Hybrid Hunter™

Version D

180911
25-0179

Hybrid Hunter™

a two-hybrid system for analysis of protein-protein interactions in the yeast, *Saccharomyces cerevisiae*

Catalog no. K5000-01

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Important Information

Kit Contents

The following reagents are included in the Hybrid Hunter™ Kit.

Zeocin™: 12 x 1.25 ml, 100 mg/ml, (1.5 g) Enough Zeocin™ is supplied for *E. coli* transformation and one large-scale library transformation. To order additional Zeocin™, see the next page.

Vectors: All vectors are supplied lyophilized. Note: pYESTrp2 is derived from pYESTrp originally supplied in the Hybrid Hunter™ Kit. The multiple cloning site has been altered to remove the *Stu* I and the second *Eco*R I site.

Vector	Amount	Purpose
pHybLex/Zeo	20 µg	Cloning vector for "bait" protein
pYESTrp2	20 µg	Cloning vector for "prey" protein or cDNA library
pHybLex/Zeo-Fos2*	10 µg	Positive control for bait plasmid
pYESTrp-Jun	10 µg	Positive control for prey plasmid
pHybLex/Zeo-Lamin	10 µg	Negative control for bait plasmid

*Replaces pHybLex/Zeo-Fos

Primers: All primers are supplied lyophilized. Primers may be used for sequencing or PCR. Additional primers are available from Invitrogen. Please see next page.

Primer	Sequence	Amount
pHybLex/Zeo Forward	5´-AGGGCTGGCGGTTGGGGTTATTCGC-3´	2 µg (257 pmoles)
pHybLex/Zeo Reverse	5´-GAGTCACTTTAAAATTTGTATACAC-3´	2 µg (263 pmoles)
pYESTrp Forward	5´-GATGTTAACGATACCAGCC-3´	2 µg (346 pmoles)
pYESTrp Reverse	5´-GCGTGAATGTAAGCGTGAC-3´	2 µg (340 pmoles)

Yeast Strains: Supplied as 20% glycerol stocks in 0.5 ml volumes.

Strain	Genotype	Phenotype
L40*	*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2::(4lex*Aop-*HIS3)URA3*::(8*lex*Aop-*lacZ*) *GAL4*	His-, Trp-, Leu-, Ade-
EGY48	MATα *ura3 trp1 his3* 6*lex*Aop-*LEU2*	Ura-, Trp-, His-, Leu-
EGY48/ pSH18-34	EGY48 transformed with pSH18-34	Ura+, Trp-, His-, Leu-

*The complete genotype for this strain is unknown (Hollenberg *et al*., 1996; Vojtek *et al*., 1993).

continued on next page

Shipping and Storage

The Hybrid Hunter™ Kit is shipped on dry ice. Upon receipt, store the glycerol stocks of L40, EGY48, and EGY48/pSH18-34 at -80°C. Store the remainder of the kit at -20°C, away from light.

Additional Products

Additional reagents are available from Invitrogen. See the table below for ordering information.

Item	Amount	Catalog no.
Zeocin™	1 gram	R250-01
	5 gram	R250-05
S.c. EasyComp Kit	20 reactions	K5050-01
pHybLex/Zeo Forward Primer	2 µg	N820-01
pHybLex/Zeo Reverse Primer	2 µg	N821-01
pYESTrp Forward Primer	2 µg	N830-01
pYESTrp Reverse Primer	2 µg	N831-01
pJG4-5 Forward Primer	2 µg	N810-01
pJG4-5 Reverse Primer	2 µg	N811-01
Anti-LexA Antibody	25 westerns	R990-25
L40 yeast strain	0.5 ml	C830-00
EGY48 yeast strain	0.5 ml	C835-00
EGY48/pSH18-34 yeast strain	0.5 ml	C836-00
EGY191 yeast strain	0.5 ml	C837-00
EGY191/pSH18-34 yeast strain	0.5 ml	C838-00
pSH18-34 *lacZ* reporter plasmid, 8 LexA ops	20 µg	V611-20
pJK103 *lacZ* reporter plasmid, 2 LexA ops	20 µg	V613-20
pRB1840 *lacZ* reporter plasmid, 1 LexA op	20 µg	V612-20
Anti-V5 Antibody	25 westerns	R960-25
Anti-V5-HRP Antibody	25 westerns	R961-25

Technical Service

If you have any questions, please call, write, or email Technical Service.

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Purchaser Notification

Introduction

The Hybrid Hunter™ System includes technologies licensed under several U.S. patents:

- No. 4,833,080, issued May 23, 1989 entitled "Regulation of Eukaryotic Gene Expression" owned by Harvard University
- Nos. 5,283,173 and 5,468,614 covering a "System to Detect Protein-Protein Interactions" owned by the State University of New York.

The Hybrid Hunter™ System also employs technologies on which there are patents pending. Invitrogen has licensed these technologies from their owners in order to make them available to research scientists.

The Invitrogen License

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(a) any use of Products in a Commercial Product
(b) any use of Products in the manufacture of a Commercial Product
(c) any sale of Products
(d) any use of Products, to facilitate or advance research or development of a Commercial Product
(e) any use of Products to facilitate or advance any research or development program the results of which will be applied to the development of Commercial Products.

Obtaining a Commercial License

Practice of the two-hybrid system is covered by U.S. Patents Nos. 5,283,173 and 5,468,614 assigned to The Research Foundation of the State University of New York. Purchase of any Invitrogen two-hybrid reagents does not imply or convey a license to practice the two-hybrid system covered by these patents, beyond use of the enclosed kit for non-commercial research. Commercial entities purchasing these reagents must obtain a license from The Research Foundation of the State University of New York before using them. For license information, please contact

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Introduction

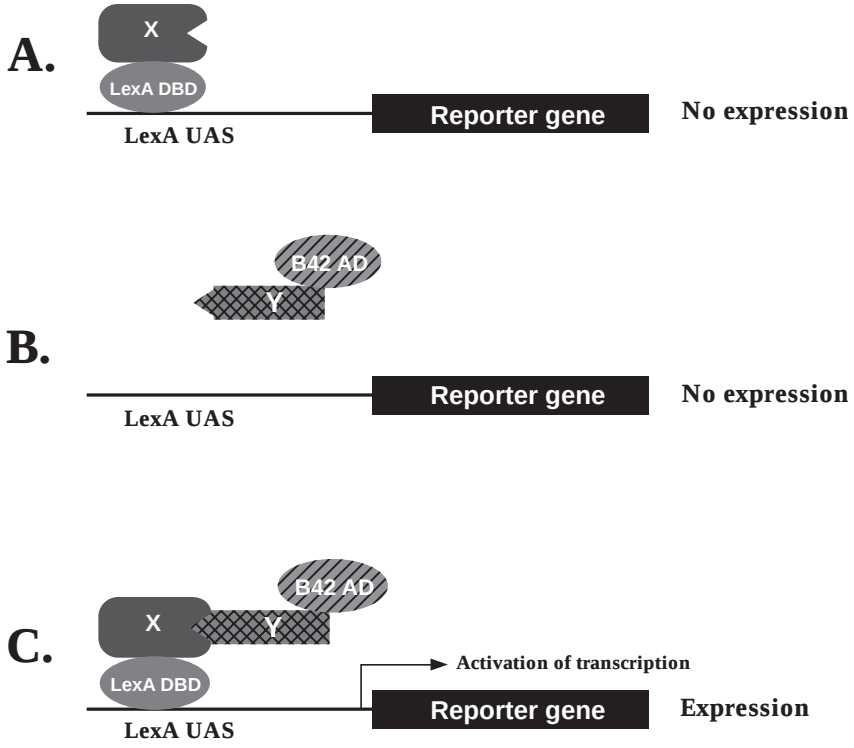
Overview

Introduction

The Hybrid Hunter™ Two Hybrid System, modified from the interactive trap system developed by Roger Brent and coworkers, is a genetic method for detecting interactions between proteins *in vivo* in the yeast *Saccharomyces cerevisiae* (Golemis *et al*., 1996; Gyuris *et al*., 1993). The system can be used to screen a library for novel proteins that specifically interact with a known bait protein of interest or to test complex formation between two known proteins or protein domains for which there is a prior reason to expect an interaction.

General Description

Two-hybrid or interaction trap systems exploit the fact that transcription factors are comprised of two domains, a DNA binding domain (DBD) and an activation domain (AD). In Hybrid Hunter™, two separate hybrid proteins are constructed (see figure, below). The first hybrid protein is the LexA DBD/protein X fusion known as the "bait" (Figure A, below) while the second hybrid protein is the B42 AD/protein Y fusion known as the "prey"(Figure B, below). Protein Y can be replaced with a cDNA library in order to screen for unknown proteins that interact with the bait of interest. These two hybrids are on separate plasmids and are transformed into a yeast strain that contains two reporter genes (*lac*Z and an auxotrophic marker). The regulatory regions for these two reporters contain the LexA DNA binding sites (operator sequences) that act as upstream activating sequences (UAS) in yeast. If protein X interacts with protein Y in the nucleus, this will bring the activation domain together with the DNA-binding domain to reconstitute transcriptional activation and result in expression of the reporter genes (Figure C). Positive interactions can be detected by selecting on plates lacking the auxotrophic marker, followed by a second screen for β-galactosidase expression.



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Experimental Outline for a Library Screen

The table below outlines the major steps required to isolate positive clones from a cDNA library using Hybrid Hunter™. Approximate time required for each step is also provided. For more information on each step, please see the indicated page.

Step	Action	Time Required	Page
Construct or purchase interactive trap library in pYESTrp, pYESTrp2, or pJG4-5	Construct a library in pYESTrp2 using standard techniques	1-2 weeks	8
	Use previously purchased, LexA-compatible two-hybrid library	0 days	8
	Purchase a Hybrid Hunter™ Library in pYESTrp, pYESTrp2, or pJG4-5	2 days	5
	Prepare large-scale preparation of library plasmid for transformation	2 days	9
Construct the bait plasmid	Ligate gene of interest in frame with LexA to create bait protein fusion	1-2 days	13
	Transform into competent *E. coli* and select on Zeocin™	2 days	14
	Sequence to confirm that gene is cloned in frame with LexA	2 days	14
Transform bait plasmid into yeast to create bait strain and test for expression	Prepare competent L40 or EGY48/pSH18-34 using a small-scale preparation and transform bait plasmid into L40 or EGY48/pSH18-34	3-5 days	15
	Test transformants for expression of bait protein by immunoblot analysis	2 days	16
Test bait plasmid for non-specific activation	Test for histidine (L40) or leucine (EGY48/pSH18-34) prototrophy Test for β-galactosidase activity	5 days	18-20
Perform a library screen for proteins that interact with the bait plasmid	Prepare competent L40 or EGY48/pSH18-34 using a large-scale preparation and transform the bait strain with the library and select directly for His^+ or Leu^+ transformants	1-2 weeks	21
	Test transformants for β-galactosidase activity	2-3 days	25
	Retrieve library plasmids encoding putative interactors and classify by restriction analysis	1 week	30
	Test for true positives, if desired	1 week	35
	Sequence selected library plasmids to identify the interacting protein	1 week	32

continued on next page

Experimental Outline for Testing Two Proteins for Interaction

The table below outlines the major steps required to clone and test two known proteins for a potential interaction. Approximate time required for each step is also provided. For more information on each step, please see the indicated page.

Step	Action	Time Required	Page
Construct prey plasmid	Clone the gene for a known protein into pYESTrp2	2 days	9
	Transform into competent *E. coli* and select on ampicillin	2 days	8
	Sequence to confirm gene is in frame with the V5-NLS-B42 peptide	2 days	8
Construct the bait plasmid	Clone gene of interest in frame with LexA to create bait protein fusion	2 days	13
	Transform into competent *E. coli* and select on Zeocin™	2 days	14
	Sequence to confirm that gene is cloned in frame with LexA	2 days	14
Cotransform bait plasmid and prey plasmid into yeast	Prepare competent yeast for small-scale transformation	0.5 day	15
	Use a small-scale transformation to cotransform bait plasmid and prey plasmid into L40 or EGY48/pSH18-34 with appropriate controls and select on histidine or leucine deficient medium	2 days	18
	Test for β-galactosidase activity	2-3 days	20

Important

The Hybrid Hunter™ Two-Hybrid System manual is designed to help you isolate positive clones in the simplest, most direct fashion, while providing references for more sophisticated uses of two-hybrid systems in general.

The user should be familiar with basic yeast molecular biology and microbiological techniques. A general reference is provided below:

Current Protocols in Molecular Biology (1996) *Saccharomyces cerevisiae*, pp. 13.01 to 13.2.12. These sections describe how to prepare yeast media and to grow and manipulate yeast.

Guthrie, C. and G. R. Fink (1991) Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, Academic Press, San Diego, CA.

