HybriZAP-2.1 XR Library Construction Kit and HybriZAP-2.1 XR cDNA Synthesis Kit

Instruction Manual

Catalog #235612 (HybriZAP-2.1 XR Library Construction Kit) and
#235614 (HybriZAP-2.1 XR cDNA Synthesis Kit)
Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

235612-12

Agilent Technologies

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.genomics.agilent.com* and click **Contact Us**

HybriZAP-2.1 XR Library Construction Kit and HybriZAP-2.1 XR cDNA Synthesis Kit

CONTENTS

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

HybriZAP-2.1 XR Library Construction Kit and HybriZAP-2.1 XR cDNA Synthesis Kit

MATERIALS PROVIDED

Caution ***DO NOT*** *substitute the components in this kit with components from another kit. Component substitution may result in lower efficiency library construction*

Materials Provided	Quantity	
	Catalog #235612	**Catalog #235614**
cDNA Synthesis Kit (see table below for component list)	1 kit	1 kit
HybriZAP-2.1 vector predigested with *Eco*R I and *Xho* I, SAP treated (1 µg/µl)	10 µg	10 µg
pBR322 test insert (4.4 kb, 0.25 µg/µl)	2.5 µg	2.5 µg
pBD-GAL4 Cam phagemid vector, undigested	20 µg	20 µg
Two-Hybrid Control Plasmids		
pGAL4 control plasmid (1 µg/µl)	20 µg	20 µg
pBD-WT control plasmid (1 µg/µl)	20 µg	20 µg
pAD-WT control plasmid (1 µg/µl)	20 µg	20 µg
pBD-MUT control plasmid (1 µg/µl)	20 µg	20 µg
pAD-MUT control plasmid (1 µg/µl)	20 µg	20 µg
pLamin C control plasmid (1 µg/µl)	20 µg	20 µg
XL1-Blue MRF´ strain[a]	glycerol stock	glycerol stock
XLOLR strain[a]	glycerol stock	glycerol stock
ExAssist interference-resistant helper phage (~1× 10^{10} pfu/ml)	1 ml	1 ml
Gigapack III Gold packaging extract[b,c]		
Gigapack III Gold-11 packaging extract	11 × 25 µl	—
λcI857 *Sam*7 wild-type lambda control DNA	1.05 µg	—
VCS257 host strain[d]	glycerol stock	—
XL1-Blue subcloning-grade competent cells[e]	8 × 0.5-ml aliquots	8 × 0.5-ml aliquots
pUC18 transformation control plasmid (0.1 ng/µl)	10 µl	10 µl
YRG-2 yeast host strain	glycerol stock	glycerol stock

[a]Use the XLOLR strain for plating excised phagemids and the XL1-Blue MRF´ strain for all other manipulations.

[b]Gigapack III packaging extract is very sensitive to slight variations in temperature. Store the packaging extracts at the bottom of a –80°C freezer directly from the dry ice shipping container in order to prevent a loss of packaging efficiency. Transferring tubes from one freezer to another may result in a loss of efficiency. **Do not allow the packaging extracts to thaw!** Do not store the packaging extracts in liquid nitrogen as the tubes may explode.

[c] Depending on the cloning efficiencies achieved, purchase of additional Gigapack III Gold packaging extract may be necessary. Enough reagents are included to generate five vector-ligated constructs.

[d]The VCS257 host strain is included for plating the λcI857 *Sam*7 positive control. This control host strain is a derivative of DP50 *supF* and should be used only when plating the packaged test DNA. The control DNA used with Gigapack III Gold packaging extract requires a *supF* mutation in the bacterial host to plate efficiently.

[e]Expect a transformation efficiency of 1 × 10^6 cfu/µg of pUC18 DNA.

Revision C

cDNA Synthesis Kit

Reagents provided[a]	Quantity	Storage temperature
First-strand reagents		
AccuScript reverse transcriptase (AccuScript RT)	15 µl	–20°C
RNase Block Ribonuclease Inhibitor (40 U/µl)	200 U	–20°C
First-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP)	15 µl	–20°C
First-strand buffer (10×)	75 µl	–20°C
Linker–primer (1.4 µg/µl)	10 µl	–20°C
Test poly(A)$^+$ RNA (0.2 µg/µl)	5 µg	–20°C
Diethylpyrocarbonate (DEPC)-treated water	500 µl	–20°C
Second-strand reagents		
Second-strand buffer (10×)	150 µl	–20°C
Second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP)	30 µl	–20°C
Escherichia coli RNase H (1.5 U/µl)	15 U	–20°C
Escherichia coli DNA polymerase I (9.0 U/µl)	500 U	–20°C
Sodium acetate (3 M)	250 µl	–20°C
Blunting reagents		
Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)	115 µl	–20°C
Cloned *Pfu* DNA polymerase (2.5 U/µl)	25 U	–20°C
Ligation reagents		
*Eco*R I adapters (0.4 µg/µl)	18 µg	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
T4 DNA ligase[b] (4 U/µl)	140 U	–20°C
Phosphorylation reagents		
T4 polynucleotide kinase (5 U/µl)	50 U	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
Xho I digestion reagents		
Xho I (40 U/µl)	600 U	–20°C
Xho I buffer supplement	250 µl	–20°C
Column reagents[d]		
Connecting tubing d (1/8-inch i.d., 3/16-inch o.d., and 1/32-inch wall)	1 × 4 cm	Room temperature or 4°C
Sepharose® CL-2B gel filtration medium	10 ml	4°C
Column-loading dye[c]	17.5 µl	4°C
STE buffer[c] (10×)	10 ml	4°C

[a] Enough reagents are included to generate five vector-ligated constructs.

[b] These reagents are used more than once in the reaction.

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

[d] The column reagents are shipped separately at 4°C.

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.)

Control Plasmids: –20°C

Helper Phage: –80°C. Retiter prior to each use if passed through more than one freeze–thaw cycle.

Test Insert: –20°C

Bacterial Glycerol Stocks: –80°C

Yeast Glycerol Stock: –80°C

Packaging Extracts: –80°C. **Do not allow the packaging extracts to thaw**. Transfer the packaging extracts to the bottom of a –80°C freezer directly from the dry ice shipping container. Do not store the packaging extracts in liquid nitrogen as the tubes may explode.

lambda control DNA (λ*c*I857 *Sam*7): –80°C

Subcloning-Grade Competent Cells: –80°C. Do not place the competent cells in liquid nitrogen.

cDNA Synthesis Kit Column Reagents: 4°C

cDNA Synthesis Kit Other Reagents: –20°C

ADDITIONAL MATERIALS REQUIRED

Reagents and Solutions

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

Note *Do not use the low-pH phenol from the Agilent 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.*

[α-^{32}P]Deoxynucleoside triphosphate ([α-^{32}P]dNTP) (800 Ci/mmol) ([α-^{32}P]dATP, [α-^{32}P]dGTP, or [α-^{32}P]dTTP may be used; do not use [α-^{32}P]dCTP)

Salmon sperm DNA

Equipment and Supplies

Ribonuclease (RNase)-free microcentrifuge tubes

Wide-bore pipet tips

Disposable plastic 10-ml syringes, sterile

Disposable 18-gauge, 1½-inch needles, sterile

Disposable plastic 1-ml pipets, negatively graduated and sterile [e.g., BD Falcon 1-ml serological pipet, Catalog #357520 or equivalent]

Portable radiation monitor (Geiger counter)

Water baths (4°, 8°, 12°, 16°, 30°, 37°, 42°, 65°, 70°, and 72°C)

Vacuum evaporator

Incubator (30° and 37°C)

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences, Catalog #352059)

Acid-washed glass beads (425–600 μm)

Whatman® No. 1 qualitative filter paper, Grade 1 {Fisher Scientific, Pittsburgh, Pennsylvania [Catalog #09-805C (7 cm diameter) and #09-805F (12.5 cm diameter)]}

VWRbrand™ qualitative filter papers, Grade No. 413 [VWR Scientific, Westchester, Pennsylvania, Catalog #28310-026 (7.5 cm diameter)] and #28310-106 (12.5 cm diameter)]

NOTICE TO PURCHASER

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

INTRODUCTION

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

The 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 and 5,468,614.

