presence and size of a DNA insert in the HybriZAP-2.1 vector may be determined by PCR amplification of DNA from individual plaques.

1. Prepare plates containing individual plaques as described in *Amplification of the HybriZAP-2.1 Library*.

2. Select a well-isolated individual plaque for analysis using a sterile pipet tip and place the plaque into 100 µl of SM buffer. Vortex.

3. In order to amplify the DNA from an individual plaque, prepare a PCR amplification reaction containing the following components:

   1.0 µl of SM buffer containing the plaque generated by the HybriZAP-2.1 two-hybrid vector system
   4.0 µl of 10× *Taq* DNA polymerase buffer (see *Preparation of Media and Reagents*)
   0.4 µl of dNTP mix (25 mM each dNTP)
   40.0 ng of 5′ AD primer
   40.0 ng of 3′ AD primer
   0.4 µl of 10% (v/v) Tween® 20
   1.0 U of *Taq* DNA polymerase
   dH_2O to a final volume of 40 µl

4. Amplify the DNA using the following PCR cycling conditions:

Number of cycles	Temperature	Length of time
1 cycle	93°C	5 minutes
	48°C	5 minutes
30 cycles	72°C	3 minutes
	93°C	1 minute
	48°C	1 minute
1 cycle	72°C	10 minutes

5. Analyze the PCR products on a 1% (w/v) agarose gel. The HybriZAP-2.1 vector without an insert will produce a PCR product which is 204 bp in length. PCR products which are >204 bp in length represent the HybriZAP-2.1 vector with an insert.

Vector	Primer	Binds to nucleotide	Nucleotide sequence (5′ to 3′)
HybriZAP-2.1 vector (AD)	5′ AD	745–765	AGGGATGTTTAATACCACTAC
	3′ AD	929–949	GCACAGTTGAAGTGAACTTGC

TROUBLESHOOTING

Mass Excision

Observation	Suggestion
The number of Amp^r colonies in the mass excision are greater than expected	All of the host cells are not infected with helper phage during the mass excision, allowing lambda phage to replicate lytically before conversion to the phagemid. Verify the titer of lambda phage and helper phage prior to the mass excision; infect the host cells with helper phage prior to growing to mid log phase on *Day 2* (cells infected with the helper phage will grow more slowly than uninfected cells).
Absence of Amp^r colonies	Ensure that XL1-Blue MRF′ host cells are used for the mass excision.

Two-Hybrid Vector System Screening

Observation	Suggestion
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 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 Strain Genotype and Phenotypic Verification* 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.
	Verify that the correct control plasmid pair is used.

(*table continues on the next page*)

(*table continues from the previous page*)

Observation	Suggestion
The transformants of the pLamin C and pAD-WT pair of control plasmids or the pLamin C and pAD-MUT pair of control plasmids turn blue	Verify that the correct control plasmid pair is used.
No His^+–$LacZ^+$ transformants are present	Verify the nucleotide sequence of the GAL4 BD and insert DNA to ensure the bait protein is expressed.
	The frequency of target proteins in the library may be low. Prepare and screen additional cotransformants; screen a different library.
	To verify that the screening procedure is working, screen a library in which expression of the bait protein is known.
	Verify the pH of the SD agar plates using a pH indicator strip.
	Vary the fusion point of the GAL4 BD and the bait protein to highlight possible steric inhibition.
	Verify that the competent cells containing the bait plasmid were used to transform the target plasmid(s).
	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 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.

Verification of Interaction

Observation	Suggestion
Presence of His^+–$LacZ^+$ colonies when the target plasmid alone is transformed into yeast	If the target protein is capable of initiating transcription of both reporter genes in the absence of the bait protein, the target plasmid is a false positive and should be discarded.
Presence of His^+–$LacZ^+$ colonies when the target plasmid and the pBD-WT and pLamin C pair of control plasmids, the pBD-MUT and pLamin C pair of control plasmids, or the pBD-GAL4 Cam phagemid vector is transformed into yeast	If the target protein is capable of initiating transcription of both reporter genes in the presence of the BD vector irrespective of the expression of a bait protein, the target plasmid is a false positive and should be discarded.
Absence of His^+–$LacZ^+$ colonies when target and bait plasmids are transformed into yeast	Determine if the original yeast host contained more than one target plasmid by miniprep analysis of target plasmid DNAs.

PREPARATION OF MEDIA AND REAGENTS

Standard Media and Reagents

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

10× Fill-In Buffer

60 mM Tris-HCl (pH 7.5)
60 mM NaCl
60 mM $MgCl_2$
0.5% (w/v) gelatin
10 mM dithiothreitol (DTT)

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
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

LB–Ampicillin Broth (per Liter)

1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

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 Broth with Supplements

Prepare 1 liter of LB broth
Autoclave
Add the following filter-sterilized supplements prior to use
10 ml of 1 M $MgSO_4$
3 ml of a 2 M maltose solution or 10 ml of 20% (w/v) maltose

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 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

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave

NZY Top Agar (per Liter)

Prepare 1 liter of NZY broth
Add 0.7% (w/v) agarose
Autoclave

NZY Agar (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
15 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate or ~80 ml/150-mm plate)

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*

TE Buffer

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

SM Buffer (per Liter)

5.8 g of NaCl
2.0 g of $MgSO_4 \cdot 7H_2O$
50.0 ml of 1 M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin
Add deionized H_2O to a final volume of 1 liter
Autoclave

10× STE Buffer

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

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

10× *Taq* DNA Polymerase Buffer

100 mM Tris-HCl (pH 8.8)
15 mM $MgCl_2$
500 mM KCl
0.01% (w/v) gelatin

[‖] 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 27)
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

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ENDNOTES

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STRATAGENE

An **Agilent Technologies** Division

HybriZAP-2.1 Two-Hybrid Predigested Vector

Catalog #235601

QUICK-REFERENCE PROTOCOL

- Ligate the bait DNA insert into the pBD-GAL4 Cam phagemid vector
- Transform into yeast and assay for reporter gene expression
- Produce a cDNA or genomic library in the HybriZAP-2.1 vector
- Mass excise to form the pAD-GAL4-2.1 library (target plasmid)
- Transform yeast containing the pBD-GAL4 Cam phagemid vector (bait plasmid) with the pAD-GAL4-2.1 library
- Assay cotransformants for reporter gene expression
- Restreak putative positives and reassay for reporter gene expression
- Isolate plasmids from yeast and transform into *E. coli*
- Isolate the pAD-GAL4-2.1 phagemid vector (target plasmid)
- Cotransform the target plasmid with the pBD-GAL4 Cam control plasmids into yeast and assay for expression of reporter genes
- Discard the target plasmids that induce expression of reporter genes with the pBD-GAL4 Cam control plasmids
- Perform secondary assays with those target plasmids that do not induce expression of reporter genes with the pBD-GAL4 Cam control plasmids