Methods

Choosing a Library

Introduction

As noted earlier, you can use the Hybrid Hunter™ Two-Hybrid Kit for the following applications:

- Screening a cDNA library for potential interactors with your bait protein
- Testing two known proteins for a potential interaction

If you wish to screen a library, you may use any of the following choices:

- Use a compatible library you already have, **or**
- Construct your own library in pYESTrp2, which is included in the Hybrid Hunter™ Kit, **or**
- Purchase a Hybrid Hunter™ library (see page 5)

Lastly, you may clone the gene of a known protein into pYESTrp2 to test for a potential interaction with a second known protein (see page 8).

Using Your Own Library

pHybLex/Zeo contains a novel gene that confers resistance to the antibiotic Zeocin™. By eliminating auxotrophic selection for the bait plasmid, you have more flexibility in using different two-hybrid libraries. In principle, you can use any activating domain with the LexA DNA binding domain (e.g. VP16, Gal4) (Brent and Ptashne, 1985). You may use any library that is compatible with a host strain that uses LexA operator sites as upstream activation sequences for the reporter genes (e.g. L40 or EGY48/pSH18-34) and that has an auxotrophic marker different from *HIS3* (L40) or *LEU2* (EGY48/pSH18-34). The table below indicates which strain will grow without certain nutrients (+ indicates growth; -- indicates no growth).

Missing Nutrient	L40	EGY48/pSH18-34
Uracil	+	+
Adenine	--	+
Histidine	--	--
Leucine	--	--
Lysine	+	+
Tryptophan	--	--

Constructing a Two-Hybrid cDNA Library

pYESTrp2 can be used to construct a two-hybrid cDNA library of your own choosing using standard methods (Ausubel *et al*., 1994). Please turn to page 9 for a few general guidelines.

continued on next page

Hybrid Hunter™ Libraries

The following libraries in pYESTrp, pYESTrp2, and pJG4-5 are available from Invitrogen. All libraries are amplified once on plates. Libraries in pJG4-5 are amplified twice. We are always adding to our line of premade libraries. Please call Technical Service (page 51) for more information or visit our web site (http://www.invitrogen.com).

Source	Plasmid	Primary Clones	Size Selection	Catalog no.
Cell Lines				
HeLa cells (Human cervical carcinoma)	pYESTrp	3.66 x 10^6	0.3 to 1.2 kb	A201-01
HeLa cells (Human cervical carcinoma)	pJG4-5*	9.6 x 10^6	0.5 to 2 kb	A211-01
BeWo cells (Human fetal placental choriocarcinoma)	pYESTrp	5.35 x 10^6	0.3 to 0.8 kb	A208-01
BeWo cells (Human fetal placental choriocarcinoma)	pYESTrp	5.5 x 10^6	0.3 to 1.2 kb	A208-02
Jurkat cells (Human T cell leukemia)	pYESTrp	3.2 x 10^6	0.3 to 1.2 kb	A209-01
A20 cells (Mouse B cell lymphoma)	pYESTrp	3.11 x 10^6	0.3 to 1.2 kb	A210-01
Human Adult Tissue				
Liver	pYESTrp	2.21 x 10^6	0.3 to 1.2 kb	A203-01
Brain	pYESTrp	8.85 x 10^6	0.3 to 0.8 kb	A204-01
Breast	pYESTrp2	9.00 x 10^6	0.4 to 1.2 kb	A217-01
Breast Tumor	pYESTrp2	8.84 x 10^6	0.4 to 1.2 kb	A216-01
Lung	pYESTrp2	5.95 x 10^6	0.4 to 1.2 kb	A213-01
Lung Tumor	pYESTrp2	1.85 x 10^6	0.4 to 1.2 kb	A215-01
Ovary	pYESTrp	4.54 x 10^6	0.3 to 1.2 kb	A206-01
Placenta	pYESTrp	4.75 x 10^6	0.3 to 1.2 kb	A207-01
Prostate	pYESTrp2	5.46 x 10^6	0.4 to 1.2 kb	A218-01
Spleen	pYESTrp2	11.4 x 10^6	0.4 to 1.2 kb	A214-01
Testis	pYESTrp	6.4 x 10^6	0.3 to 1.2 kb	A205-01
Human Fetal Tissue				
Fetal Liver	pYESTrp	2.37 x 10^6	0.3 to 1.2 kb	A202-01

*Please see page 44 for information on pJG4-5.

Cloning into pYESTrp2

pYESTrp2

pYESTrp2 (5828 bp) can be used to make two-hybrid cDNA libraries or to clone genes encoding known proteins. The table below describes the features of this vector. All elements have been functionally tested. To clone known proteins or your own library into pYESTrp2, please see page 9.

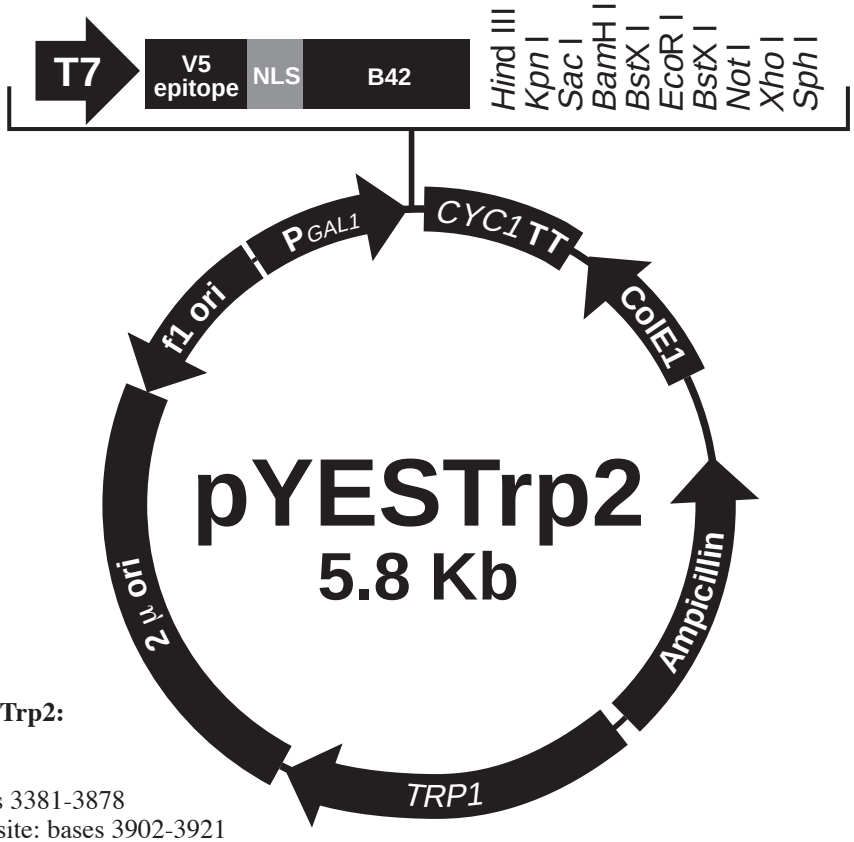
Feature	Benefit
GAL1 promoter	Expression of genes cloned into pYESTrp2. Expression is constitutive in L40 and inducible in EGY48/pSH18-34
T7 promoter/priming site	Permits sequencing of insert or *in vitro* transcription of sense strand
V5 epitope	Allows detection of fusion protein(s) using the Anti-V5 Antibody (Catalog no. R960-25) or Anti-V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991)
SV40 large T antigen nuclear localization sequence (NLS)	Localizes fusions to the nucleus for potential interaction with LexA fusions
B42 activation domain (AD)	Transcriptional activation domain that allows expression of reporter genes when brought into proximity with the LexA DNA binding domain (DBD) by two interacting proteins (Ma and Ptashne, 1987)
Multiple cloning site with 8 unique sites, plus two *Bst*X I sites.	Allows in-frame cloning of a cDNA library or a single gene with the B42 activation domain
CYC1 transcription termination signal	Permits efficient termination and stabilization of mRNA
ColE1 origin (pUC derived)	Maintenance and high-copy replication in *E. coli*
Ampicillin resistance gene	Selection in *E. coli*.
TRP1 gene	Auxotrophic selection of the plasmid in Trp^- yeast hosts (e.g. L40 or EGY48/pSH18-34)
2 μ origin	Maintenance and high-copy replication in yeast
f1 origin	Rescue of single-stranded DNA

continued on next page

Cloning into pYESTrp2, continued

Map of pYESTrp2

A map of pYESTrp2 is provided below and a diagram of the multiple cloning site is provided on the next page. **The sequence is available for downloading from our Web site (http://www.invitrogen.com)** or from Technical Service (see page 51).



Comments for pYESTrp2:
5828 nucleotides

GAL1 promoter: bases 3381-3878
T7 promoter/priming site: bases 3902-3921
Initiation ATG: bases 3942-3944
V5 epitope: bases 3945-3986
Nuclear localization signal (NLS): bases 4002-4028
B42 activation domain: bases 4032-4269
pYESTrp Forward priming site: bases 4233-4251
Multiple cloning site: bases 4275-4368
pYESTrp Reverse priming site: bases 4400-4418
CYC1 transcription termination region: bases 4383-4631
ColE1 origin: bases 4813-5486
Ampicillin resistance gene: bases 5631-663
TRP gene: bases 973-1647
2 µ origin: bases 2051-2885
f1 origin: bases 2954-3326

continued on next page

Ligating a Gene into pYESTrp2

Propagate pYESTrp2 in *E. coli* as described on page 10. Decide on a cloning strategy using the diagram on the next page and ligate your gene into pYESTrp2.

E. coli Transformation

Once you have set up your ligation reactions, transform your ligation mixtures into *E. coli* and select on 50 to 100 µg/ml ampicillin. Plasmid DNA from selected transformants is analyzed for the presence and orientation of insert.

We recommend that you sequence your construct with the supplied pYESTrp Forward and pYESTrp Reverse primers to confirm that your gene is fused in frame with the B42 activation domain.

Testing Two Known Proteins for Interaction

After ligating the first gene into pYESTrp2, please refer to page 13 to ligate a second gene into pHybLex/Zeo. Once you have cloned your two proteins, use a small-scale transformation to cotransform your constructs in pHybLex/Zeo and pYESTrp2 and select for histidine (or leucine) prototrophy. Score resulting positives for β-galactosidase activity. If the proteins interact, the majority of His^+ transformants should also be $LacZ^+$.

Constructing a cDNA Library

Review the general guidelines listed below to generate a unidirectional cDNA library in pYESTrp2. Please refer to Current Protocols in Molecular Biology, Unit 5 for the details of cDNA library construction.

- Isolate mRNA from the source of interest.
- Prime mRNA with random primers, Oligo dT(*Not* I) primer (Catalog no. N430-01), or an Oligo dT (*Xho* I) primer to prepare first strand cDNA.
- After second strand synthesis, be sure the ends are blunt prior to adding *Bst*X I or *Eco*R I adaptors.
- Digest with *Not* I or *Xho* I and electrophorese on an agarose gel for size selection.
- Isolate cDNA for ligation into pYESTrp2.
- Digest pYESTrp2 with either *Bst*X I (or *Eco*R I) and *Not* I (or *Xho* I) to complement the ends on the cDNA.
- Ligate cDNA into digested vector and transform into *E. coli*.
- Determine number of primary recombinants. You may wish to amplify the library prior to large-scale isolation of plasmid DNA for the library screen (page 22 or page 26).

Large-Scale Isolation of Plasmid

You will need 500 µg of library plasmid DNA to perform a large-scale library transformation. To isolate plasmid DNA, perform the following steps. If you are using a Hybrid Hunter™ Premade Library, follow the directions that come with your library.

1. Inoculate 1-2 liters of LB medium containing the appropriate antibiotic (for pYESTrp2, use 50 to 100 µg/ml ampicillin) with sufficient bacterial library stock to insure 2-3 times the number of independent clones in the library.
2. Incubate at 37°C overnight with shaking.
3. After incubation, pellet the cells and proceed with large- or mega-scale isolation of plasmid DNA. Any standard method is suitable. You may have to adjust the plasmid preparation protocol to account for the density of the culture.
4. Store the plasmid at -20°C until ready for use.

continued on next page

Cloning a Single Gene into pYESTrp2

The sequence of the multiple cloning site has been provided below to help you clone the gene of interest into pYESTrp2. You must clone your gene in frame with the sequence encoding the V5 epitope-NLS-B42 fusion protein in order to create a "prey" fusion protein with a nuclear localization signal, activation domain, and an epitope for detection. This leader peptide adds 12 kDa to your prey protein. Restriction sites are labeled to indicate the cleavage site. The complete sequence may be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (see page 51).