Overview of HybriZAP-2.1 Two-Hybrid Library Screens

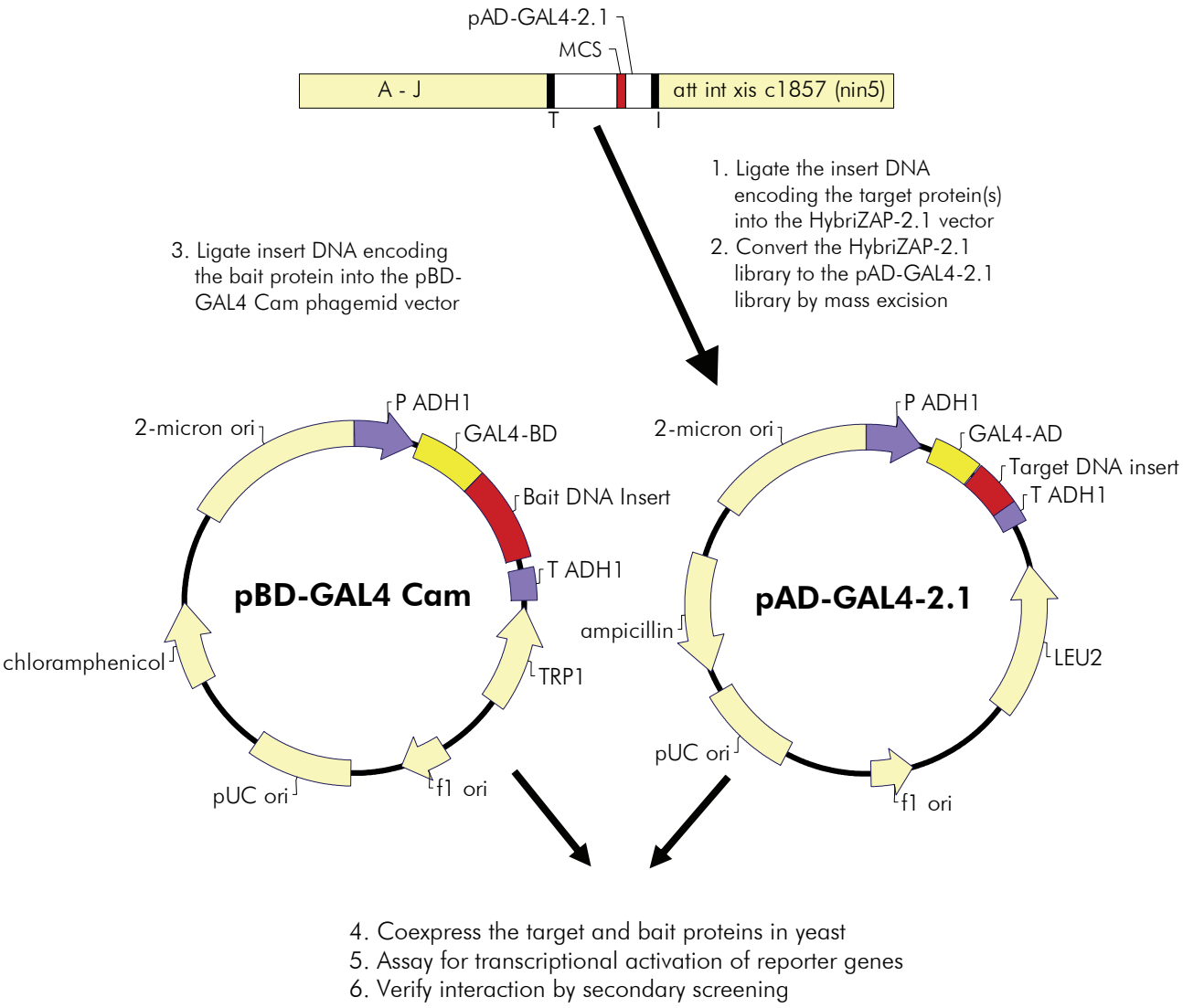


FIGURE 1 The HybriZAP-2.1 two-hybrid vector system. DNA inserts are ligated into the HybriZAP-2.1 vector to generate the primary lambda library. This primary lambda library is amplified and converted by *in vivo* mass excision to a pAD-GAL4-2.1 library. DNA that expresses a library of the GAL4 AD hybrid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

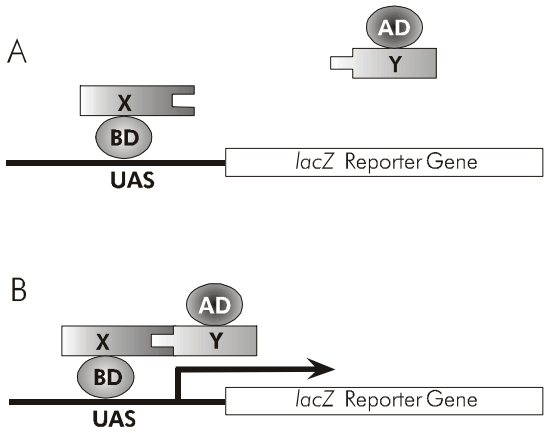


FIGURE 2 Detection of interacting proteins by transcription of the *lacZ* reporter gene. The GAL4 UAS and the *lacZ* reporter gene are integrated into the yeast chromosome. (A) The GAL4 BD hybrid protein (BD and the bait protein *X*) binds to the GAL4 UAS present upstream of the *lacZ* reporter gene. 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 Agilent 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

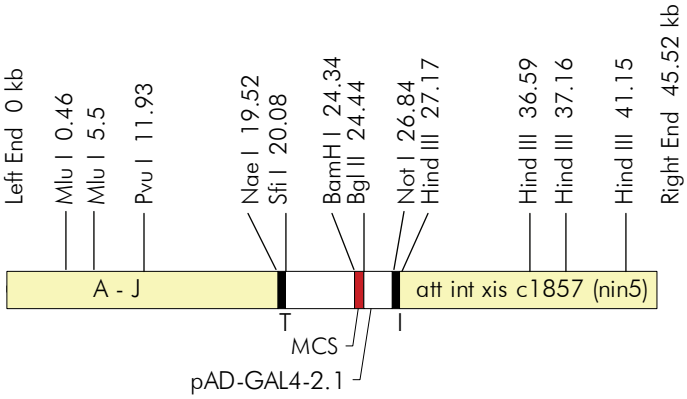
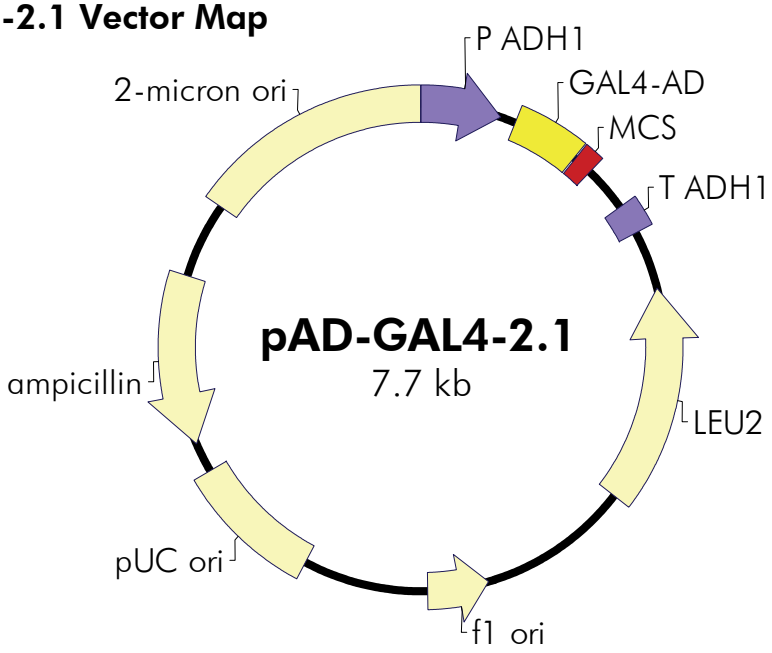


FIGURE 3 Restriction map of the HybriZAP-2.1 vector. The HybriZAP-2.1 vector contains lambda genes *A* through *J* in the left arm and *att*, *int*, *xis*, and cI857 in the right arm. The f1 initiator (I) and terminator (T) allow efficient *in vivo* excision of the pAD-GAL4-2.1 phagemid vector from the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

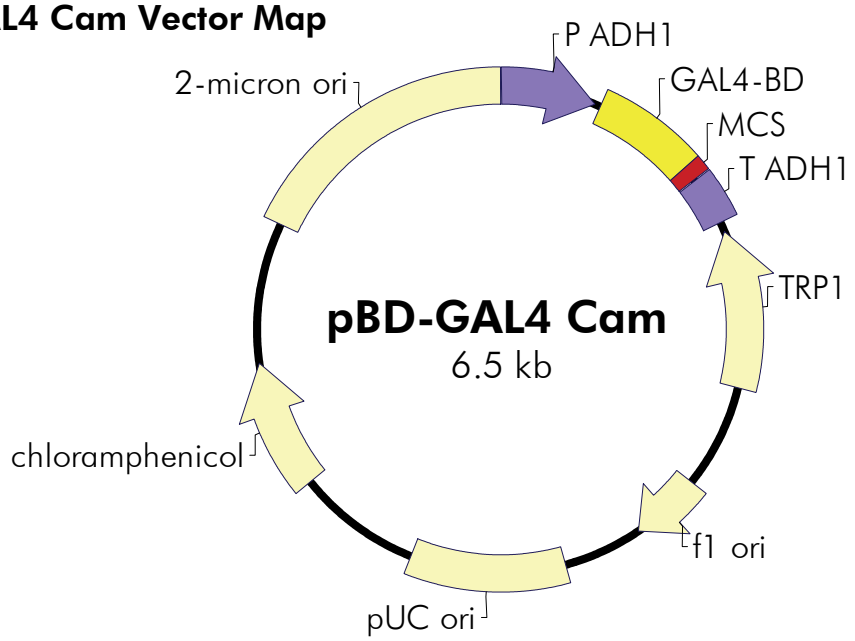
...ACT GCA GAG ATC TAT GAA TCG TAG ATA CTG AAA AAC 3´

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.genomics.agilent.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.genomics.agilent.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 XL1-Blue MRF′ compatible with cloning both cDNA constructed using the Agilent cDNA Synthesis Kit and genomic DNA.*

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]
VCS257 strain[d]	LB[a]	LB broth with supplements[a-c]	NZY[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.

[d] For use with Gigapack III Gold packaging extract and wild-type lambda DNA control only. Supplied with Gigapack III Gold packaging extract.

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 that 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.