5′ end of *GAL1* promoter

```
3381  CGCGCTTAAT GGGGCGCTAC AGGGCGCGTG GGGATGATCC ACTAGTACGG ATTAGAAGCC

                  GAL4 binding site                GAL4 binding site
3441  GCCGAGCGGG TGACAGCCCT CCGAAGGAAG ACTCTCCTCC GTGCGTCCTC GTCCTCACCG

3501  GTCGCGTTCC TGAAACGCAG ATGTGCCTCG CGCCGCACTG CTCCGAACAA TAAAGATTCT

3561  ACAATACTAG CTTTTATGGT TATGAAGAGG AAAAATTGGC AGTAACCTGG CCCCACAAAC

                                                                  TATA Box
3681  TTATTTCTGG GGTAATTAAT CAGCGAAGCG ATGATTTTTG ATCTATTAAC AGATATATAA

3741  ATGCAAAAAC TGCATTAACC ACTTTAACTA ATACTTTCAA CATTTTCGGT TTGTATTACT

                          transcriptional start
3801  TCTTATTCAA ATGTAATAAA AGTATCAACA AAAAATTGTT AATATACCTC TATACTTTAA

                                                  T7 promoter/priming site
3861  CGTCAAGGAG AAAAAACCCC GGATCGGACT ACTAGCAGCT GTAATACGAC TCACTATAGG

                                                  V5 epitope
3921  GAATATTAAG CTAAGCTCAC C ATG GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT
                              Met Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly

                                                  SV40 NLS
3975  CTC GAT TCT ACA CAA GCT ATG GGT GCT CCT CCA AAA AAG AAG AGA AAG GTA
      Leu Asp Ser Thr Gln Ala Met Gly Ala Pro Pro Lys Lys Lys Arg Lys Val

4026  GCT GGT ATC AAT AAA GAT ATC GAG GAG TGC AAT GCC ATC ATT GAG CAG TTT
      Ala Gly Ile Asn Lys Asp Ile Glu Glu Cys Asn Ala Ile Ile Glu Gln Phe

4077  ATC GAC TAC CTG CGC ACC GGA CAG GAG ATG CCG ATG GAA ATG GCG GAT CAG
      Ile Asp Tyr Leu Arg Thr Gly Gln Glu Met Pro Met Glu Met Ala Asp Gln
                                  B42 transactivation domain
4128  GCG ATT AAC GTG GTG CCG GGC ATG ACG CCG AAA ACC ATT CTT CAC GCC GGG
      Ala Ile Asn Val Val Pro Gly Met Thr Pro Lys Thr Ile Leu His Ala Gly

4179  CCG CCG ATC CAG CCT GAC TGG CTG AAA TCG AAT GGT TTT CAT GAA ATT GAA
      Pro Pro Ile Gln Pro Asp Trp Leu Lys Ser Asn Gly Phe His Glu Ile Glu
                pYESTrp Forward priming site                        HindIII
4230  GCG GAT GTT AAC GAT ACC AGC CTC TTG CTG AGT GGA GAT GCC TCC AAG CTT
      Ala Asp Val Asn Asp Thr Ser Leu Leu Leu Ser Gly Asp Ala Ser Lys Leu
           KpnI      SacI  BamHI                                BstXI  EcoRI
4281  GGT ACC GAG CTC GGA TCC ACT AGT AAC GGC CGC CAG TGT GCT GGA ATT CTG
      Gly Thr Glu Leu Gly Ser Thr Ser Asn Gly Arg Gln Cys Ala Gly Ile Leu
                          BstXI  NotI      XhoI            SphI
4332  CAG ATA TCC ATC ACA CTG GCG GCC GCT CGA GGC ATG CAT CTA GAG GGC CGC
      Gln Ile Ser Ile Thr Leu Ala Ala Ala Arg Gly Met His Leu Glu Gly Arg
                                pYESTrp Reverse priming site
4381  ATC ATG TAA TTAGTTA TGTCACGCTT ACATTCACGC CCTCCCCCCA
      Ile Met ***
```

Constructing the Bait Plasmid

Introduction

This section provides information on cloning the gene for the protein of interest into the bait plasmid, pHybLex/Zeo, to create a LexA fusion protein. The user should be familiar with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry. For more information on these topics, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al*., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al*., 1994) (See **References**, page 53).

Bait Protein Criteria

The first step in construction of pHybLex/Zeo bait plasmids is to decide whether to fuse a full length protein or a particular domain of that protein in frame to LexA. Screens employing full length protein fused to LexA tend to have a lower background of false positives. However, in the event that the full length protein activates the reporter genes, then domains or fragments of the protein should be fused to LexA and tested as possible baits. Avoid proteins with extensive transmembrane domains or signal sequences that would direct it to places other than the nucleus.

Maintenance and Propagation of Hybrid Hunter™ Plasmids

To propagate and maintain the Hybrid Hunter™ vectors, follow the steps below.

1. Prepare 1 μg/μl stock solutions of each vector:

   Resuspend pHybLex/Zeo-Fos2, pYESTrp-Jun, and pHybLex/Zeo-Lamin in 10 μl sterile water.

   Resuspend pHybLex/Zeo and pYESTrp2 in 20 μl sterile water.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, TOP10F´, DH5α, JM109, or equivalent. Use 10 ng of each plasmid for transformation of *E. coli*.
3. Select transformants as follows:

   Use LB plates containing 50 to 100 μg/ml ampicillin for pYESTrp2 and pYESTrp-Jun transformants

   Use Low Salt LB containing 25 to 50 μg/ml Zeocin™ for pHybLex/Zeo pHybLex/Zeo-Fos2, pHybLex/Zeo-Lamin, and transformants (see page 42 for Zeocin™ selective medium for *E. coli*).
4. Store the stock solution at -20°C when finished.
5. Be sure to prepare a glycerol stock of each strain containing plasmid for long-term storage (see below).

Preparing a Glycerol Stock

Once you have transformed the Hybrid Hunter™ plasmids into a suitable *E. coli* strain (see above), purify a single transformant and prepare a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at -20°C.

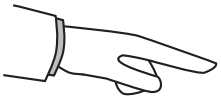
- Streak the original colony out on an LB plate containing 50 µg/ml ampicillin. Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin.
- Grow the culture to mid-log phase (OD_{600}=0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

continued on next page

Features of pHybLex/Zeo

The table below summarizes the features of pHybLex/Zeo (4768 bp). All features have been functionally tested.

Feature	Purpose
Alcohol dehydrogenase (*ADH1*) promoter	Strong, constitutive promoter for expression of LexA fusions
LexA ORF	Complete *lex*A gene (202 amino acids; 606 bp) for creation of fusion proteins with the LexA DNA binding domain (Horii *et al*., 1981; Markham *et al*., 1981)
Multiple cloning site with 10 unique restriction sites	Allows insertion of your gene into the expression vector
ADH1 transcription termination (TT)	Provides efficient transcription termination and stabilizes the mRNA
2 μ origin	Allows replication of the plasmid in yeast strains
TEF1 promoter (GenBank accession numbers D12478, D01130)	Transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives expression of the *Sh ble* gene in yeast, conferring Zeocin™ resistance
EM-7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the *Sh ble* gene in *E. coli*, conferring Zeocin™ resistance
Sh ble gene (*Streptoalloteichus hindustanus ble* gene)	Zeocin™ resistance gene (Calmels *et al*., 1991; Drocourt *et al*., 1990; Gatignol *et al*., 1988).
CYC1 transcription termination region (GenBank accession number M34014)	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the *Sh ble* gene for increased stability
ColE1 origin (pUC-derived)	Allows high-copy replication and maintenance of the plasmid in *E. coli*



NOTE

pHybLex/Zeo does not contain a nuclear localization signal. LexA fusions appear to be produced in sufficient amounts to allow entry into the nucleus by mass action.

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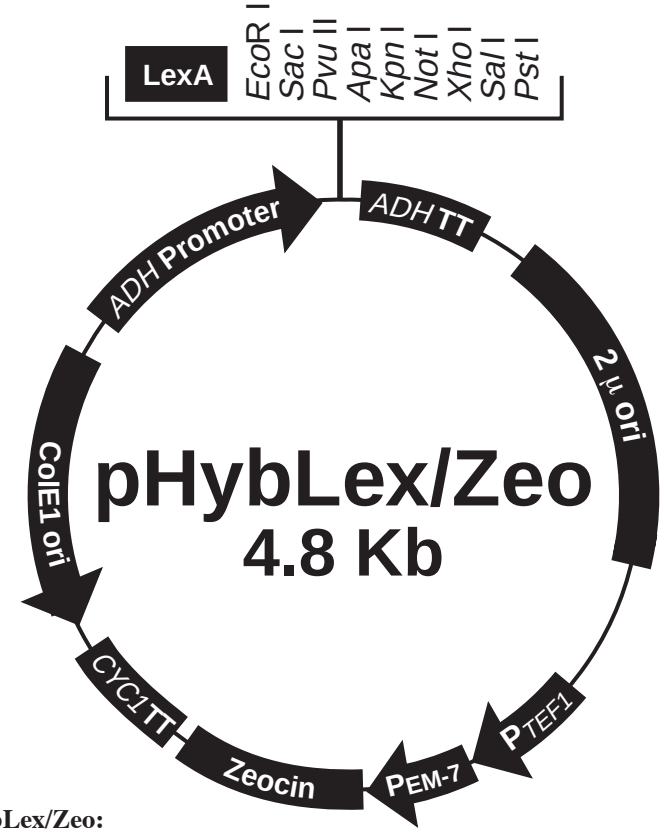
Map of pHybLex/Zeo

The figure below summarizes the features of pHybLex/Zeo vector. The complete nucleotide sequence for pHybLex/Zeo is provided after page 53. The sequence is also available for downloading from our World Wide Web site (http://www.invitrogen.com) or from Technical Service (see page 51). Details of the multiple cloning site are shown on page 13.



Comments for pHybLex/Zeo:
4768 nucleotides

ADH Promoter: bases 1-399
LexA Open Reading Frame: bases 420-1029
pHybLex/Zeo Forward priming site: bases 986-1010
Multiple Cloning Site: bases 1030-1093
pHybLex/Zeo Reverse priming site: bases 1161-1185
ADH Transcription Termination Region: bases 1144-1301
2 µ Origin of Replication: bases 1602-2311
TEF1 Promoter: bases 2858-3266
EM-7 Promoter: bases 3270-3337
Zeocin™ Resistance Gene: bases 3338-3712
CYC1 Transcription Termination Region: bases 3713-4030
ColE1 Origin: bases 4041-4714

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Constructing the Bait Plasmid, continued

Cloning into pHybLex/Zeo

To ensure proper expression of your bait protein, you must clone your bait gene in frame with the LexA protein. See the sequence below to develop a cloning strategy. Restriction sites are labeled to indicate the cleavage site.

LexA ORF

```
420  ATG AAA GCG TTA ACG GCC AGG CAA CAA GAG GTG TTT GAT CTC ATC CGT GAT
     Met Lys Ala Leu Thr Ala Arg Gln Gln Glu Val Phe Asp Leu Ile Arg Asp

471  CAC ATC AGC CAG ACA GGT ATG CCG CCG ACG CGT GCG GAA ATC GCG CAG CGT
     His Ile Ser Gln Thr Gly Met Pro Pro Thr Arg Ala Glu Ile Ala Gln Arg

522  TTG GGG TTC CGT TCC CCA AAC GCG GCT GAA GAA CAT CTG AAG GCG CTG GCA
     Leu Gly Phe Arg Ser Pro Asn Ala Ala Glu Glu His Leu Lys Ala Leu Ala

573  CGC AAA GGC GTT ATT GAA ATT GTT TCC GGC GCA TCA CGC GGG ATT CGT CTG
     Arg Lys Gly Val Ile Glu Ile Val Ser Gly Ala Ser Arg Gly Ile Arg Leu

624  TTG CAG GAA GAG GAA GAA GGG TTG CCG CTG GTA GGT CGT GTG GCT GCC GGT
     Leu Gln Glu Glu Glu Glu Gly Leu Pro Leu Val Gly Arg Val Ala Ala Gly

675  GAA CCA CTT CTG GCG CAA CAG CAT ATT GAA GGT CAT TAT CAG GTC GAT CCT
     Glu Pro Leu Leu Ala Gln Gln His Ile Glu Gly His Tyr Gln Val Asp Pro

726  TCC TTA TTC AAG CCG AAT GCT GAT TTC CTG CTG CGC GTC AGC GGG ATG TCG
     Ser Leu Phe Lys Pro Asn Ala Asp Phe Leu Leu Arg Val Ser Gly Met Ser

777  ATG AAA GAT ATC GGC ATT ATG GAT GGT GAC TTG CTG GCA GTG CAT AAA ACT
     Met Lys Asp Ile Gly Ile Met Asp Gly Asp Leu Leu Ala Val His Lys Thr

828  CAG GAT GTA CGT AAC GGT CAG GTC GTT GTC GCA CGT ATT GAT GAC GAA GTT
     Gln Asp Val Arg Asn Gly Gln Val Val Val Ala Arg Ile Asp Asp Glu Val

879  ACC GTT AAG CGC CTG AAA AAA CAG GGC AAT AAA GTC GAA CTG TTG CCA GAA
     Thr Val Lys Arg Leu Lys Lys Gln Gly Asn Lys Val Glu Leu Leu Pro Glu

930  AAT AGC GAG TTT AAA CCA ATT GTC GTA GAT CTT CGT CAG CAG AGC TTC ACC
     Asn Ser Glu Phe Lys Pro Ile Val Val Asp Leu Arg Gln Gln Ser Phe Thr

          pHybLex/Zeo Forward priming site                          EcoR I
981  ATT GAA GGG CTG GCG GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG GAA TTC
     Ile Glu Gly Leu Ala Val Gly Val Ile Arg Asn Gly Asp Trp Leu Glu Phe

               Sac I              Pvu II      Apa I      Kpn I  Not I      Xho I      Sal I
1032 AAG CTT GAG CTC AGA TCT CAG CTG GGC CCG GTA CCG CGG CCG CTC GAG TCG
     Lys Leu Glu Leu Arg Ser Gln Leu Gly Pro Val Pro Arg Pro Leu Glu Ser

          Pst I
1083 ACC TGC AGC CAAGCTAATTC CGGGCGAATT TCTTATGATT TATGATTTTT ATTATTAAAT
     Thr Cys Ser
```

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Constructing the Bait Plasmid, continued

E. coli Transformation

Once you have set up your ligation reactions, please refer to page 42 to transform your ligation mixtures into *E. coli* and select on Zeocin™. Selection requires plating the transformation mixture on **Low Salt** LB medium containing 25 to 50 μg/ml Zeocin™. Plasmid DNA from selected transformants is analyzed for the presence and orientation of insert.

We recommend that you sequence your construct with the supplied pHybLex/Zeo Forward and pHybLex/Zeo Reverse primers to confirm that your gene is fused in frame with the LexA protein.

The Next Step

Once you are satisfied that you have fused your bait protein correctly in frame to LexA, you are ready to transform your construct into the yeast strain L40 or EGY48/pSH18-34 to confirm expression of the bait (next page).

Yeast Transformation with Bait Plasmid

Introduction

In this section, you will use a small-scale yeast transformation protocol to transform your bait plasmid into L40 or EGY48/pSH18-34 to create the bait strain. We recommend that the bait strain be tested for proper expression of the LexA fusion (page 16) and for any non-specific activation of reporter constructs (next section, pages 18-20).

Choosing the Yeast Host

Two yeast hosts, L40 and EGY48/pSH18-34, are provided in the Hybrid Hunter™ Kit. To assist you in choosing a host strain, a table of features is provided below. For most purposes, L40 works very well; however, if you are concerned about potentially toxic interactors, use EGY48/pSH18-34. Other yeast host strains are available from Invitrogen. For more information, please see page 17.

L40	EGY48/pSH18-34
Contains reporter constructs integrated into the genome	Contains pSH18-34 already transformed. For more information on pSH18-34, see page 45
GAL1 promoters are constitutive in this host strain	*GAL1* promoters are inducible by galactose and repressed by glucose. Helps eliminate false positives and express potentially toxic interactors
Wild-type for GAL4	Wild-type for GAL4
Will express libraries in pYESTrp, pYESTrp2, or pJG4-5	Will express libraries in pYESTrp, pYESTrp2, or pJG4-5

Basic Yeast Molecular Biology

The user should be familiar with basic yeast molecular biology and microbiological techniques. Please refer to Current Protocols in Molecular Biology (1996) *Saccharomyces cerevisiae*, pp. 13.01 to 13.2.12 for information on preparing yeast media and handling yeast.

Important

To initiate cultures from frozen yeast stocks, streak a small amount of frozen stock on a YPD plate. Once growth is established, you may check the phenotype of each strain by streaking the strain on a minimal plate supplemented with the appropriate amino acids.

Be sure to make glycerol stocks of all strains including transformed strains. If you use strains or transformants directly from plates be sure the plates are less than 4 days old.

Materials Required

Be sure to have the following reagents and equipment on hand before proceeding:

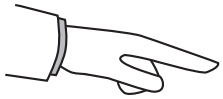
- 30°C incubator and shaking incubator
- 60°C and 70°C water baths or temperature blocks and a boiling water bath
- Clinical centrifuge and low-speed centrifuge
- Reagents for yeast transformation (see next page)
- YPD containing 0.1% adenine and 300 µg/ml Zeocin™ (YPAD Z300) for medium and plates (see page 39 for recipe)
- Cracking buffer (see page 40 for recipe), prewarmed to 60°C
- Acid washed glass beads (Sigma G-8772, 425-600 microns)
- Reagents for SDS-PAGE and immunoblotting
- Antibody to your bait protein or Anti-LexA antibody (see page v)

continued on next page

Reagents for Yeast Transformation

The *S. c.* EasyComp™ Kit (Catalog no. K5050-01) provides a quick and easy method for the preparation of competent yeast cells that can be used immediately or stored frozen for future use. Transformation efficiency is guaranteed at $>10^3$ transformants per μg DNA.

For your convenience, a small-scale protocol is included in the **Appendix**, page 43. Alternatively, there are published references for other small-scale transformation methods (Gietz *et al*., 1992; Gietz and Schiestl, 1996; Hill *et al*., 1991; Schiestl and Gietz, 1989).



NOTE

EGY48/pSH18-34 is more sensitive to Zeocin™ than L40. Use 200 μg/ml in plates and medium.

Yeast Transformation

Using one of the methods described above (or one of your own choosing), transform your bait plasmid into competent L40 (or EGY48/pSH18-34).

- Select transformants on YPAD Z300 (or YPD Z200) plates.
- Grow for 2 to 3 days at 30°C.
- Select several Zeocin™-resistant transformants to characterize for expression of the bait.

  Note: Be sure to keep your transformation plates in the event you need to select another transformant. Plates are stable for 4 days when wrapped with parafilm and stored at +4°C.

Expression of Bait

Use the protocol below to prepare lysates from your Zeocin™-resistant transformants (putative bait strain) and untransformed L40 (or EGY48/pSH18-34) for immunoblot analysis. Be sure to test several transformants because of the heterogeneity of LexA fusion expression levels.

1. Inoculate 5 ml of YPAD Z300 (or YPD Z200) with a single colony of your bait strain (above) and inoculate 5 ml of YPAD (YPD) with L40 (EGY48/pSH18-34) as a negative control. Grow overnight with shaking at 30°C.
2. Streak a sample from each culture onto a fresh plate. After checking for expression, you can return to this plate and use it as a source of your bait strain.
3. Pellet the cells by centrifuging 5 minutes at 2500 rpm (room temperature). Decant the medium.
4. Transfer the tubes to a -80°C freezer for 10 minutes.
5. Thaw cell pellet in 100 μl of prewarmed (60°C) cracking buffer and resuspend by pipetting the cell pellet in the buffer.
6. Transfer cell suspension to a 1.5 ml microcentrifuge tube containing 100 μl of glass beads.
7. Incubate the solution at 70°C for 10 minutes.
8. Vortex solution for 1 minute.
9. Centrifuge at 14,000 rpm for 5 minutes (room temperature) and transfer supernatant to new tube.
10. Add SDS-PAGE sample buffer and boil sample for 5 minutes. Use 30 to 50 μl for immunoblot analysis. Detect LexA fusion using antibody to your protein or the Anti-LexA Antibody (page v).

continued on next page

What You Should See

The calculated molecular weight of the LexA protein expressed from pHybLex/Zeo is 26 kDa. This includes additional amino acids encoded by the multiple cloning site. We have observed that the LexA protein expressed from pHybLex/Zeo migrates at 32 kDa on SDS-PAGE. The results from your immunoblotting experiment should show that the bait strain expresses an intact LexA fusion whereas the untransformed L40 strain should not show any cross-reactivity. Please note that the background may be increased when using crude lysates.

Once your bait strain has been shown to express the LexA fusion, proceed to the next section to ensure that the bait behaves correctly in order to perform an interactor hunt.

Other Yeast Host Strains

If you wish to use a yeast host strain that allows more stringent selection of interactors and exhibits lower background with baits, the EGY191 yeast strain is available from Invitrogen. The EGY191 strain is related to EGY48, but contains only 2 *LexA* operators to direct transcription of the *LEU2* reporter gene. Use of both strains in the Hybrid Hunter™ System will enable you to differentiate between strong and weak interactors with your protein of interest. The EGY191 strain is also available pretransformed with the pSH18-34 *lacZ* reporter plasmid (see below and page 45 for more information).

Strain	Genotype	Phenotype	Catalog no.
EGY191	MATα *ura3 trp1 his3* 2*lex*Aop-*LEU2*	Ura$^-$, Trp$^-$, His$^-$, Leu$^-$	C837-00
EGY191/ pSH18-34	EGY191 transformed with pSH18-34	Ura$^+$, Trp$^-$, His$^-$, Leu$^-$	C838-00

LacZ Reporter Plasmids

Invitrogen offers a number of *lacZ* reporter plasmids to allow creation of your own yeast reporter strain. The reporter plasmids contain varying numbers of *LexA* operators directing expression of the *lacZ* reporter gene, and may be used to generate reporter strains with varying degrees of sensitivity for transcriptional activation of β-galactosidase expression in the Hybrid Hunter™ System. Please note that sensitivity increases as the number of *LexA* operators increases. A map and brief description of pSH18-34 is provided on page 45. For more information about the pJK103 and pRB1840 *lacZ* reporter plasmids, please refer to (Estojak *et al.*, 1995), Current Protocols in Molecular Biology (1994), pp. 20.1.5, or call Technical Service (see page 51). Ordering information is provided below.

Plasmid	No. of LexA operators	Catalog no.
pSH18-34	8	V611-20
pJK103	2	V613-20
pRB1840	1	V612-20

Testing the Bait Plasmid

Introduction

In this section you will test the LexA fusion (bait) for nonspecific activation. A well-behaved bait (i.e., a protein fused to LexA) **should not:**

- Non-specifically transactivate the reporter constructs in the L40 or EGY48/pSH18-34 strains.
- Interact with either the nuclear localization signal (NLS) or with the acidic activation domain in the library plasmid (e.g. pYESTrp2).

The bait plasmid is transformed alone into L40 (or EGY48/pSH18-34) and together with pYESTrp2 (or pJG4-5) and the resulting strains tested for growth on histidine (L40) or leucine (EGY48/pSH18-34) and β-galactosidase activity. The transformed strains **should not** grow in the absence of histidine (or leucine) **OR** exhibit detectable β-galactosidase activity.

Important

If you use EGY48 as host--

Be sure to use EGY48/pSH18-34. This strain contains the LacZ reporter plasmid (see page 45). Since pSH18-34 contains the wild-type *URA3* gene, the strain should grow on uracil-deficient medium (YC-U).

Add galactose to the medium to induce expression of the activation domain in pYESTrp2 (see below and Steps 4 and 5, next page).

Materials Required

Be sure to have the following reagents and equipment on hand before proceeding. Please see pages 37-41 for specific media recipes.

- 30°C incubator and shaking incubator
- Reagents for yeast transformation
- Centrifuges
- Bait plasmid
- pYESTrp2 (or pJG4-5)
- YPAD medium
- 3-aminotriazole (optional, Sigma)
- β-galactosidase activity reagents (see page 40)

Media Requirements

The table below describes the media used for testing the bait plasmid in L40 or EGY48/pSH18-34. Please refer to the protocol on the next page to determine which media you will need. Please see page 37 for more detail on these media.

Experiment	Plasmids	L40	EGY48/pSH18-34
Selection	Bait Plasmid	YPAD Z300	YC-U Z200
	Bait Plasmid + pYESTrp2	YC-W Z300	YC-WU Z200/Gal
Assay for Prototrophy (histidine or leucine)	Bait Plasmid	YC-HUK Z300	YC-UL Z200
	Bait Plasmid + pYESTrp2	YC-WHUK Z300	YC-WUL Z200/Gal
Assay for β-galacto-sidase Activity	Bait Plasmid	YPAD Z300	YC-U Z200
	Bait Plasmid + pYESTrp2	YC-W Z300	YC-WU Z200/Gal

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Testing the Bait Plasmid, continued

Controls

In addition to pHybLex/Zeo containing your bait and pYESTrp2, you may wish to transform the following plasmids into L40 (or EGY48/pSH18-34) as controls.

Plasmid	Control
pHybLex/Zeo (no insert)	Positive control for low activation of the reporter constructs*
pHybLex/Zeo-Lamin	Negative control for activation

*The activity of unfused LexA from pHybLex/Zeo is high enough to weakly activate the *HIS3* (or *LEU2*) and *lac*Z reporters. Fusions to LexA generally decrease this background activation. When L40 (or EGY48/pSH18-34) containing pHybLex/Zeo is patched onto His^- plates, only a few colonies are apparent as opposed to a solid streak.