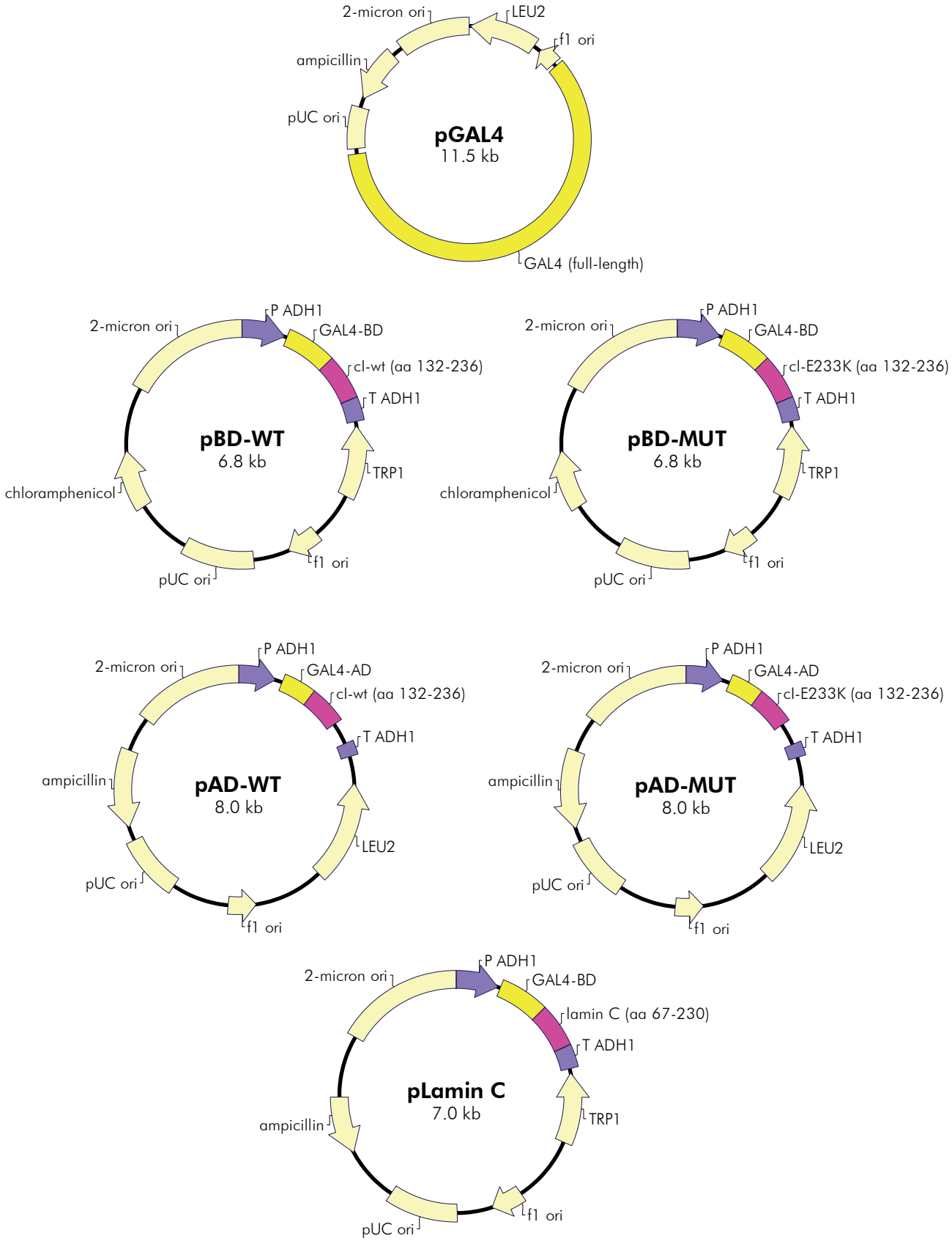


FIGURE 6 Circular maps of the control plasmids.

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 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.

cDNA Libraries

DNA inserts to be ligated into the HybriZAP-2.1 vector are prepared from mRNA. The cDNA Synthesis Kit provides the reagents required to convert mRNA to cDNA inserts prior to unidirectional insertion into the HybriZAP-2.1 vector. The protocols for preparing cDNA inserts are found in *cDNA Insert Preparation*.

cDNA Insert Preparation

Background for Preparation of cDNA Libraries

Complementary DNA libraries represent the information encoded in the mRNA of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then is inserted into a self-replicating lambda vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease. Therefore, cDNA library construction provides a method by which the transcription and processing of mRNA can be examined and interpreted.

The cDNA Synthesis Kit uses a hybrid oligo(dT) linker–primer that contains an *Xho* I restriction site. First-strand synthesis is primed with the linker–primer and the mRNA is reverse-transcribed using AccuScript reverse transcriptase (AccuScript RT) and 5-methyl dCTP.

AccuScript RT is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3'-5' exonuclease. AccuScript reverse transcriptase delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript reverse transcriptase delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy. These advantages make AccuScript RT the enzyme of choice for applications involving the preparation of accurate, full-length, cDNA transcripts, including first-strand cDNA synthesis and library construction.

The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, which protects the cDNA from digestion with certain restriction endonucleases such as *Xho* I. Therefore, on *Xho* I digestion of the cDNA, only the unmethylated site within the linker–primer is cleaved.

Hemimethylated DNA introduced into an McrA$^+$ McrB$^+$ strain would be subject to digestion by the *mcrA* and *mcrB* restriction systems. Therefore, it is necessary to initially infect an McrA$^-$ McrB$^-$ strain (e.g., the XL1-Blue MRF´ strain supplied with the HybriZAP-2.1 vector) when using the cDNA Synthesis Kit. After passing the library through XL1-Blue MRF´ cells, the DNA is no longer hemimethylated and can be grown on McrA$^+$ McrB$^+$ strains (e.g., XL1-Blue strain).

Note *Use high-efficiency Gigapack III Gold packaging extract[6, 20] since it is McrA$^-$, McrB$^-$, and Mrr$^-$. Other commercially available packaging extracts can restrict hemimethylated DNA, therefore producing low-titer libraries.*

cDNA Synthesis

The yield, length, and accuracy of cDNA transcripts is enhanced with the use of AccuScript RT, an engineered version of the Moloney murine leukemia virus reverse transcriptase combined with a proofreading 3'-5' exonuclease. First-strand cDNA synthesis begins when AccuScript RT, in the presence of nucleotides and buffer, finds a template and a primer. The template is mRNA and the primer is a 50-base oligonucleotide with the following sequence:

5´-GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTTT-3´
"GAGA" Sequence *Xho* I Poly(dT)

This oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the HybriZAP-2.1 vector in a sense orientation (*Eco*R I–*Xho* I) with respect to the P *AHD1* The poly(dT) region binds to the 3´ poly(A) region of the mRNA template, and AccuScript RT begins to synthesize the first-strand cDNA.

The nucleotide mixture for the first strand contains normal dATP, dGTP, and dTTP plus the analog 5-methyl dCTP. The complete first strand will have a methyl group on each cytosine base, which will protect the cDNA from restriction enzymes used in subsequent cloning steps.

During second-strand synthesis, RNase H nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. DNA polymerase I "nick-translates" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture has been supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. This ensures that the restriction sites in the linker–primer will be susceptible to restriction enzyme digestion. The uneven termini of the double-stranded cDNA are nibbled back or filled in with cloned *Pfu* DNA polymerase, and *Eco*R I adapters are ligated to the blunt ends. The adapters have the sequence shown below.

```
5´-OH-AATTCGGCACGAGG-3´
      3´-GCCGTGCTCCp-5´
```

These adapters are composed of 10- and 14-mer oligonucleotides, which are complementary to each other with an *Eco*R I cohesive end. The 10-mer oligonucleotide is phosphorylated, which allows it to ligate to other blunt termini available in the form of cDNA and other adapters. The 14-mer oligonucleotide is kept dephosphorylated to prevent it from ligating to other cohesive ends. After adapter ligation is complete and the ligase has been heat inactivated, the 14-mer oligonucleotide is phosphorylated to enable its ligation into the dephosphorylated vector arms.

The *Xho* I digestion releases the *Eco*R I adapter and residual linker–primer from the 3´ end of the cDNA. These two fragments are separated on a drip column containing Sepharose® CL-2B gel filtration medium. The size-fractionated cDNA is then precipitated and ligated to the HybriZAP-2.1 vector.

The lambda library is packaged in a high-efficiency system such as Gigapack III Gold packaging extract [4, 20] and is plated on the *E. coli* cell line XL1-Blue MRF´. **Since most *E. coli* strains digest DNA containing 5´-methyl dCTP, it is important to plate on this McrA⁻ McrB⁻ strain.**

Note *An outline of the Agilent cDNA Synthesis Kit is provided (see Figure 7). If you plan to be away from the project for 1 or 2 days, We recommend scheduling the synthesis such that the cDNA remains in the ligation reaction. Even though the majority of ligation is complete in the time recommended by the procedure, the ligase is extremely active and will continue to find and ligate available ends. Although most investigators wish to produce their cDNA libraries as rapidly as possible, it is important to remember that extended ligations and overnight precipitations can increase the yield.*

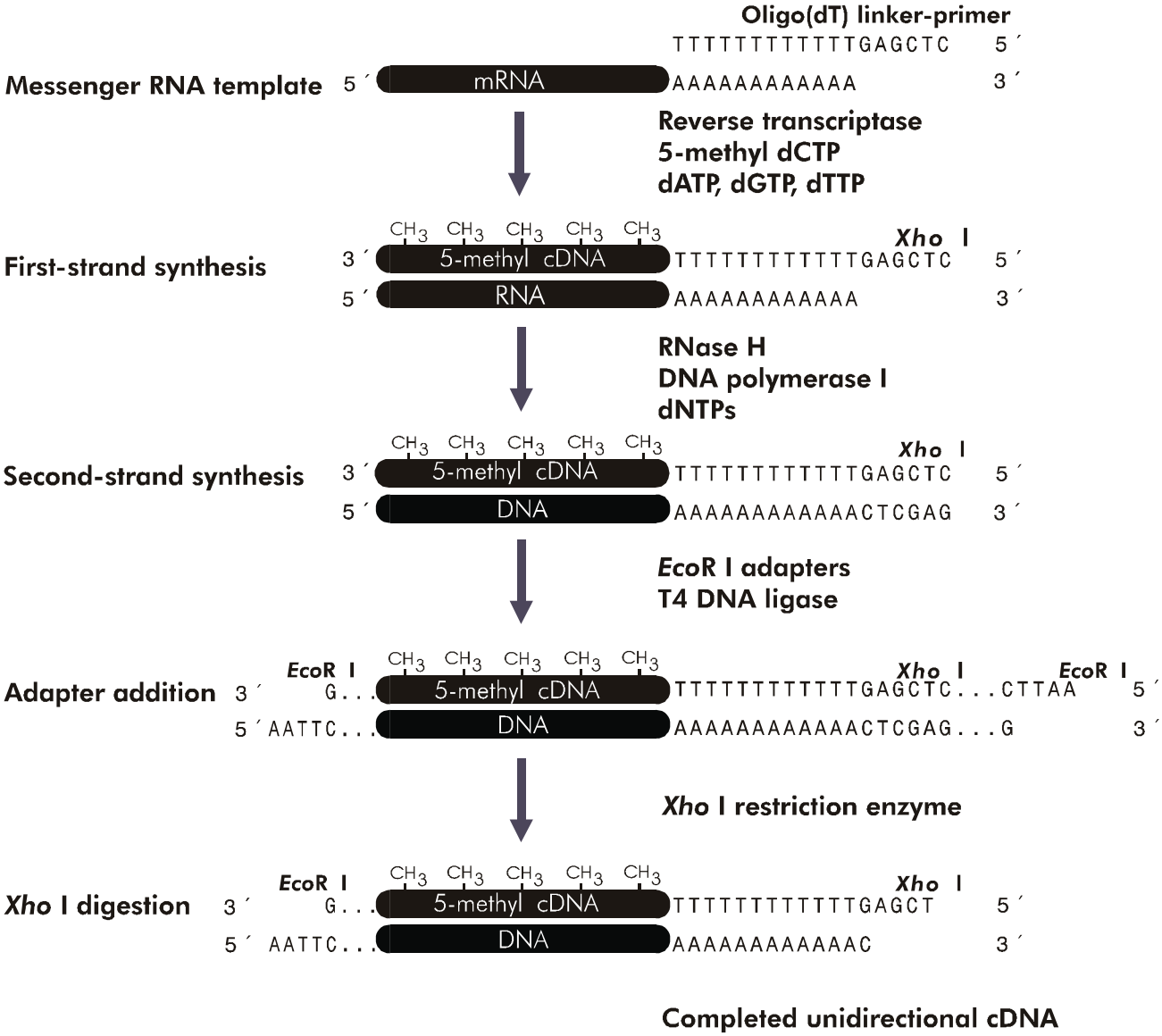


FIGURE 7 cDNA synthesis flow chart.