In addition to determining the degree of non-specific reporter activation by the bait plasmid, it is recommended that you test the interaction of the bait with a known partner, if available. This step ensures that a functional fusion has been made and that at least some of the protein is localized to the nucleus.

pHybLex/Zeo-Lamin

This plasmid was created by ligating a 500 bp *Eco*R I-*Sal* I DNA fragment containing the lamin gene into pHybLex/Zeo.

Transforming and Testing Bait Plasmid

Use the protocol below to test for non-specific activation. You will need special media for selection of transformants, assay of prototrophy, and assay of β-galactosidase. Please refer to the table on the previous page for the correct medium.

1. Transform the bait plasmid alone and together with pYESTrp2 into L40 (EGY48/pSH18-34) using a small-scale transformation protocol.

   Note: You may also use the bait strain you constructed on page 16. However, you must make competent cells of the bait strain before transforming with pYESTrp2. You may wish to keep a batch of frozen, competent L40 or EGY48/pSH18-34 (prepared using the *S.c*. EasyComp™ Kit) on hand for testing the bait plasmid.
2. Select transformants on the appropriate media (see previous page).
3. Incubate plates at 30°C for 3 days or until single colonies appear.
4. To assay for histidine prototrophy (L40), patch individual transformants from Step 3 onto the appropriate plates.

   Note: To test for leucine prototrophy (EGY48/pSH18-34) you will need different plates. Please refer to the table on the previous page.
5. To assay for β-galactosidase activity, patch individual colonies from Step 3 and arrange in a grid pattern on the appropriate plates.
6. Incubate all plates at 30°C for 2 to 3 days until colonies form. Assay for β-galactosidase activity using the β-galactosidase filter assay (see next page).

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β-Galactosidase Filter Assay

We recommend using a filter lift assay to detect β-galactosidase activity (Breeden and Nasmyth, 1985). This assay is more sensitive and can detect lower levels of enzyme activity. This protocol describes a convenient method to assay colonies on a plate for β-galactosidase activity. Prepare or have on hand the following reagents and supplies.

- Nitrocellulose filters sized to fit 100 mm or 150 mm plates
- Liquid nitrogen
- Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0)
- 50 mg/ml X-Gal
- Petri dishes (100 or 150 mm)
- #1 Whatman filter paper circles sized to fit plates
- Foil
- 30°C incubator

1. Add liquid nitrogen to a styrofoam container and float a foil boat large enough to hold a nitrocellulose filter on top of the liquid nitrogen.
2. Lay a dry nitrocellulose filter onto the yeast colonies that are on selective medium.
3. Prepare a petri dish for the reaction. In the lid, place 1.5 ml (4.5 ml for 150 mm plates) Z buffer containing 30 μl (100 μl for 150 mm plates) of 50 mg/ml X-gal.

   Note: If you are assaying several plates you can make a cocktail of Z buffer and X-gal, vortex, and add to all the plates.
4. Carefully lay one #1 Whatman filter circle on the Z buffer containing X-gal. Try to avoid forming air bubbles.
5. Carefully remove the filter from the colonies using filter forceps and place it colony side up in the foil boat.
6. After 30 seconds of floating, immerse the boat with the filter for 5 seconds in the liquid nitrogen.
7. Remove the boat with the filter and place at room temperature, until the filter is thawed. **Note**: The filter is extremely brittle.
8. Lay the nitrocellulose filter on top of the wet filter paper with the colonies facing up. Try to avoid forming air bubbles between the nitrocellulose and filter paper.
9. Cover with the bottom of the dish, cover dish with foil, and place at 30°C. Strong interactions yield detectable color in less than 30 minutes. If longer incubations are required for positive signals to be visualized, the petri dish should be placed in a humidified chamber.

Analysis

A well-behaved bait should **NOT** allow the yeast to grow in the absence of histidine or express detectable β-galactosidase activity, **UNLESS** there is an interactor present. If the bait strain becomes His^+ $LacZ^+$ upon transformation of the bait plasmid, please see the **Troubleshooting** section, page 33.

The Next Step

Once you have confirmed that your bait is behaving properly, keep a fresh plate and make a glycerol stock of L40 or EGY48/pSH18-34 containing the bait plasmid (bait strain). Proceed to the interactor hunt (next page).

Interactor Hunt Overview

Introduction

Now that you have a well-behaved bait plasmid transformed into L40 or EGY48/pSH18-34 (bait strain), you are ready to perform one of two applications:

1) an interactor hunt to identify proteins that interact with your bait.

2) test for an interaction with a known protein.

You may use your own library or one of the libraries available in pYESTrp or pYESTrp2 (see page 5). **Please note that the pHybLex/Zeo bait plasmid can be used with any library and the appropriate host strain.** Alternatively, you may clone a second protein into pYESTrp2 for coexpression with your bait in L40 or EGY48/pSH18-34 (see page 8).

Experimental Outline

The table below provides a simplified outline of how a 1-step interactor hunt is performed using either L40 (next page) or EGY48/pSH18-34 (page 26).

Step	L40	EGY48/pSH18-34
1	Transform bait strain with library in pYESTrp, pYESTrp2 or pJG4-5 (or other compatible vector).	
Note	Library is constitutively expressed.	Library is induced with galactose (no glucose).
2	Transformed cells tested for histidine prototrophy. Cells that contain an interactor will grow in the absence of histidine.	Transformed cells tested for leucine prototrophy. Cells that contain an interactor will grow in the absence of leucine.
3	Transformed cells are tested for β-galactosidase activity.	

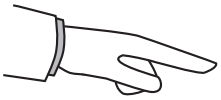
While both library plasmids use the full-length *GAL1* promoter to express proteins as fusions to a B42 activation domain, this promoter expresses equally well on either glucose or galactose in the strain L40. If you use EGY48/pSH18-34, the *GAL1* promoter is inducible, and galactose needs to be included in the medium to express the library.

Interactor Hunt Using L40

Materials Required

Be sure to have the following reagents and equipment on hand before proceeding with the interactor hunt.

- 30°C incubator and shaking incubator
- 42°C water bath
- Centrifuges
- Bait strain (L40 + bait plasmid)
- YPAD medium
- YPAD Z300 medium
- 1X TE
- 1X LiAc/0.5X TE (see **Recipes**, page 37)
- Denatured salmon sperm DNA (see page 50) or yeast tRNA
- pYESTrp or pYESTrp2 library DNA or other DNA library (For more information on Hybrid Hunter™ premade libraries, see page 5)
- 1X LiAc/40% PEG/1X TE (see **Recipes**, page 37)
- DMSO
- YPA (contains no glucose)
- YC-WU medium
- YC-WU Z300 medium and plates
- YC-WHUK medium
- YC-WHUK Z300 medium and plates



NOTE

In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = ~2.0 x 10^7 yeast cells.

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Large-Scale Library Transformation

The 1-step protocol described below should be performed straight through except at the indicated incubations. Review the procedure carefully and make sure you have all the necessary reagents before starting. The whole procedure will take 4 days with an additional 2 to 5 days for colonies to come up.

Before Starting

- Prepare (page 50) or purchase denatured salmon sperm DNA or use yeast tRNA carrier
- Prepare 500 μg library plasmid DNA using cesium chloride ultracentrifugation or DNA binding resin. Alternatively, DNA can be prepared by alkaline lysis, followed by phenol-chloroform extraction; RNase treatment is not required.
- 2 150 mm YC-WU Z300 plates
- 40 150 mm YC-WHUK Z300 plates

Preparation of Bait Strain for Transformation.

For the large-scale library transformation of the L40 bait strain, we utilize a protocol that is a modification of published methods (Gietz *et al*., 1992; Gietz and Schiestl, 1996; Hill *et al*., 1991; Schiestl and Gietz, 1989). The bait strain is prepared for transformation as follows.

1. Inoculate the L40 bait strain into 5 ml of YPAD Z300. Grow overnight at 30°C.
2. Prepare a culture flask containing 100 ml of YPAD Z300 and inoculate with sufficient overnight culture to bring the culture to an OD_{600} of 2 to 3 (mid-log phase) in 16 hours (overnight).

   To calculate the amount of overnight culture needed to bring a 100 ml culture to an OD_{600} of 3 per ml in 16 hours, assume that yeast double every 2 hours when grown in Zeocin™-containing medium. In 16 hours, the OD_{600} will increase by a factor of 2^8 or 256. Therefore, you will need a starting OD_{600} of 0.012 per ml (3 ÷ 256). If your overnight culture is 3 OD_{600} per ml, then for a 100 ml culture, add

   $$\frac{(0.012\ \text{OD/ml})\ (100\ \text{ml})}{3\ \text{OD/ml}} = 0.40\ \text{ml}$$
3. Grow 16 hours (overnight) at 30°C with constant shaking.
4. Using the overnight culture from Step 3, inoculate 1 liter of YPAD to a final OD_{600} of 0.3.
5. Grow at 30°C with constant shaking for 3 hours.
6. Pellet cells at 5000 rpm for 10 minutes at room temperature. Decant supernatant.
7. Wash cell pellet in 500 ml of sterile TE buffer.
8. Resuspend cells in 20 ml of 1X lithium acetate/0.5X TE and transfer to a sterile 1 liter flask. Proceed immediately to the next section.

continued on next page

Large-Scale Library Transformation, continued

Transformation of Bait Strain with library DNA.

9. Mix together 1 ml of 10 mg/ml denatured salmon sperm DNA and 500 μg library DNA.
10. Add DNA mixture to cell suspension from Step 8, previous page.
11. Add 140 ml 1X lithium acetate/40% PEG-3350/TE. Swirl to mix and incubate at 30°C for 30 minutes.
12. Add 17.6 ml DMSO and swirl to mix.
13. Heat shock at 42°C for 6 minutes with occasional swirling to facilitate heat transfer.
14. Immediately dilute with 400 ml of YPA (or YPAD) and rapidly cool to room temperature in a water bath.
15. Pellet cells at 5000 rpm for 10 minutes (room temperature).
16. Resuspend the pellet with 500 ml YPA (or YPAD) and pellet cells at 5000 rpm for 10 minutes.
17. Resuspend pellet in 1 liter YPAD and incubate at 30°C for 1 hour with constant shaking. Proceed to the next section.

Plating Transformation

First you will plate out a small sample of transformed cells to determine the primary transformation efficiency. The transformed cells will be allowed to grow before plating on selective medium.

18. Remove 1 ml of cells from culture in Step 17, above, and pellet.
19. Resuspend cells in 1 ml YC-WU Z300 and plate 10 μl and 1 μl aliquots ($1/10^5$ and $1/10^6$ of total) on YC-WU Z300 to measure the primary transformation efficiency. This protocol should yield 10 to 100 million transformants.
20. Pellet remaining cells from Step 17, above.
21. Resuspend the pellet in 500 ml YC-WU, pellet cells, and resuspend in 1 liter of prewarmed YC-WU Z300.
22. Incubate, with shaking, at 30˚C for 16 hours.
23. Pellet cells and wash with 500 ml YC-WHUK.
24. Repeat Step 23 (optional).
25. Pellet cells and resuspend final pellet in 10 ml YC-WHUK Z300.
26. Prepare a $1:10^5$ and a $1:10^6$ dilutions of the cell suspension from Step 25. Use YC-WHUK Z300 as the diluent. Plate 100 μl of each dilution on YC-WU Z300 plates to compare to the number of primary transformants you calculated in Step 2. This allows determination of the number of doublings and the number of His^+ colonies that should be screened to roughly cover the number of primary transformants.
27. Plate 10 YC-WHUK Z300 plates each of 5 μl, 10 μl, 25 μl, and 50 μl from the culture in Step 25 (40 plates total). **Note**: Because His^+ colonies grow poorly if the density on the plates is too high, the optimal plating volume is dependent on the recovery time in YC-WU Z300 and on the efficiency of the transformation.

    Save the remainder of the 10 ml at +4°C as a backup in the event the dilutions are inappropriate. Yeast are stable for at least one week at +4°C.
28. Incubate all plates at 30°C for 2 to 5 days.

continued on next page

Analysis of Transformants

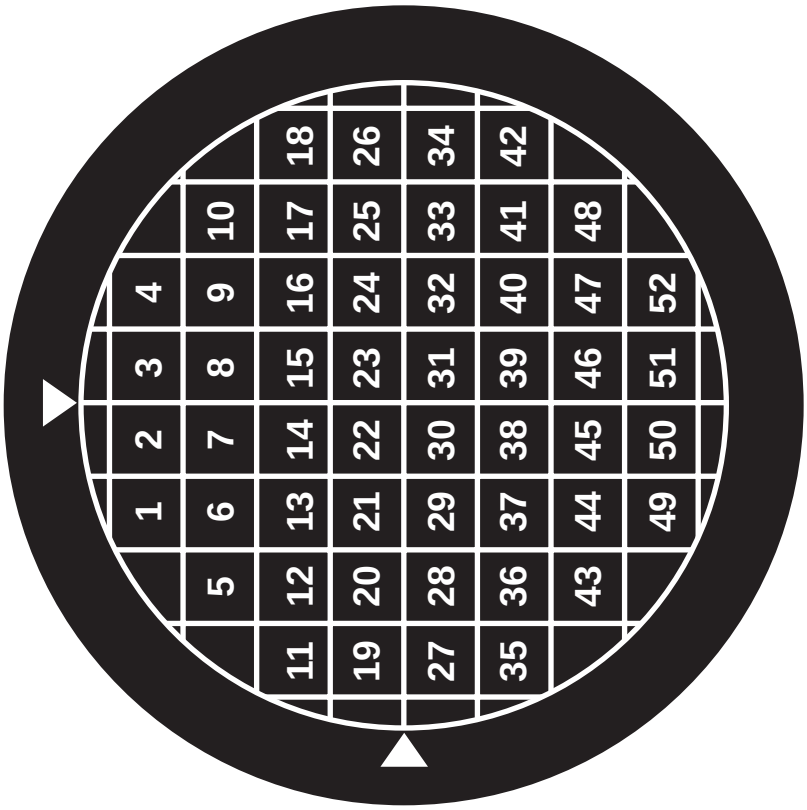
The His^+ colonies that grow on selective plates represent colonies that were transformed with a library plasmid that encodes a potential interactor. We recommend that you screen as many His^+ colonies as possible. At Invitrogen, most screens yield ~50% His^+, $LacZ^+$ colonies. In the original paper by Brent and coworkers, they used leucine prototrophy and β-galactosidase activity to select potential interactors out of a library screen. Of the 412 Leu^+ colonies obtained, 55 showed β-galactosidase activity. Hint: You can do filter lifts of the entire plate for the whole library, if you desire.

1. Patch His^+ colonies to two YC-WHUK Z300 plates, e.g. create a master plate and a duplicate plate for β-galactosidase filter assay. Arrange the colonies in a grid-like pattern. A grid is provided below to help you patch your colonies.
2. Grow at 30°C for 1 to 2 days and analyze the duplicate plate for β-galactosidase activity using the filter assay (page 20).

   You should obtain several colonies that are His^+ and yield β-galactosidase activity ($LacZ^+$). You are now ready to analyze your positive clones by PCR or shuttle the plasmids into *E. coli* for further analysis.

Colony Grid Pattern

The grid below is provided for your convenience. Other grids may be used if you so desire.



Interactor Hunt Using EGY48/pSH18-34

Introduction

You may wish to use EGY48 as a host for your library screen. This strain allows libraries to be induced by galactose. This is particularly important if expression of potential interactors is toxic to yeast. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene, required in the biosynthetic pathway for leucine (Leu), are replaced with LexA DNA binding sites. EGY48 is supplied transformed with the *lac*Z reporter plasmid, pSH18-34. pSH18-34 contains 8 LexA operators upstream of the *lac*Z gene. These two reporters allow selection for transcriptional activation by permitting growth when the cells are plated on medium lacking leucine, and discrimination based on color when the yeast is assayed for β-galactosidase activity.

Using EGY48/pSH18-34

In an interactor hunt, the strain EGY48/pSH18-34 containing the bait plasmid is transformed with a library made in the vector pYESTrp, pYESTrp2, or pJG4-5. Libraries in these vectors use the inducible yeast *GAL1* promoter to express proteins as fusions to the activation domain B42. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose (and raffinose, if desired). The plates lack leucine, so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow. Yeast cells containing library proteins that interact with the bait will form colonies within 2 to 5 days. Colonies are further screened by assaying for β-galactosidase activity.

You can use the large scale transformation protocol given for use with the L40 strain (substituting the appropriate media and plates). Alternatively you can use a small-scale transformation protocol (see page 46), but expect a lower transformation efficiency.

Materials Needed

Be sure to have the following materials and reagents on hand before starting.

- EGY48/pSH18-34 containing pHybLex/Zeo construct
- YC-U Z200 medium and plates
- 30°C incubator and shaking incubator
- YPD
- Centrifuge
- Sterile TE buffer
- 50 ml conical centrifuge tubes
- Denatured salmon sperm DNA
- 1X LiAc/0.5X TE
- 1X LiAc/40% PEG-3350/1X TE
- DMSO
- YP (contains no glucose)
- YC-WU medium
- YC-WU Z200 medium and plates
- 42°C water bath
- YC-WUL medium
- YC-WUL Z200 medium and plates
- YC-WUL Z200 Gal/Raff dropout medium and 150 mm plates
- Glycerol solution
- pYESTrp or pYESTrp2 library DNA or other DNA library

continued on next page

Large-Scale Library Transformation

The 1-step protocol described below should be performed straight through except at the indicated incubations. Review the procedure carefully and make sure you have all the necessary reagents before starting. The whole procedure will take 4 days with an additional 2 to 5 days for colonies to come up.

Note: EGY48/pSH18-34 appears to be more sensitive to Zeocin™ than L40; therefore, the Zeocin™ concentration is decreased to 200 μg/ml.

Before Starting

- Prepare (page 50) or purchase denatured salmon sperm DNA or use yeast carrier tRNA.
- Prepare 500 μg library plasmid DNA using cesium chloride ultracentrifugation or large-scale preparations of DNA binding resin. Alternatively, DNA can be prepared by alkaline lysis and followed by phenol-chloroform extraction. RNase treatment is not required.
- 2 150 mm YC-WU Z200 plates.
- 40 150 mm YC-WUL Z200 plates.

Preparation of Bait Strain for Transformation.

For the large-scale library transformation of the EGY48/pSH18-34 bait strain, we utilize a protocol that is a modification of published methods (Gietz *et al*., 1992; Gietz and Schiestl, 1996; Hill *et al*., 1991; Schiestl and Gietz, 1989). The bait strain is prepared for transformation as follows:

1. Inoculate EGY48/pSH18-34 bait strain into 5 ml of YC-U Z200. Grow overnight at 30°C.
2. Prepare a culture flask containing 100 ml of YC-U Z200 and inoculate with sufficient overnight culture to bring the culture to an OD_{600} of 2 to 3 (mid-log phase) in 16 hours (overnight).

   To calculate the amount of overnight culture needed to bring a 100 ml culture to an OD_{600} of 3 per ml in 16 hours, assume that yeast double every 2 hours when grown in Zeocin™-containing medium. In 16 hours, the OD_{600} will increase by a factor of 2^8 or 256. Therefore, you will need a starting OD_{600} of 0.012 per ml (3 ÷ 256). If your overnight culture is 3 OD_{600} per ml, then for a 100 ml culture, add

   (0.012 OD/ml) (100 ml)/3 OD/ml = 0.40 ml
3. Using the overnight culture from Step 2, inoculate 1 liter of YPD to a final OD_{600} of 0.3.
4. Grow at 30°C with constant shaking for 3 hours.
5. Pellet cells at room temperature by centrifugation at 5000 rpm for 10 minutes. Decant supernatant.
6. Wash pellet in 500 ml of sterile 1X TE.
7. Resuspend pellet in 20 ml of 1X LiAc/ 0.5X TE and transfer to a sterile 1 liter flask. Proceed immediately to the next section.

continued on next page

Large-Scale Library Transformation, cont.

Transformation of Bait Strain with Library DNA

8. Mix together 1.0 ml of 10 mg/ml denatured salmon sperm DNA and 500 μg library DNA.
9. Add DNA mixture to cell suspension from Step 7, above.
10. Add 140 ml 1X LiAc/40% PEG-3350/1X TE. Swirl to mix and incubate at 30°C for 30 minutes.
11. Add 17.6 ml DMSO and swirl to mix.
12. Heat shock at 42°C for 6 minutes with occasional swirling to facilitate heat transfer.
13. Immediately dilute with 400 ml of YP (or YPD) and rapidly cool to room temperature in a water bath.
14. Pellet cells at 5000 rpm for 10 minutes at room temperature.
15. Wash the pellet with 500 ml YP (YPD).
16. Resuspend the pellet in 1 liter YPD and incubate at 30°C for 1 hour with constant shaking. Proceed to next section.

Plating Transformation

First you will plate out a small sample of transformed cells to determine the primary transformation efficiency. The transformed cells will be allowed to grow before plating on selective medium.

17. Remove 1 ml of cells from the culture in Step 16, previous page, and pellet the cells.
18. Resuspend the cells in 1 ml YC-WU Z200 and plate 10 and 1 μl aliquots ($1/10^5$ and $1/10^6$ of total) on YC-WU Z200 plates to measure the primary transformation efficiency. Incubate plates at 30°C for 2 to 3 days. This protocol should yield 10 to 100 million transformants.
19. Pellet remaining cells from Step 16, previous page.
20. Wash pellet in 500 ml YC-WU and resuspend it in 1 liter of prewarmed YC-WU Z200.
21. Incubate, with shaking, at 30°C for 16 hours.
22. Pellet cells and wash with 500 ml YC-WUL.
23. Repeat Step 22 (optional).
24. Pellet cells and resuspend final pellet in 10 ml YC-WUL Z200.

25 Take 1 ml and dilute 1:10 with YC-WUL Z200 Gal/Raff medium. Save the remainder of the 10 ml at +4°C as a backup in the event the dilutions are inappropriate. Yeast are stable for at least one week when stored in this fashion.

26. Grow at 30°C for 4 hours, pellet cells, and resuspend in 1 ml YC-WUL Z200 Gal/Raff.
27. Plate $1/10^6$ and $1/10^7$ of the total on YC-WU Z200 plates to compare to the number of primary transformants from Step 18. This allows a calculation of the number of doublings and the number of Leu^+ colonies which should be screened to roughly cover the number of primary transformants.
28. Plate 10 YC-WUL Z200 Gal/Raff plates each with 5 μl, 10 μl, 25 μl, and 50 μl from the culture in Step 24 (40 plates total) and incubate at 30°C for 2 to 3 days. **Note**: Because Leu^+ colonies grow poorly if the density on the plates is too high, the optimal plating volume is dependent on the recovery time in YC-WU Z200 and on the efficiency of the transformation.

continued on next page

Analysis of Transformants

The Leu$^+$ colonies that grow on selective plates represent colonies that were transformed with a library plasmid that encodes a potential interactor. We recommend that you screen as many Leu$^+$ colonies as possible.

1. Patch Leu$^+$ colonies to two YC-WUL Z200 Gal/Raff plates, e.g. a master plate and a duplicate plate for β-galactosidase filter assay. Arrange the colonies in a grid-like pattern.
2. Grow at 30°C for 1 to 2 days and analyze the duplicate plate for β-galactosidase activity using the filter assay (page 20).

   You should obtain several colonies that are Leu$^+$ and yield β-galactosidase activity (LacZ$^+$). You are now ready to analyze your positive clones by PCR or shuttle the plasmids into *E. coli* (next page).

Retrieving Putative Interactors

Introduction

There are a number of methods to retrieve the library plasmid or the gene encoding a putative interactor. You may use one of the following procedures to isolate the library plasmid or gene.

- Isolate plasmid DNA from yeast and shuttle it into *E. coli*
- Use plasmid segregation to remove the bait plasmid, then isolate the library plasmid
- Use PCR to isolate the DNA of interest from a single yeast colony and clone it into a PCR cloning vector

The first two methods allow you to obtain the original library plasmid and use it for additional analyses, but the quality and quantity of DNA isolated from yeast is poor. The DNA has to passed through *E. coli* to generate sufficient plasmid for additional characterizations (see page 30). We routinely use PCR to extract out the DNA of interest and clone it (TA Cloning®). Protocols for each of these methods are provided below.

Materials Required

Be sure you have the following reagents and equipment on hand before starting.

- YC-W medium (see **Recipes**, page 37)
- 30°C shaking incubator
- Clinical centrifuge
- Yeast Lysis buffer (see **Recipes**, page 37)
- Acid washed glass beads (Sigma G-8772, 425-600 microns)
- Phenol/Chloroform
- 80% and 100% Ethanol
- TE

Isolation of Plasmid DNA from Yeast

Yeast plasmid DNA can be extracted from double positive colonies (His^+ $LacZ^+$) and transferred to *E. coli* to facilitate further analysis. Please note that the yield and quality of the DNA is very poor from yeast extraction, so expect to see very few *E. coli* transformants.

1. Inoculate 5 ml of YC-W with a single colony of your double positive and incubate overnight at 30°C with shaking.
2. Pellet cells at 2500 rpm for 5 minutes in a clinical centrifuge.
3. Resuspend the pellet in 0.3 ml of Yeast Lysis buffer.
4. Transfer to a 1.5 ml microcentrifuge tube and add approximately 150 µl of glass beads and 0.3 ml of phenol/chloroform.