Generation of cDNA Inserts

Notes ***DO NOT*** *substitute the reagents in this kit with reagents from another kit. Component substitution may result in lower efficiency library construction.*

The following protocol has been optimized for 5 μg of poly(A)$^+$ RNA.

Protocol Guidelines

- The quality and quantity of the mRNA used is of fundamental importance to the construction of a large, representative cDNA library (see *Appendix II: RNA Purification and Quantitation*). Agilent's RNA Isolation Kit uses the guanidinium thiocyanate–phenol–chloroform extraction method,[21] which quickly produces large amounts of undegraded RNA. To isolate mRNA, Agilent offers the Poly(A) Quik mRNA isolation kit.

- Secondary structure of mRNA may cause the synthesis of truncated cDNAs. To relax secondary structure, treatment with methylmercury hydroxide (CH_3HgOH) is recommended (see *Appendix III: Methylmercury Hydroxide Treatment*).

- It is imperative to protect the RNA from any contaminating RNases until the first-strand cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

- When removing aliquots of any of the enzymes used in the HybriZAP-2.1 cDNA synthesis protocol, flick the bottom of the tube to thoroughly mix the enzyme solution. Do not vortex the enzyme stock tubes.

Synthesizing First-Strand cDNA

1. Prepare water baths at 16°, 42°, and 72°C.

2. Thaw the radioactive [α-^{32}P]dNTP (do not use [^{32}P]dCTP) and all nonenzymatic first-strand components. Keep the radioactive dNTP on ice for use in step 6 and in the second-strand synthesis. Briefly vortex and spin down the contents of the nonenzymatic tubes. Place the tubes on ice.

   Note *AccuScript RT is temperature sensitive and should remain at –20°C until the last moment.*

3. The final volume of the first-strand synthesis reaction is 50 µl. The volume of added reagents and enzymes is 14 µl, thus the mRNA template and DEPC-treated water should be added in a combined volume of 36 µl. For the control reaction, prepare the following annealing reaction with 25 µl (5 µg) of test RNA and 11 µl of DEPC-treated water.

4. In an RNase-free microcentrifuge tube, add the following reagents in order:

   5 µl of 10× first-strand buffer
   3 µl of first-strand methyl nucleotide mixture
   2 µl of linker–primer (1.4 µg/µl)
   X µl of DEPC-treated water
   1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)

5. Mix the reaction and then add *X* µl of poly(A)$^+$ RNA (5 µg). Mix gently.

6. Allow the primer to anneal to the template for 10 minutes at room temperature. During the incubation, aliquot 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol) into a separate tube for the control.

7. Add 3 µl of AccuScript RT to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction should now be 50 µl.

8. Mix the sample gently and spin down the contents in a microcentrifuge.

9. Transfer 5 µl of the first-strand synthesis reaction to the separate tube containing the 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol). This radioactive sample is the first-strand synthesis control reaction.

10. Incubate the first-strand synthesis reactions, including the control reaction, at 42°C for 1 hour.

11. Prepare a 16°C water bath for second-strand synthesis. If a water bath with a cooling unit is not available, use a large Styrofoam® container with a lid. Fill the container three-quarters full with water and adjust the temperature to 16°C with ice. Cover the container with a lid.

12. After 1 hour, remove the first-strand synthesis reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reaction on ice. Store the radioactive first-strand synthesis control reaction at –20°C until ready to resolve by electrophoresis on an alkaline agarose gel (see *Appendix IV: Alkaline Agarose Gels*). Agilent advises running the radioactive first-strand reaction after the second-strand reaction has been blunted and resuspended in the *Eco*R I adapters (see step 17 in *Blunting the cDNA Termini*).

Synthesizing Second-Strand cDNA

1. Thaw all nonenzymatic second-strand components. Briefly vortex and spin in a microcentrifuge before placing the tubes on ice.

   Note *It is important that all reagents be <16°C when the DNA polymerase I is added.*

2. Add the following components in order to the 45-µl nonradioactive, first-strand synthesis reaction on ice:

   20 µl of 10× second-strand buffer
   6 µl of second-strand dNTP mixture
   114 µl of sterile dH_2O (DEPC-treated water is not required)
   2 µl of [α-^{32}P]dNTP (800 Ci/mmol)

3. Add the following enzymes to the second-strand synthesis reaction:

   2 µl of RNase H (1.5 U/µl)
   11 µl of DNA polymerase I (9.0 U/µl)

4. Gently vortex the contents of the tube, spin the reaction in a microcentrifuge, and incubate for 2.5 hours in a 16°C water bath. Check the water bath occasionally to ensure that the temperature does not rise above 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector.

5. After second-strand synthesis for 2.5 hours at 16°C, immediately place the tube *on ice.*

Blunting the cDNA Termini

1. Add the following to the second-strand synthesis reaction:

   23 µl of blunting dNTP mix
   2 µl of cloned *Pfu* DNA polymerase

2. Quickly vortex the reaction and spin down in a microcentrifuge. Incubate the reaction at 72°C for 30 minutes. **Do not exceed 30 minutes!!**

3. Thaw the 3 M sodium acetate.

   Note *Since radioactivity can leak out between the lid and body of some micro-centrifuge tubes during the vortexing and precipitation steps, wrap a small piece of Parafilm laboratory film around the rim of the microcentrifuge tube to prevent leakage.*

4. Remove the reaction and add 200 µl of phenol–chloroform [1:1 (v/v)] and vortex.

   Note ***Do not use the low-pH phenol from Agilent's RNA Isolation Kit because this phenol is too acidic and may denature the DNA.*** *The phenol must be equilibrated to pH 7–8.*

5. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube. Be careful to avoid removing any interface that may be present.

6. Add an equal volume of chloroform and vortex.

7. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube.

8. Precipitate the cDNA by adding the following to the saved aqueous layer:

   20 µl of 3 M sodium acetate
   400 µl of 100% (v/v) ethanol

   Vortex the reaction.

9. Precipitate overnight at –20°C.

10. In order to orient the direction of precipitate accumulation, place a mark on the microcentrifuge tube or point the tube hinge away from the center of the microcentrifuge as an indicator of where the pellet will form.

11. Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C.

12. Avoid disturbing the pellet and carefully remove and discard the radioactive supernatant in a radioactive waste container.

    Note *The conditions of synthesis and precipitation produce a large white pellet. The pellet accumulates near the bottom of the microcentrifuge tube and may taper up along the marked side of the tube.*

13. Gently wash the pellet by adding 500 µl of 70% (v/v) ethanol to the side of the tube away from the precipitate. **Do not mix or vortex!**

14. Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature with the orientation marked as in step 10.

15. Aspirate the ethanol wash and dry the pellet by vacuum centrifugation.

16. Resuspend the pellet in 9 µl of *Eco*R I adapters and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend. To ensure that the cDNA is completely in solution, transfer the cDNA to a fresh microcentrifuge tube. Monitor the now empty tube with a handheld Geiger counter. If the cDNA is in solution, few counts should remain in the empty tube.

17. **Transfer 1 µl of this second-strand synthesis reaction to a separate tube. This radioactive sample is the second-strand synthesis control reaction.** Agilent strongly recommends running the samples of the first- and second-strand synthesis reactions on an alkaline agarose gel at this point. It is important to determine the size range of the cDNA and the presence of any secondary structure (see *Appendix IV: Alkaline Agarose Gels*).

    Note *The second-strand synthesis reaction can be stored overnight at –20°C.*

Ligating the *Eco*R I Adapters

1. Add the following components to the tube containing the blunted cDNA and the *Eco*R I adapters:

   1 µl of 10× ligase buffer
   1 µl of 10 mM rATP
   1 µl of T4 DNA ligase (4 U/µl)

2. Spin down the volume in a microcentrifuge and incubate overnight at 8°C. Alternatively, the ligations can be incubated at 4°C for 2 days.

3. In the morning, heat inactivate the ligase by placing the tubes in a 70°C water bath for 30 minutes.

Phosphorylating the *Eco*R I Ends

1. After the ligase is heat inactivated, spin the reaction in a microcentrifuge for 2 seconds. Cool the reaction at room temperature for 5 minutes.

2. Phosphorylate the adapter ends by adding the following components:

   1 µl of 10× ligase buffer
   2 µl of 10 mM rATP
   5 µl of sterile water
   2 µl of T4 polynucleotide kinase (5 U/µl)

3. Incubate the reaction for 30 minutes at 37°C.

4. Heat inactivate the kinase for 30 minutes at 70°C.

5. Spin down the condensation in a microcentrifuge for 2 seconds and allow the reaction to equilibrate to room temperature for 5 minutes.

Digesting with *Xho* I

1. Add the following components to the reaction:

   28 µl of *Xho* I buffer supplement
   3 µl of *Xho* I (40 U/µl)

2. Incubate the reaction for 1.5 hours at 37°C.

3. Add 5 µl of 10× STE buffer and 125 µl of 100% (v/v) ethanol to the microcentrifuge tube.

4. Precipitate the reaction overnight at –20°C.

5. Following precipitation, spin the reaction in a microcentrifuge at maximum speed for 60 minutes at 4°C.

6. Discard the supernatant, dry the pellet completely, and resuspend the pellet in 14 µl of 1× STE buffer.

7. Add 3.5 µl of the column-loading dye to each sample.

The sample is now ready to be run through a drip column containing Sepharose CL-2B gel filtration medium (see *Size Fractionating*).

Size Fractionating

Before attempting the experimental protocols outlined within this section, please read this section in its entirety in order to become familiar with the procedures. Review of the *Troubleshooting* section may also prove helpful. The drip columns should be prepared and the cDNA should be eluted in 1 day. Because a full day is required to complete these procedures, gathering all necessary materials in advance is recommended (see *Additional Materials Required*).