   Note: Remove any beads adhering near the top of the tube as they can be caught when the lid is closed and cause phenol to leak out of the tube.
5. Vortex vigorously for 1 minute. Place a drop of the solution on a microscope slide and check for the extent of lysis. Continue to vortex until 80% of the cells are lysed.
6. Centrifuge in a microcentrifuge at 1400 rpm for 1 minute.
7. Transfer aqueous phase to a fresh 1.5 ml tube.
8. Precipitate plasmid DNA with 0.1 volume 3 M sodium acetate and 1.5 volume of ethanol and resuspend in 25 µl of TE. Proceed immediately to transformation (next page).

continued on next page

Isolation of Plasmid DNA from Yeast, continued

9. Transform competent *E. coli* with 5 µl of the DNA suspension and plate out the whole transformation on LB plates containing 50 to 100 µg/ml ampicillin.
10. Isolate plasmid DNA from ampicillin-resistant transformants and analyze by agarose gel electrophoresis. You should only see one plasmid with a size >5.8 kb.
11. Characterize each plasmid by restriction analysis before sequencing. This will allow you to classify plasmids into groups to avoid sequencing identical clones. Use the empty vector as a negative control.
12. Sequence using primers (e.g. pYESTrp or pJG4-5 Forward and Reverse primers) to identify interactor.

Plasmid Segregation

Instead of physically isolating the putative interactor plasmid, you can let yeast do the work by plating on selective medium. Prepare the following reagents:

- YC-W
- YC-W plates
- YC-W Z300 plates

1. For each double positive colony, inoculate 5 ml of YC-W with a His^+ $LacZ^+$ transformant. Grow 2 days at 30˚C.
2. Plate on YC-W plates to achieve a density of 100 to 200 cells per plate (approximately 100 µl of a 1:10,000 dilution of the 2 day cultures).
3. Incubate plates 2 days at 30˚C.
4. Replica-plate the YC-W plates from Step 3 first to YC-W Z300 and then to YC-W plates.
5. Incubate 1 to 2 days at 30˚C.
6. Identify colonies that are sensitive to Zeocin™ (i.e., colonies that grow on YC-W but not on YC-W Z300). These colonies have segregated the bait plasmid.
7. Isolate plasmid DNA from yeast using the protocol on the previous page, transform into *E. coli,* isolate plasmid DNA, and sequence.

PCR Cloning of Interactor

If you do not wish to isolate DNA from your yeast colonies and shuttle into *E. coli*, an alternative approach is to do PCR analysis. You can perform PCR directly from the yeast colony to easily verify the size of the insert in the pYESTrp2 and obtain a hard copy of the cDNA by simply cloning the PCR product. You will then have a source of DNA to sequence from or subclone into other expression vectors.

1. Set up the following 20 µl PCR cocktail in a 0.6 ml microcentrifuge tube. The 5X PCR buffer we use yields a final concentration of 60 mM Tris-HCl, 15 mM ammonium sulfate, 2 mM $MgCl_2$, pH 9.5 (at 22°C).

5X PCR buffer	4.0 µl
100 mM dNTPs	0.2 µl
pYESTrp Forward (100 ng/µl)	0.5 µl
pYESTrp Reverse (100 ng/µl)	0.5 µl
Water	14.3 µl
Taq polymerase 1 U	0.5 µl

continued on next page

PCR Cloning of Interactor, cont.

2. Add a single yeast colony to the cocktail and overlay with mineral oil.

   Note: We just use a yellow pipette tip to scrape up a small bit of the colony and shake it into a microcentrifuge tube containing the cocktail.

3. Use the following cycling parameters to amplify the DNA:

Temperature	Time	Cycles
94°C	10 minutes	1
94°C	1	25
56°C	1	
72°C	1	
72°C	10	1

4. Analyze 10 µl on a 1% agarose gel.
5. Ligate 2 µl into a TA Cloning® vector or other PCR cloning vector. Sequence your clone using Universal M13 primers.

After Obtaining Sequence

Once you have the sequences of a few of your clones, you can do a sequence comparison with known sequences using the database of choice. You can search GenBank through the World Wide Web by using the following URL:

http://www.ncbi.nlm.nih.gov/BLAST

Technical Assistance

General Troubleshooting

Inability to select single colonies on minimal defined medium. Check the yeast host strain phenotype. The phenotype of L40 is His^-, Trp^-, Leu^-, Ade^-. This strain is unable to grow without inclusion of histidine, tryptophan, leucine and adenine in the medium. Confirm the phenotype of this strain by streaking on YPAD (or YC), YC-H, YC-W, YC-L, and YC-Ade. L40 should only grow on YPAD or YC.

The phenotype of EGY48 is Ura^-, Trp^-, His^-, and Leu^-. This strain is unable to grow without inclusion of uracil, tryptophan, histidine, and leucine in the medium. Confirm the phenotype of this strain by streaking on YPD (or YC), YC-Ura, YC-W, YC-H, and YC-L. The strain should only grow on YPD or YC. EGY48/pSH18-34 is the same as EGY48 except that pSH18-34 confers a Ura^+ phenotype.

Testing Reporter Function

Two control plasmids, pHybLex/Zeo-Fos2 and pYESTrp-Jun, are provided to test reporter function. Since Fos and Jun are known to interact, cotransformation of these two plasmids into L40 or EGY48/pSH18-34 should activate β-galactosidase expression and allow histidine or leucine prototrophy, respectively. Transform both plasmids into L40 and select on YC-W Z300 medium. (For EGY48/pSH18-34, use YC-W Z200 medium). Test each transformant for histidine (or leucine) prototrophy and β-galactosidase activity as described on pages 18-20.

pHybLex/Zeo-Fos2 and pYESTrp-Jun

Fos (300 bp) is cloned into pHybLex/Zeo between the first *Bgl* II site (in LexA) and the *Not* I site. The *Bgl* II site is filled in to maintain the reading frame and is destroyed upon subcloning of Fos. Please note that this eliminates the pHybLex/Zeo Forward priming site and 20 amino acids from LexA. Fusion is still active by functional testing.

Jun (160 bp) is cloned into pYESTrp between the *Hin*d III and *Sph* I sites.

Troubleshooting the Bait Plasmid

Transformation of the bait plasmid alone results in a His^+ $LacZ^+$ phenotype. The bait may have some nonspecific activation activity, especially if the bait protein is a transcription factor. Try the following suggestions to alleviate this activity:

> **Construct additional protein fusions to LexA.** You may have to truncate the protein or use specific domains to avoid activating transcription when there are no interactors present. Identifying a fusion construct that does not activate will require less effort than analyzing clones arising from a screen with a high background of false positives (although see below).
>
> **Switch the bait protein with the prey protein.** If you are testing for an interaction between two known proteins, try switching the proteins around between the bait plasmid and the prey plasmid.

Yeast cells transformed with bait plasmid fail to grow. If L40 or EGY48/pSH18-34 fail to grow or grow poorly on selective medium after transformation with the bait plasmid, then the bait may be toxic to the cell. Truncating the protein may alleviate toxicity.

Unusually large or small colonies, rapid growth, or strange colony morphology. If you observe any of these three things, it is probably because of bait overexpression in the nucleus. In most cases, you should proceed on with the experiment. Unexpected behavior may give some clues as to function, particularly if working with unknown proteins.

continued on next page

Using 3-Aminotriazole

If you find that truncating or altering the bait protein destroys the interacting domain, you will have to return to your original LexA fusion and use 3-aminotriazole (3-AT) to suppress the growth of false positives. 3-AT is a competitive inhibitor of imidazole-glycerolphosphate dehydratase, the product of the *HIS3* gene (Kanazawa *et al*., 1988). Non-specific (weak) interactions will produce some dehydratase which is subsequently inhibited by 3-AT, resulting in no growth on histidine-deficient medium. Strong interactions (i.e. true interactions) will produce more dehydratase, resulting in histidine prototrophy. Screens have been successfully completed with the inclusion of 3-AT at concentrations of 1 to 5 mM, but 3-AT does not completely eliminate false positives. Use 3-AT at the lowest possible concentration. Add 1 to 5 mM of 3-AT to selective plates after autoclaving. If you observe microsatellite colonies on 3-AT, inhibition is incomplete. **Note**: 3-AT is very toxic to humans. Be sure to wear protective clothing.

Troubleshooting the Library Screen

Very few histidine or leucine prototrophs. Make sure your transformation is working by performing a 2-step selection. Use the small-scale transformation described on pages 46-49 and select on tryptophan-deficient, Zeocin™ containing medium (page 47) before screening for histidine or leucine prototrophy. In addition, you may find that your interactor is toxic and you need to use EGY48/pSH18-34 as a host.

Excessive background growth on library screening medium. Check to make sure you use the correct dropout medium for screening. If you use L40, you need YC-WHUK Z300 medium; if you use EGY48/pSH18-34, you need YC-WUL Z200 medium.

Low transformation efficiency. The transformation efficiency should be between 10^3 and 10^4 cfu/μg for a library-scale transformation. This number is determined in Step 19 of the **Large-Scale Transformation** protocol on page 24 if you are using L40 and in Step 18 on page 28 if you are using EGY48/pSH18-34. To improve the transformation efficiency:

- Use clean DNA. Ethanol precipitate the DNA to ensure a clean preparation.
- Use sufficient carrier DNA.
- Do a small-scale transformation.
- Plate on YC-W Zeocin™ first, then replica-plate to YC-L or YC-H.

False Negative Results

False negative results occur when there is a failure to detect interactions between two proteins that normally interact i*n vivo*. False negative results may occur because of one or some of the following:

- High-level expression of the bait is toxic to the cell (see **Troubleshooting the Bait Plasmid**, above).
- Transformation efficiency is too low. (see **Troubleshooting the Library Screen**, above)
- Failure of the proteins to interact because of one of the following reasons:
  - Hybrid proteins not stably expressed
  - The site of interaction is blocked by the act of fusing bait or prey proteins
  - Improper folding of hybrid proteins
  - Hybrid protein cannot be localized to the nucleus

  In the above cases, it may be helpful to construct hybrids using different domains of the bait protein.

continued on next page

False Positives

Additional analyses may be performed to eliminate false positives. False positives are considered to be putative interactors that do not specifically interact with the bait protein, but activate transcription in some other non-specific manner (Bartel *et al*., 1993b).

If you isolate the library plasmid from yeast as described on page 30, then you can test for specific interactions by retransforming into the host strain as described below and testing for prototrophy and β-galactosidase activity.

a. Library plasmid alone
b. Library plasmid with pHybLex/Zeo
c. Library plasmid with pHybLex/Zeo-Bait protein construct
d. Library plasmid with pHybLex/Zeo-Lamin (included in the kit)

True positives should only interact with the bait protein in pHybLex/Zeo to yield β-galactosidase activity or prototrophy.

Technical Service

If you have any questions, please call, write, or email Technical Service.

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Invitrogen Corporation
1600 Faraday Avenue
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Tel: (800) 955-6288
Fax: (760) 603-7201
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Resources for Two-Hybrid Technology

Introduction

The Hybrid Hunter™ Two-Hybrid System is designed to quickly help you get started with two-hybrid (interactive trap) system. You may find that you need additional resources to help you isolate putative interactors. A number of resources are listed below ranging from informative web sites to additional applications to review papers.

Web Sites

Both Roger Brent's laboratory at Harvard and Erica Golemis' laboratory at Fox Chase Cancer Center maintain Web sites on the latest in Interactive Trap technology. Use the following URLs to connect:

Roger Brent Laboratory: http://xanadu.mgh.harvard.edu/
Erica Golemis Laboratory: http://www.fccc.edu/research/labs/golemis/

In addition, Brent's laboratory maintains a gopher site:

gopher://weeds.mgh.harvard.edu:70/11/dept_molbiol/molbiol/brent/interaction_trap

Review Articles

Selected reviews are available below.

Bartel, P. L., Chien, C.-T., Sternglanz, R., and Fields, S. (1993a) Using the Two-Hybrid System to Detect Protein-Protein Interactions. In *Cellular Interactions in Development: A Practical Approach*, D. A. Hartley, ed. (Oxford: Oxford University Press), pp. 153-179.

Chien, C.-T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The Two-Hybrid System: A Method to Identify and Clone Genes for Proteins that Interact with a Protein of Interest. Proc. Natl. Acad. Sci. U.S.A. *88*, 9578-9582.

Fields, S., and Song, O. (1989). A Novel Genetic System to Detect Protein-Protein Interaction. Nature *340*, 245-246.

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Two-Hybrid Affinity Data

A systematic comparison of data from two-hybrid system-derived in vivo affinity determinations with in vitro determinations has been performed (Estojak *et al.*, 1995). The strength of the in vivo interaction generally correlates with that determined in vitro, but the amount of expression of a single reporter did not linearly correlate with affinity measured in vitro.

Appendix

Recipes

Media Table

The table below describes the different media used to characterize the bait plasmid in L40 or EGY48/pSH18-34.