Assembling the Drip Column

1. Perform the following preparatory steps while assembling the drip columns:

   a. Remove the Sepharose CL-2B gel filtration medium and the 10× STE buffer from refrigeration and equilibrate the two components to room temperature.

   b. Prepare 50 ml of 1× STE buffer by diluting 10× STE buffer 1:10 in sterile water.

2. Assemble the drip columns as outlined in the following steps (see Figure 8 for a diagram of the final setup):

   Note *Wear gloves while assembling the drip columns.*

   a. Remove the plastic wrapper from the top of a sterile 1-ml pipet.

b. Using a sterile needle or a pair of fine-tipped forceps, **carefully** tease the cotton plug out of each pipet, leaving a piece of the cotton plug measuring ~3–4 mm inside. Cut off the external portion of the cotton plug.

c. Push the remaining 3- to 4-mm piece of the cotton plug into the top of each pipet with the tip of the needle or forceps.

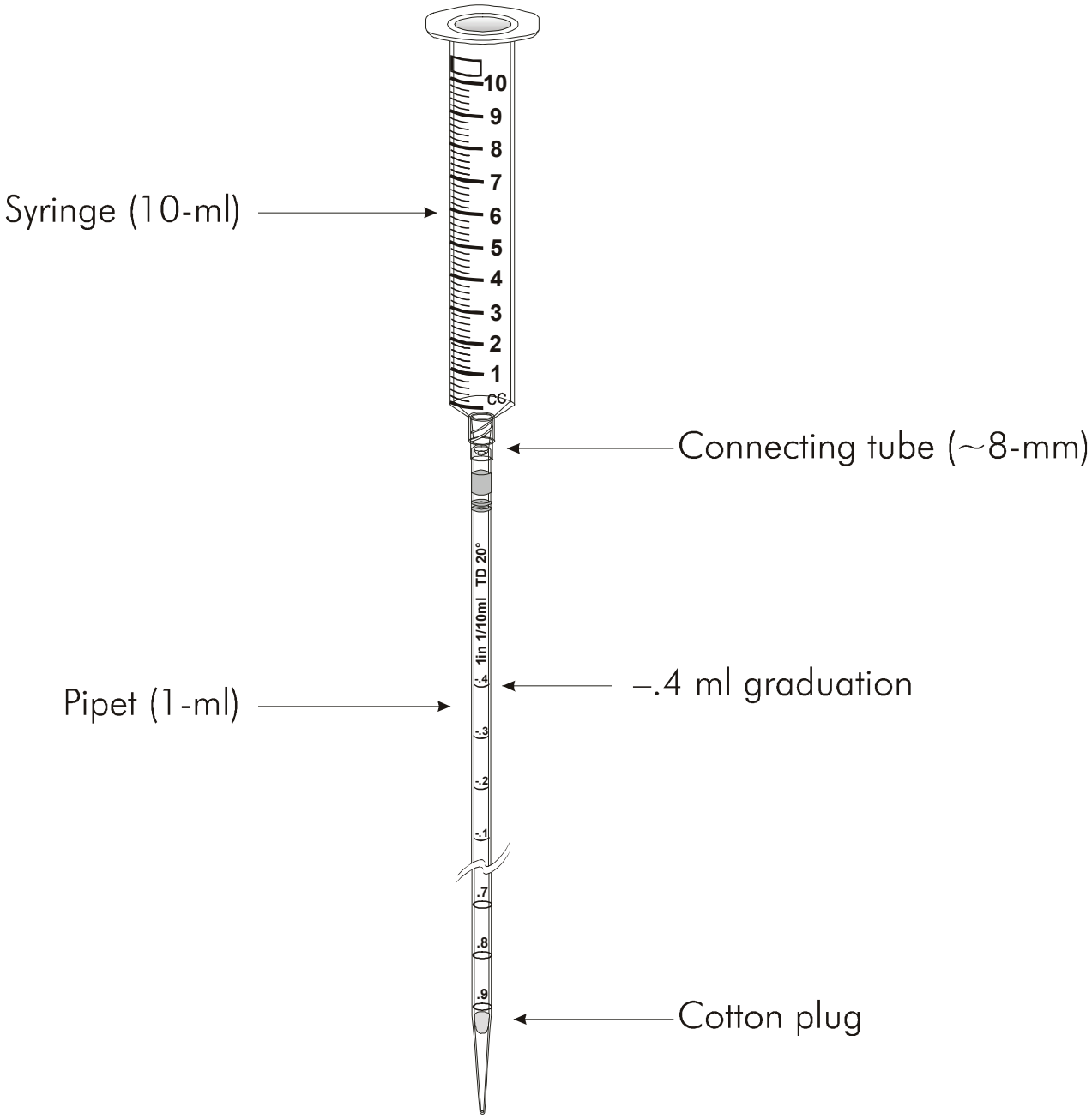


FIGURE 8 Assembly of the drip columns.

d. Cut a small piece of plastic tubing measuring ~8 mm. Use this small tube to connect the 1-ml pipet to the 10-ml syringe. First attach one end of the connecting tube to the pipet and then connect the other end to the syringe. There should be no gap between the pipet and the syringe when joined by the connecting tube.

Note *The inside diameter of the plastic tubing (~1/8-inch i.d.) snugly connects most disposable 1-ml pipets and the ends of all BD® 10-cc syringes with the Luer Lok® tips.*

e. Rapidly and forcefully push the plunger into the syringe to thrust the cotton plug down into the tip of the pipet.

Note *It may take several attempts to drive the cotton all the way down into the tip of the pipet. However, pushing the cotton plug as far down into the pipet tip as possible is important in order to achieve optimal separation of the cDNA fractions.*

f. Remove the plunger from the syringe. Because the syringe functions as a buffer reservoir for the drip column, leave the syringe firmly attached to the pipet throughout the remainder of the size fractionation procedure.

3. Locate a support for the assembled drip column. Butterfly clamps or a three-fingered clamp on a ring stand can be used.

Loading the Drip Column

1. Load the drip column with a uniform suspension of Sepharose CL-2B gel filtration medium as outlined in the following steps:

   a. Immediately prior to loading the drip column, gently mix the Sepharose CL-2B gel filtration medium by inversion until the resin is uniformly suspended.

   b. Place the column in the ring stand. Fill a glass Pasteur pipet with ~2 ml of 1× STE buffer. Insert the pipet as far into the drip column as possible and fill the column with the buffer.

Notes *If the 1× STE buffer flows too quickly through the column, stem the flow by affixing a yellow pipet tip to the end of the column. Make sure to remove the pipet tip prior to loading the column with the Sepharose CL-2B gel filtration medium.*

If bubbles or pockets of air become trapped in the STE buffer while filling the column, remove the trapped air prior to packing the column with the resin. To remove the bubbles or air, re-insert the Pasteur pipet into the top of the column and gently pipet the STE buffer in and out of the pipet until the trapped air escapes through the top of the column.

c. Immediately add a uniform suspension of Sepharose CL-2B gel filtration medium to the column with a Pasteur pipet by inserting the pipet as far into the column as possible. As the resin settles, continue adding the Sepharose CL-2B gel filtration medium. Stop adding the resin when the surface of the packed bed is ¼ inch below the "lip of the pipet." The lip of the pipet is defined as the point where the pipet and the syringe are joined.

Notes *If air bubbles form as the resin packs, use a Pasteur pipet as described in step 1b to remove the blockage. Failure to remove bubbles can impede the flow of the column and result in a loss of the cDNA.*

If the preparation of Sepharose CL-2B gel filtration medium settles and becomes too viscous to transfer from the stock tube to the column, add a small volume (~1–5 ml) of 1× STE buffer to resuspend the resin.

2. Wash the drip column by filling the buffer reservoir (i.e., the syringe) with a minimum of 10 ml of 1× STE buffer. As the column washes, the buffer should flow through the drip column at a steady rate; however, it may take at least 2 hours to complete the entire wash step. After washing, do not allow the drip column to dry out, because the resin could be damaged and cause sample loss. If this occurs, pour another column.

Note *If a free flow of buffer is not observed, then bubbles or pockets of air have become trapped in the drip column. In this case, the column must be repacked. If cDNA is loaded onto a column on which a free flow of buffer is not observed, the sample could become irretrievably lost.*

3. When ~50 µl of the STE buffer remains above the surface of the resin, immediately load the cDNA sample using a pipettor. Gently release the sample onto the surface of the column bed, but avoid disturbing the resin as this may affect cDNA separation.

4. Once the sample enters the Sepharose CL-2B gel filtration medium, fill the connecting tube with buffer using a pipettor.

Note *Do not disturb the bed while filling the connecting tube with buffer.*

Gently add 3 ml of 1× STE buffer to the buffer reservoir by trickling the buffer down the inside wall of the syringe. Do not squirt the buffer into the reservoir because this will disturb the resin, resulting in loss of the sample.

5. As the cDNA sample elutes through the column, the dye will gradually diffuse as it migrates through the resin. Because the dye is used to gauge when the sample elutes from the column, monitor the progress of the dye, or the cDNA sample could be irretrievably lost.

Collecting the Sample Fractions

The drip column containing the Sepharose CL-2B gel filtration medium separates molecules on the basis of size. Large cDNA molecules elute first followed by smaller cDNA and finally unincorporated nucleotides. Using a handheld monitor, two peaks of radioactivity can generally be detected during the course of elution. The first peak to elute from the column represents the cDNA. Due to the conditions of label incorporation during second-strand synthesis, the cDNA is not extremely radioactive; therefore, the counts per second may be barely above background levels. In contrast, the second peak to elute from the column is highly radioactive as this is the unincorporated radioactive nucleotides. Although this material elutes from the column in parallel with the dye, unincorporated nucleotides are usually not collected because the cDNA has already eluted from the column.

For standard cDNA size fractionation (>400 bp), Agilent recommends collecting ~12 fractions using the procedure described in this section. The progression of the leading edge of the dye through the column will be used as a guideline to monitor collection; however, the drops collected from the column should be monitored for radioactivity using a handheld Geiger counter. Until the fractions have been assessed for the presence of cDNA on a 5% nondenaturing acrylamide gel (see *Preparation of Media and Reagents*), do not discard any fractions based on the quantity of radioactivity detected.

1. Using a fresh microcentrifuge tube to collect each fraction, begin collecting **three drops** per fraction when the leading edge of the dye reaches the **–0.4-ml** graduation on the pipet.

2. Continue to collect fractions until the trailing edge of the dye reaches the **0.3-ml** graduation. A minimum of 12 fractions, each containing ~100 µl (i.e., three drops), should be collected. Alternatively, fractions can be collected until the radioactive, free nucleotides begin to elute. In either case, monitor the fractions for the presence of radioactivity to determine whether the cDNA has eluted successfully. If no counts are detected, continue collecting the fractions until the peak of unincorporated nucleotides is recovered.

3. Before processing the fractions and recovering the size-fractionated cDNA, **remove 8 µl of each collected fraction and save for later analysis. These aliquots will be electrophoresed on a 5% nondenaturing acrylamide gel** to assess the effectiveness of the size fractionation and to determine which fractions will be used for ligation.

Processing the cDNA Fractions

In this section of the size fractionation procedure, the fractions collected from the drip column are extracted with phenol–chloroform and are precipitated with ethanol to recover the size-selected cDNA. The purpose of the organic extractions is to remove contaminating proteins; of particular concern is kinase which can be carried over from previous steps in the synthesis. Because kinase often retains activity following heat treatment, it is necessary to follow the extraction procedures.

1. Begin extracting the remainder of the collected fractions by adding an equal volume of phenol–chloroform [1:1 (v/v)].

2. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

3. Add an equal volume of chloroform.

4. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

5. To each extracted sample, add a volume of 100% (v/v) ethanol that is equal to twice the individual sample volume.

   Note *The 1× STE buffer contains sufficient NaCl for precipitation.*

6. Precipitate overnight at –20°C.

7. Spin the sample in the microcentrifuge at maximum speed for 60 minutes at 4°C. Transfer the supernatant to another tube. To ensure that the cDNA has been recovered, use a handheld Geiger counter to check the level of radioactivity present in the pellet. If the majority of the radiation is detected in the supernatant, repeat the centrifugation step; otherwise, discard the supernatant.

8. Carefully wash the pellet with 200 µl of 80% (v/v) ethanol, ensuring that the pellet remains undisturbed. *Do not mix or vortex!* Spin the sample in a microcentrifuge at maximum speed for 2 minutes at room temperature. Remove the ethanol and verify that the pellet has been recovered by visual inspection or with the handheld Geiger counter. Vacuum evaporate the pellet for ~5 minutes or until dry. Do not dry the pellet beyond the point of initial dryness or the cDNA may be difficult to solubilize.

9. Using a handheld Geiger counter verify that the cDNA has been recovered and record the number of counts per second (cps) that is detected for each fraction.

10. If <30 cps is detected, resuspend each cDNA pellet in 3.5 µl of sterile water. If the value is >30 cps, resuspend the cDNA in 5 µl of sterile water. Mix by pipetting up and down.

Agilent strongly recommends quantitating the cDNA before proceeding (see *Appendix V: Ethidium Bromide Plate Assay—Quantitation of DNA*). Best results are usually obtained by ligating 100 ng of cDNA/1 µg of vector. Place the remaining cDNA at –20°C for short term storage only. The cDNA is most stable after ligation into vector arms and may be damaged during long-term storage.

Ligation of cDNA into the HybriZAP-2.1 Vector

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

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

   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
   0.5 µl of 10 mM rATP (pH 7.5)
   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 cDNA (~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.

4. After ligation is complete, package 1 µl of each ligation, including the test insert ligation, using Gigapack III Gold packaging extract according to the packaging instructions outlined in *Packaging Reaction*. A good representational primary library size consists of ~1 × 10^6 clones. If a low number of plaque-forming units results from packaging the 1-µl ligation, try packaging 2–3 µl of the remaining ligation mixture in one packaging reaction.

Note *Agilent recommends using high-efficiency Gigapack III Gold packaging extract[4, 20] since this packaging extract is McrA⁻, McrB⁻, and Mrr⁻. Other commercially available packaging extracts can restrict hemimethylated DNA, therefore producing low-titer libraries.*

Packaging Reaction

Packaging Protocol

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

1. Remove the appropriate number of packaging extracts from the –80°C freezer and place the extracts on dry ice.

2. Quickly thaw a packaging extract between your fingers until the extract just begins to thaw.

3. Add the test DNA **immediately** (1–4 µl containing 0.1–0.5 µg of ligated DNA) to the packaging extract and place the tube on ice.

4. Stir the contents of the tube with a pipet tip to mix well. You may **gently** pipet, but do not introduce air bubbles.

5. Spin the tube quickly (for 3–5 seconds), if desired, to ensure that all contents are at the bottom of the tube.

6. Incubate the tube at room temperature (22°C) for 2 hours.

7. Add 500 µl of SM buffer to the tube.

8. Add 20 µl of chloroform and mix the contents of the tube gently.

9. Spin the tube briefly to sediment the debris.

10. The supernatant containing the phage is now ready to be titered and may be stored at 4°C.

Titering the Packaging Reaction

Preparation of Host Bacteria

Notes *XL1-Blue MRF′ cells are RecA⁻ and consequently grow slowly.*

Prepare an overnight culture of the VCS257 cells using the appropriate media modifications (see the table in Recommended Media*) if performing the optional packaging efficiency test protocol [see* Testing the Efficiency of the Gigapack III Packaging Extract with the Wild-Type Lambda Control DNA (Optional*), below].*

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.

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 $MgSO_4$.*

Titering Protocol

6. Dilute the cells to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

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

7. 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.*

8. 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.)

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

10. 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.

11. 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.*

Testing the Efficiency of the Gigapack III Packaging Extract with the Wild-Type Lambda Control DNA (Optional)

Use the following procedure to test the efficiency of the Gigapack III packaging extract with the λ*c*I857 *Sam*7 wild-type lambda control DNA:

1. Prepare a culture of VCS257 cells as outlined in the *Preparation of Host Bacteria* subsection of *Titering the Packaging Reaction,* changing the media used for VCS257 cell growth as indicated in *Recommended Media*.

2. Thaw the frozen wild-type lambda control DNA on ice and gently mix after thawing.

3. Using 1 µl of the wild-type lambda control DNA (~0.2 µg), proceed with steps 1–10 in the *Packaging Protocol.*

    Note *Because of the high titer achieved with the wild-type lambda control DNA, Agilent recommends stopping the control packaging reaction with 1 ml of SM buffer. This should make the plaques easier to count.*

4. Prepare two consecutive 10^{-2} dilutions in SM buffer of the packaging reaction from step 10 of the *Packaging Protocol*. (The final dilution is 10^{-4}.)

5. Dilute the VCS257 cells (from step 5 of *Preparation of Host Bacteria* in *Titering the Packaging Reaction*) to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

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

6. Add 10 µl of the 10^{-4} packaging reaction dilution from step 3 to 200 µl of the VCS257 host strain from step 4. (The VCS257 strain is recommended for plating the wild-type lambda control DNA only.)

7. Incubate at 37°C for 15 minutes to allow the phage to attach to the cells.

8. Add 3 ml of NZY top agar, melted and cooled to ~48°C, and plate immediately onto dry, prewarmed NZY agar plates. Allow the plates to set for 10 minutes. Invert the plates and incubate at 37°C.

9. Plaques should be visible after 12 hours. Count the plaques. Approximately 400 plaques should be obtained on the 10^{-4} dilution plate when the reaction is stopped with 1 ml of SM buffer.

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 VI: 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.*

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. Agilent recommends storing 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, 22]

This *in vivo* excision depends on the DNA sequences that Agilent has placed 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.[23] 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. *E. 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* Titering the Packaging Reaction *subsection of* 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:

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

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, 24] 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.

Agilent suggests dephosphorylation of 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.

Preparation of the pBD-GAL4 Cam vector

1. Digest 5 µg of the pBD-GAL4 Cam phagemid vector in a final volume of 50 µl.

2. Extract with an equal volume of phenol–chloroform until a clear interface is obtained.

3. Repeat the extraction once with an equal volume of chloroform only.

4. Add an equal volume of 4 M NH_4OAc to the aqueous phase.

5. Add 2.5 volumes of 100% (v/v) ethanol equilibrated at room temperature. Immediately spin in a microcentrifuge at room temperature to precipitate the vector DNA.

6. Wash the pellet once with 70% (v/v) ethanol.

7. Resuspend the pellet 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).

Ligation Reaction

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:

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

Agilent suggests the following protocol which includes three control ligations:

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

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

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

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

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

Incubate the ligation reactions overnight at 4°C. When ligating blunt ends, incubate overnight at 12–14°C.

Transformation of XL1-Blue Subcloning-Grade Competent Cells

Transform 1–5 µl of the ligation mix into XL1-Blue subcloning-grade competent cells as outlined in the following steps:

Important *Please see* Appendix VII: Transformation Guidelines *before proceeding with the transformation protocol.*

1. Thaw the subcloning-grade competent cells on ice.

2. Gently mix the cells (do not vortex). Aliquot 50 µl of the subcloning-grade competent cells into a prechilled 14-ml BD Falcon polypropylene tube. Prepare a second 50-µlaliquot of the subcloning-grade competent cells for the pUC18 control transformation.

3. Add 1–5 µl of the ligation mix to the cells and swirl gently. Add 1 µl of the pUC18 control plasmid to a separate 50-µl aliquot of the subcloning-grade competent cells and swirl gently.

4. Incubate the tubes on ice for 30 minutes.

5. Heat pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is critical for obtaining the highest efficiencies.

6. Incubate the tubes on ice for 2 minutes.

7. Add 0.9 ml of SOC medium[§] and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm

8. Plate ≤200 µl of the ligation reaction transformation mixture on an LB–chloramphenicol agar plate[§,‖] using a sterile spreader. For the pUC18 control transformation, add 5 µl of the transformation mixture into a 200-µl pool of SOC medium on an LB-ampicillin agar plate and then spread the mixture.

   Note *If desired, the cells may be concentrated by centrifuging at 1000 rpm for 10 minutes, removing the supernatant, and resuspending the pellet in 200 µl of SOC medium.*

9. Incubate the plates at 37°C until colonies are visible.

Note *Transformants containing the pBD-GAL4 Cam phagemid vector may grow slowly and may require incubation at 37°C for up to 24 hours.*

Table VI

EXPECTED RESULTS
(USING XL1-BLUE SUBCLONING-GRADE COMPETENT CELLS)

Sample	Amount of transformation plated	Expected colony number	Efficiency (cfu/µg of DNA)
pUC18 control	5 µl	>10	$>1 \times 10^6$
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.

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

[‖] When spreading bacteria onto the plate, tilt and tap the spreader to remove the last drop of cells. If plating ≥100 µl, the cells can be spread directly onto the plates. If plating <100 µl of the transformation mixture, plate into a 200-µl pool of SOC medium.

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.

Relatively low levels of expression 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

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

TABLE VII

Selective Media for Yeast Transformations

Yeast transformations	Selective media		
		SD 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

Agilent recommends transforming 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.

Agilent recommends the use of 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 *Yeast Transformation Solutions* in *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 *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.[25] 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, 26 and 27.

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 for in vitro immunoprecipitation assays with the bait protein.

Discussion regarding further verification of protein–protein interactions can be found in numerous publications.[19, 28]

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: RNA PURIFICATION AND QUANTIFICATION

RNA Purification

Agilent highly recommends using the RNA Isolation Kit or the guanidinium thiocyanate–phenol–chloroform extraction method[21] to isolate total RNA. This method is rapid, yet it produces large amounts of high-quality, undegraded RNA.

Although AccuScript RT is not inhibited by ribosomal RNA (rRNA) and transfer RNA (tRNA) contamination, it is advisable to select the poly(A)$^+$ fraction. The amounts of rRNA and tRNA vastly outnumber the mRNA and will decrease the efficiency of the cDNA synthesis. Poly(A)$^+$ RNA is selected on oligo(dT) cellulose columns.[29] Some protocols call for the addition of SDS in the purification steps. Sodium dodecyl sulfate is a powerful enzyme inhibitor and helps prevent degradation of the RNA by RNases, but its presence can also inhibit the enzymes required for cDNA synthesis. If the mRNA intended for use with this kit is suspended in an SDS solution, the RNA must be phenol extracted and ethanol precipitated.

Ribonucleases A and T1 are widely used in almost all molecular biology labs and are nearly indestructible. Ribonucleases are produced by microbes and have also been found in the oils of the skin. Make an effort to use tubes and micropipet tips which have been handled only with gloves. Use freshly autoclaved and baked tips and tubes. Usually these precautions are sufficient, but to be absolutely certain that microcentrifuge tubes and other components intended for use with RNA are not contaminated, the components can be treated with DEPC. Diethylpyrocarbonate is extremely toxic and should be handled with care. Submerge the microcentrifuge tubes in a 0.1% (v/v) DEPC-treated water solution. Leave the beaker of submerged tubes in a fume hood overnight and then dispose of the DEPC-treated water. Autoclave the microcentrifuge tubes for at least 30 minutes. Even though the tubes may still have a sweet DEPC odor, the DEPC is completely inactivated by this procedure. Place the tubes in a drying oven overnight. Equipment which cannot be treated by DEPC can be rinsed in a freshly mixed 3% (v/v) hydrogen peroxide solution, followed by a methanol rinse. Remember, once the RNA is converted to first-strand cDNA, RNases are no longer a concern. Caution should still be exercised in maintaining a sterile, DNase-free environment.

RNA Quantification

RNA can be quantified by measuring the optical density of a dilute RNA solution. The conversion factor for RNA at the wavelength of 260 nm is 40 µg/ml/OD unit as shown in the example below.

Two microliters of an unquantified poly(A)$^+$ sample is added to 498 ml of water (OD_{260} = 0.1).

$$0.1 \text{ OD unit} \times \left(\frac{500}{2} \text{ dilution factor}\right) \times 40 \text{ µg / ml} = 1000 \text{ µg / ml or } 1 \text{ µg / µl}$$

If a sample has significant rRNA contamination, the actual amount of mRNA available for cDNA conversion will be overestimated by this procedure.

If the amount of mRNA is below 1.5 µg/synthesis reaction, the RT may synthesize unclonable hairpin structures. If the amount of mRNA is above 7 µg, the percentage of cDNAs which are full length may decrease. The cDNA Synthesis Kit provided with the HybriZAP-2.1 two-hybrid vector system has been optimized for 5 µg of mRNA, but successful libraries have been generated using the minimums and maximums described here.

Secondary structure may be a problem with certain RNAs, particularly plant and tumor mRNAs. These samples can be treated with methylmercury hydroxide (see *Appendix III: Methylmercury Hydroxide Treatment*). Treatment with methylmercury hydroxide requires heating the RNA to 65°C. If the RNA contains even a minute amount of RNase, the RNase activity will increase by several orders of magnitude with the increased temperature and significantly degrade the RNA. Treatment with methylmercury hydroxide is therefore recommended only if the RNA is free of RNases.

APPENDIX III: METHYLMERCURY HYDROXIDE TREATMENT

Warning *Methylmercury hydroxide is an extremely toxic chemical. Wear gloves and use with caution in a fume hood.*

1. Resuspend the mRNA in 20 µl of DEPC-treated water.
2. Incubate at 65°C for 5 minutes.
3. Cool to room temperature.
4. Add 2 µl of 100 mM CH_3HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 µl of 700 mM β-mercaptoethanol (see *Preparation of Media and Reagents*).
7. Incubate at room temperature for 5 minutes.

APPENDIX IV: ALKALINE AGAROSE GELS

Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix.

Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first- and second-strand cDNA.

Note *The test cDNA sample will run as a tight band at 1.8 kb and will show distinctly different intensity between the first and second strands. This is due to the relative ratio of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally the second strand will be only 1/10 to 1/20 the intensity of the first-strand band.*

Alkaline agarose gels differ from conventional gels in the following ways:

1. The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.

2. The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree which makes a clear autoradiograph difficult to interpret.

The following alternative methods help avoid these complications.

The Slide Technique

The easiest and least expensive method is to use a 5- × 7.5-cm glass slide, position a minigel comb over it with high tension clips, and add 10 ml of molten alkaline agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel which can be exposed without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. To improve the resolution, pat the gel dry with several changes of Whatman 3MM paper after electrophoresis is complete.

To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.

The Vertical Alkaline Agarose Technique

Vertical alkaline agarose gels can be produced using a vertical gel apparatus with 1.5-mm spacers. Since the alkaline agarose gels do not have sufficient friction to remain bound to ordinary glass, a frosted glass plate or gel bond must be used with the vertical apparatus. The combs normally used for acrylamide can be used with this apparatus, if the outside teeth are wrapped in tape to prevent the comb from sinking more than 1.2 cm into the agarose. The 55°C agarose will solidify almost immediately on contact with the cold glass plates, so it is essential to load the mold rapidly with a 60-ml syringe. The comb should already be in the mold, and if it is necessary to reposition the comb, do it immediately after the gel is poured. In order to reduce the possibility of destroying the wells when pulling out the comb, place the solidified gel in a –20°C freezer for 5 minutes immediately prior to removing the comb. When pulling out the comb, it is essential to avoid a vacuum between the teeth and the well. Vacuum can be detected when the well distorts from its normal square shape. When a vacuum occurs, push the comb to separate the glass plates and break the vacuum. After the samples have been run and the glass plates are ready to be opened, slide the unfrosted glass plate off of the alkaline agarose gel instead of prying the plate away from the gel. Pat the gel dry several times using several pieces of Whatman 3MM paper.

Note *To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.*

Conventional Submerged Gels

These gels will require drying either by blotting or through the use of a gel dryer.

Caution ***Even when multiple layers of absorbent paper are placed under the gel, free nucleotides can easily contaminate the drying apparatus. These gels should be poured as thin as possible and should be dried without heat, if time permits, and should never be dried above 40°C.***

Protocol

The following formula makes 80 ml of 1% (w/v) alkaline agarose for cDNAs in the 1- to 3-kb size range.

Melt 0.8 g of agarose in 72 ml of water. Allow the agarose to cool to 55°C. During this time, assemble the gel apparatus. Add 8 ml of 10× alkaline buffer (see *Preparation of Media and Reagents*) to the cooled agarose, swirl to mix, and pour the agarose immediately. If buffer is added before the correct temperature is reached, the agarose may not solidify.

Load the sample in an equal volume of alkaline agarose 2× loading buffer (see *Preparation of Media and Reagents*). Run the gel with 1× alkaline buffer at 100 mA and monitor the system for heat. If the apparatus becomes warmer than 37°C, the amperage should be reduced. The migration of the BPB in alkaline agarose is similar to the migration in regular agarose and should be run to at least one-half or three-quarters distance of the gel.

Note *The alkali conditions causes the blue dye to fade.*

APPENDIX V: 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, changing pipet tips between each standard. 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.

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. Standards and unknowns must be spotted within 10 minutes of each other. Remove the lid and photograph the plate using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates.

APPENDIX VI: 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 *Verification of Insert Percentage and Size*.

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 237 bp in length. PCR products which are >237 bp in length represent the HybriZAP-2.1 vector with an insert.

Vector	Primer	Binds to nucleotide (nt)	Nucleotide sequence (5′ to 3′)
HybriZAP-2.1 vector (AD)	5′ AD	745–765	AGGGATGTTTAATACCACTAC
	3′ AD	962–982	GCACAGTTGAAGTGAACTTGC

APPENDIX VII: TRANSFORMATION GUIDELINES

Important *Please read the transformation guidelines before proceeding with the* Transformation of XL1-Blue Subcloning-Grade Competent Cells *in* Bait Protein Insert Preparation, Ligation, and Transformation.

Storage Conditions

Subcloning-grade competent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Subcloning-grade competent cells should be placed at –80°C directly from the dry ice shipping container. Cells stored in this manner should retain their guaranteed efficiency for 6 months.

Aliquoting Cells

When aliquoting, keep the subcloning-grade competent cells on ice at all times. It is essential that the BD Falcon polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 50 µl of subcloning-grade competent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol. The incubation period during the heat-pulse step is critical and has been optimized for the thickness and shape of the 14-ml BD Falcon polypropylene round-bottom tubes.

Quantity of DNA Added

Greatest efficiencies are observed when adding 1 µl of 0.1 ng/µl of ligated DNA/100 µl of cells. A greater number of colonies will be obtained when plating up to 50 ng, although the overall efficiency may be lower.

Length of the Heat Pulse

There is a defined "window" of highest efficiency resulting from the heat pulse in step 5 of *Transformation of XL1-Blue Subcloning-Grade Competent Cells*. Optimal efficiencies are observed when cells are heat-pulsed for 45–50 seconds. Heat-pulsing for at least 45 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease sharply when incubating for <45 seconds or for >60 seconds.

TROUBLESHOOTING

cDNA Synthesis

Observations	Suggestions
Poor first-strand synthesis	Ensure enzymes are mixed and buffers are not precipitated. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	Minute amounts of SDS or lithium in the RNA will inhibit the first-strand synthesis reaction. Do not use these in the RNA preparations. Multiple phenol–chloroform extractions will sometimes remove the inhibitors.
	Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	Ensure that [α-^{32}P]dNTP is not degraded. If [α-^{32}P]dNTP is heated, is left at room temperature too long, or is contaminated, it may not incorporate into cDNA, giving a false indication that synthesis is not occurring.
Poor second-strand synthesis	It is important to interpret the gel results correctly. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally, the second strand will have only 1/10 to 1/20 the intensity of the first-strand band.
	Ensure enzymes are mixed and buffers are not precipitated. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously.
	No first-strand synthesis. See the previous suggestions for *Poor first-strand synthesis*.
No first-strand synthesis, but good second-strand synthesis	Ensure that there is no DNA contamination within the RNA preparation.
Hairpinning	Hairpinning can occur if incubation temperatures are higher than 16°C . Add second-strand synthesis reaction components to the first-strand reaction mix on ice and then transfer the reaction mixture directly to 16°C for incubation. After incubation, place the samples on ice immediately.
	Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure (see *Appendix III: Methyl-mercury Hydroxide Treatment*).
	Ensure that excessive DNA polymerase is not used. Use a calibrated pipet to measure the enzyme. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.
Low counts in the drip column fractions	The number of counts per second per fraction may vary from 0 to 250 cps and yield primary libraries of $>1 \times 10^6$ pfu. Most of the counts remaining in the drip column are from unincorporated [α-^{32}P]dNTP. Verify the quantity of cDNA on the EtBr plate.
Poor ligation	This can result from excessive glycerol in the reaction. Do not use excess ligase. Also, do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.

Mass or Single-Clone 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 or low number of Amp^r colonies	The molar ratios of lambda phage-to-cells-to-helper phage is critical. Verify that the titer of lambda and helper phage stocks and use only calibrated pipettor.
	Excision efficiencies are directly related to the HybriZAP-2.1 phage titer. If an excision is unsuccessful, prepare a high-titer stock of the phage and repeat the excision procedure.
	Chloroform, which is added after packaging to prevent bacterial contamination, may lyse the *E. coli* before the helper phage can infect and excise. Briefly spin the lambda phage stock prior to removing an aliquot for *in vivo* excision in order to ensure that the chloroform is separated completely.
	Ensure that XL1-Blue MRF´ host cells are used for the mass excision.

Two-Hybrid Vector System Screening

Observation	Suggestion
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 Host Strain Phenotype* and prepare new yeast competent cells using the same yeast colony.
	Use sterile technique when preparing and transforming the yeast competent cells to avoid contamination with a different yeast strain or *E. coli*.
	Ensure that the filter paper makes good contact with the yeast colonies.
	Verify that the colony material has been transferred by visual inspection of the filter paper and the colonies on the plate.
The 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.
	Insert DNA is expressed at levels insufficient for detection 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.

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.
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.

PREPARATION OF MEDIA AND REAGENTS

Standard Media and Reagents

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

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 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 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–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–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–Chloramphenicol Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 3 ml of 10 mg/ml chloramphenicol (filter-sterilized)
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 1.5 ml of 10 mg/ml tetracycline (filter-sterilized)
Store broth in a dark, cool place as tetracycline is light-sensitive

LB–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 1.5 ml of 10 mg/ml tetracycline (filter-sterilized)
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

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)

Nondenaturing Acrylamide Gel (5%)

Mix the following in a vacuum flask

5 ml of 10× TBE buffer
8.33 ml of a 29:1 acrylamide–bis-acrylamide solution
36.67 ml of sterile deionized H_2O

De-gas this mixture under vacuum for several minutes
Add the following reagents
25 µl of TEMED
250 µl of 10% ammonium persulfate

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× *Taq* DNA Polymerase Buffer

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

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Autoclave
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ prior to use
Filter sterilize

SOC Medium (per 100 ml)

Note *This medium should be prepared immediately before use*

1 ml of a 2 M filter-sterilized glucose solution **or** 2 ml of 20% (w/v) glucose
SOB medium to a final volume of 100 ml
Filter sterilize

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*

10× MOPS Buffer

200 mM 3-[*N*-morpholino]propane-sulfonic acid (MOPS)
50 mM sodium acetate
10 mM EDTA
Adjust to a final pH of 6.5–7.0 with NaOH
Do not autoclave

Alkaline Agarose 2× Loading Buffer

200 µl of glycerol
750 µl of water
46 µl of saturated BPB‖
5 µl of 5 M NaOH

700 mM β-Mercaptoethanol

5 µl of 14 M β-mercaptoethanol
95 µl of DEPC-treated water
Do not autoclave

TE Buffer

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

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× Alkaline Buffer (per 50 ml)

3 ml of 5.0 M NaOH
2 ml of 0.5 M EDTA
45 ml of water

Column-Loading Dye

50% (v/v) glycerol
10% (v/v) 10× STE buffer
40% (w/v) saturated BPB‖

‖ To make saturated BPB, add a small amount of BPB crystals to water and vortex. Centrifuge the sample briefly and look for the presence of an orange pellet. If a pellet is seen, the solution is saturated. If not, add more crystals and repeat the procedure.

‡ 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 threonine and aspartic acid, which must 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 30)
48 ml of chloroform
2 ml of isoamyl alcohol
Store at 4°C

Yeast Lysis Solution

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

REFERENCES

1. Fields, S. and Song, O. (1989) *Nature* 340(6230):245-6.
2. Mullinax, R. L. and Sorge, J. A. (1995) *Strategies* 8(1):3-5.
3. Ma, J. and Ptashne, M. (1987) *Cell* 51(1):113-9.
4. Alting-Mees, M., Hoener, P., Ardourel, D., Sorge, J. and Short, J. M. (1992) *Strategies* 5(3):58-61.
5. Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988) *Nucleic Acids Res* 16(15):7583-600.
6. Short, J. M. and Sorge, J. A. (1992) *Methods Enzymol* 216:495-508.
7. Jerpseth, B., Greener, A., Short, J. M., Viola, J. and Kretz, P. L. (1992) *Strategies* 5(3):81–83.
8. Feilotter, H. E., Hannon, G. J., Ruddell, C. J. and Beach, D. (1994) *Nucleic Acids Res* 22(8):1502-3.
9. Callahan, M., Jerpseth, B., Mullinax, R. L. and Greener, A. (1995) *Strategies* 8(2):45–46.
10. Guthrie, C. and Fink, G. R. (Eds.). (1991). *Guide to Yeast Genetics and Molecular Biology*, Vol. 194. Academic Press, Inc., Boston.
11. Kohler, S. W., Provost, G. S., Kretz, P. L., Dycaico, M. J., Sorge, J. A. et al. (1990) *Nucleic Acids Res* 18(10):3007-13.
12. Kretz, P. L., Reid, C. H., Greener, A. and Short, J. M. (1989) *Nucleic Acids Res* 17(13):5409.
13. Kretz, P. L. and Short, J. M. (1990) *Strategies* 3(2):21-22.
14. Kretz, P. L. and Short, J. M. (1989) *Strategies in Molecular Biology* 2(2):25-26.
15. Estojak, J., Brent, R. and Golemis, E. A. (1995) *Mol Cell Biol* 15(10):5820-9.
16. Ptashne, M., Backman, K., Humayun, M. Z., Jeffrey, A., Maurer, R. et al. (1976) *Science* 194(4261):156-61.
17. Cohen, S., Knoll, B. J., Little, J. W. and Mount, D. W. (1981) *Nature* 294(5837):182-4.
18. Gimble, F. S. and Sauer, R. T. (1989) *J Mol Biol* 206(1):29-39.
19. Bartel, P. L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993). Using the Two-hybrid System to Detect Protein-Protein Interactions. In *Cellular Interactions in Development: A Practical Approach*,D. A. Hartley (Ed.), pp. 153–179. Oxford Univ. Press, Oxford, England.
20. Kretz, P. L., Danylchuk, T., Hareld, W., Wells, S., Provost, G. S. et al. (1994) *Strategies* 7(2):44-45.
21. Chomczynski, P. and Sacchi, N. (1987) *Anal Biochem* 162(1):156-9.
22. Alting-Mees, M. A. and Short, J. M. (1989) *Nucleic Acids Res* 17(22):9494.
23. Dotto, G. P., Horiuchi, K. and Zinder, N. D. (1984) *J Mol Biol* 172(4):507-21.
24. Hogrefe, H. H., Mullinax, R. L., Lovejoy, A. E., Hay, B. N. and Sorge, J. A. (1993) *Gene* 128(1):119-26.
25. Hoffman, C. S. and Winston, F. (1987) *Gene* 57(2-3):267-72.
26. Bartel, P., Chien, C. T., Sternglanz, R. and Fields, S. (1993) *Biotechniques* 14(6):920-4.
27. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y. et al. (1993) *Genes Dev* 7(4):555-69.
28. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993) *Cell* 75(4):805-16.
29. Krug, M. S. and Berger, S. L. (1987) *Methods Enzymol* 152:316-25.
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. et al. (1987). *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

ENDNOTES

Difco® is a registered trademark of Difco Laboratories.
GenBank® is a registered trademark of U.S. Department of Health and Human Services.
Parafilm® is a registered trademark of American Can Company.
Sepharose® is a registered trademark of Pharmacia Biotech AB.
Styrofoam® is a registered trademark of Dow Chemical Co.
Triton® is a registered trademark of Rohm and Haas Co.
Tween® is a registered trademark of ICI Americas, Inc.
Whatman® is a registered trademark of Whatman Ltd.
VWRbrand is a trademark of VWR Scientific.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

HybriZAP-2.1 XR Library Construction Kit and HybriZAP-2.1 XR cDNA Synthesis Kit

Catalog #235612 (HybriZAP-2.1 XR Library Construction Kit) and
#235614 (HybriZAP-2.1 XR cDNA Synthesis Kit)

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 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