Medium	Use with.....	Purpose
YPAD or YPD	All Strains	Complex medium for general, non-selective growth. L40 requires adenine for growth.
YPAD Z300	L40 + pHybLex/Zeo	Inclusion of Zeocin™ selects for bait plasmid
YC	All Strains	Minimal defined medium for selective growth
YC-HUK Z300	L40 + pHybLex/Zeo	Minimal medium for test of bait plasmid. Does not contain histidine, uracil or lysine.
YC-W	L40 + pYESTrp2	Minimal medium for growing up library plasmid to isolate DNA
YC-WU Z300	L40 + pYESTrp2 + pHybLex/Zeo	Minimal medium for selection of bait and prey plasmids. Does not contain tryptophan or uracil.
YC-WHUK Z300	L40 + pYESTrp2 + pHybLex/Zeo	Tests for histidine prototrophy. Does not contain tryptophan, histidine, uracil, or lysine
YC-U	EGY48/pSH18-34	Minimal medium for selection of pSH18-34. Does not contain uracil.
YC-U Z200	EGY48/pSH18-34 + pHybLex/Zeo	Minimal medium for selection of pSH18-34 and pHybLex/Zeo. Does not contain uracil.
YC-WU Z200	EGY48/pSH18-34 + pHybLex/Zeo + pYESTrp2	Minimal medium for selection of pSH18-34, pHybLex/Zeo, and pYESTrp2. Does not contain uracil or tryptophan.
YC-WUL Z200	EGY48/pSH18-34 + pHybLex/Zeo + pYESTrp2	Tests for leucine prototrophy. Does not contain tryptophan, leucine, or uracil

continued on next page

YC Medium and Plates

YC is minimal defined medium for yeast.

0.12% yeast nitrogen base (**without either** amino acids or ammonium sulfate)
0.5% ammonium sulfate
1% succinic acid
0.6% NaOH
2% glucose
0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil)
0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine)
2% agar (for plates)

1. Dissolve the following reagents in 900 ml deionized water. **Note**: We make medium and plates as we need them and weigh out each amino acid. Many researchers prepare 100X solutions of each amino acid mix that they need.

1.2 g Yeast Nitrogen Base	**0.1 g each**	**0.05 g each**
5 g Ammonium sulfate	adenine	aspartic acid
10 g Succinic acid	arginine	histidine (H)
6 g NaOH	cysteine	isoleucine
	leucine (L)	methionine
	lysine (K)	phenylalanine
	threonine	proline
	tryptophan (W)	serine
	uracil (U)	tyrosine
		valine

   Note: The amino acids with the one letter code are those you need to omit to make selective plates, depending on the genotype of the host, plasmid markers, and reporters.

2. If you are making plates, add the agar after dissolving the reagents above.
3. Autoclave at 15 psi, 121°C for 20 minutes.
4. Cool to 50°C and add 100 ml of filter-sterilized 20% glucose. **Note**: You may add the sugar before autoclaving; however, the medium will be darker in color because of heating the glucose.

   If you need to add Zeocin™, add it at this point to a final concentration of 300 μg/ml (3 ml per liter).

   If you need to add 3-AT to the medium, add it at this point from a filter-sterilized stock.

   For plates that contain galactose and raffinose, add 100 ml 20% galactose and 50 ml 20% raffinose instead of glucose.
5. Pour plates and allow to harden. Invert the plates and store at +4°C. Plates are stable for 6 months unless they contain Zeocin™. Plates containing Zeocin™ are stable for about a month.

NOTE

The recipe for YC medium has been optimized for use with the Hybrid Hunter™ Kit. Other recipes may be suitable, but should be tested with the host strain, plasmid markers, and reporters.

Recipes, continued

YPD (YPAD) ± Zeocin™

Yeast Extract Peptone Dextrose Medium (± Adenine) (1 liter)

1% yeast extract
2% peptone
2% dextrose (D-glucose)
± 0.1 g adenine
± 300 μg/ml Zeocin™

1. Dissolve the following in 900 ml of water:
   10 g yeast extract
   20 g of peptone
   (0.1 g adenine, if desired)
2. Optional: Add 20 g agar, if making plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 ml of 20% dextrose.
5. If desired, cool the solution to <50°C and add 3.0 ml of 100 mg/ml Zeocin™ just prior to use.

Store medium at room temperature. The shelf life is approximately one to two months.

10X TE

100 mM Tris pH 7.5
10 mM EDTA

1. For 100 ml, dissolve 1.21 g of Tris base and 0.37 g of EDTA in 90 ml of deionized water.
2. Adjust the pH to 7.5 with concentrated HCl and bring the volume up to 100 ml.
3. Filter sterilize and store at room temperature.

Alternatively, you can make the solution using 1 M Tris-HCl, pH 7.5 and 0.5 M EDTA.

1X TE

10 mM Tris pH 7.5
1 mM EDTA

Dilute 10X TE 10-fold with sterile water.

10X LiAc

1 M Lithium Acetate, pH 7.5

1. For 100 ml, dissolve 10.2 g of lithium acetate in 90 ml of deionized water.
2. Adjust pH to 7.5 with dilute glacial acetic acid and bring up the volume to 100 ml.
3. Filter-sterilize and store at room temperature.

1X LiAc

100 mM Lithium Acetate, pH 7.5

Dilute 10X LiAc solution 10-fold with sterile, deionized water.

continued on next page

Recipes, continued

1X LiAc/ 40% PEG-3350/ 1X TE

100 mM Lithium acetate, pH 7.5
40% PEG-3350
10 mM Tris-HCl, pH 7.5

1. For 200 ml, mix together 20 ml 10X LiAc, 20 ml 10X TE, and 80 g PEG 3350.
2. Add deionized water to 200 ml and dissolve the PEG. You may have to heat the solution.
3. Autoclave at 121°C, 15 psi for 20 minutes. Store at room temperature.

Cracking Buffer

8 M urea
5% SDS
40 mM Tris-HCl pH 6.8
0.1 mM EDTA
1% β-mercaptoethanol
0.4 mg/ml bromophenol blue

1. Prepare a 1 M Tris-HCl, pH 6.8 stock. (12.11 g in 90 ml deionized water and adjust pH to 6.8. Bring the volume to 100 ml).
2. Mix together the following reagents:

Urea	48.0 g
SDS	5 g
1 M Tris-HCl, pH 6.8	4 ml
EDTA	3.72 mg (or 20 µl of a 0.5 M stock)
β-mercaptoethanol	1 ml
Bromophenol blue	40 mg

   Bring up in 100 ml deionized water and dissolve reagents.

3. Store at +4°C or -20°C.

Z Buffer

60 mM Na_2HPO_4
40 mM NaH_2PO_4
10 mM KCl
1 mM $MgSO_4$-$7H_2O$
pH 7.0

1. For 100 ml, dissolve the following reagents in 90 ml deionized water:

Na_2HPO_4	0.85 g
NaH_2PO_4	0.48 g
KCl	0.075 g
$MgSO_4$-$7H_2O$	24.7 mg

2. Adjust pH to 7.0 with NaOH or phosphoric acid. Bring up to 100 ml.
3. Autoclave or filter sterilize. Store at room temperature.

continued on next page

Glycerol solution

65% glycerol
0.1 M $MgSO_4$
25 mM Tris-HCl, pH 8.0

1. Prepare 1 M $MgSO_4$ and 1 M Tris-HCl, pH 8.0 solutions.
2. For 100 ml, mix together the following reagents:

1 M Tris-HCl, pH 8.0	2.5 ml
1 M $MgSO_4$	10 ml
Glycerol	65 g

3. Bring up the volume to 100 ml with deionized water.
4. Autoclave the solution and store at room temperature.

Yeast Lysis Buffer

2.5 M LiCl
50 mM Tris-HCl, pH 8.0
4% Triton X-100
62.5 mM EDTA

1. For 100 ml, dissolve the following reagents in 90 ml deionized water.

1 M Tris-HCl, pH 8.0	5.0 ml
LiCl	10.6 g
Triton X-100	4 ml
$Na_2EDTA\text{-}2H_2O$	2.33 g

2. Adjust the pH if necessary with NaOH or HCl and bring the volume to 100 ml.
3. Store at room temperature.

E. coli Transformation

Introduction

For maximal activity of Zeocin™, the salt concentration of LB medium must remain low (< 90 mM) and the pH must be 7.5. Prepare LB broth and plates using the following recipe. Please note the lower salt content of this medium. **Failure to lower the salt content of your LB medium will result in non-selection because of inactivation of the drug.**

IMPORTANT! Low Salt LB Medium with Zeocin™

Low Salt LB Medium:

10 g Tryptone
5 g NaCl
5 g Yeast Extract

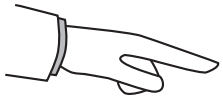
1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration (250 µl per liter).
4. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Note: Pre-mixed Low Salt LB Medium is available from Invitrogen in convenient pouches or in bulk. Please contact Technical Service for more information (page 51).

Transformation

Transform pHybLex/Zeo containing your insert into TOP10F´ (or similar *E. coli* strain) using your preferred method. Remember the following important points:

- Add either Low Salt LB or LB medium to the cells after heat shock or electroporation to allow them to recover.
- Plate on **Low Salt LB medium** with 25 µg/ml Zeocin™ and incubate overnight at 37°C.
- Analyze 10-20 clones for the presence of insert.
- Sequence to confirm fusion to LexA (see page 14).



NOTE

If you see a haze or satellite colonies, increase the Zeocin™ concentration to 50 µg/ml.

Small-Scale Yeast Transformation

Introduction

A small-scale yeast transformation protocol for routine transformations is provided below.

Materials Needed

Be sure to have the following reagents on hand before starting.

- YPAD
- 1X TE
- 1X LiAc/0.5X TE
- Denatured sheared salmon sperm DNA
- Plasmid DNA to be transformed
- 1X LiAc/40% PEG-3350/1X TE
- DMSO
- Selective plates

Protocol

1. Inoculate 10 ml of YPAD with a colony of L40 or EGY48/pSH18-34 and shake overnight at 30°C.
2. Determine the OD_{600} of your overnight culture. Dilute culture to an OD_{600} of 0.4 in 50 ml of YPAD and grow an additional 2-4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml 1X TE.
4. Pellet the cells at 2500 rpm and resuspend pellet in 2 ml of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 μg plasmid DNA and 100 μg denatured sheared salmon sperm DNA with 100 μl of the yeast suspension from Step 5.
7. Add 700 μl of 1X LiAc/40% PEG-3350/1X TE and mix well.
8. Incubate solution at 30°C for 30 minutes.
9. Add 88 μl DMSO, mix well, and heat shock at 42°C for 7 minutes.
10. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 ml 1X TE and re-pellet.
12. Resuspend the pellet in 50-100 μl TE and plate on a selective plate.

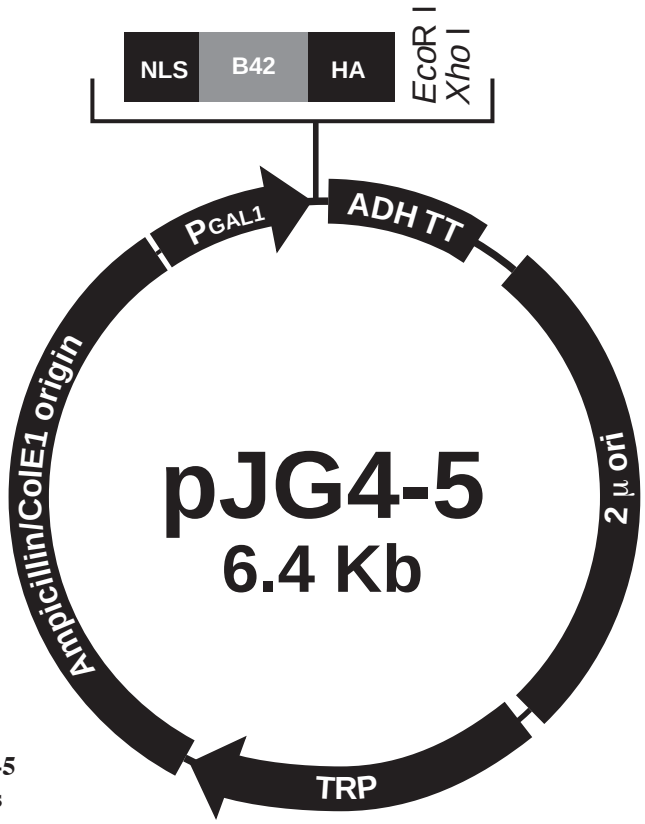
pJG4-5

Description

pJG4-5 is the library plasmid developed by Roger Brent and coworkers (Gyuris *et al*., 1993). Many libraries designed for two-hybrid screening are made with this vector. It contains unique *Eco*R I and *Xho* I sites for in-frame fusion of cDNA with the NLS, the activation domain B42, and the hemagglutinin epitope tag. In addition, it contains the *TRP1* selectable marker, 2 μ origin to allow propagation in yeast, ampicillin resistance gene, and the pUC origin for propagation in *E. coli*. Primers are available that flank the multiple cloning site for PCR analysis of inserts and sequencing. Please see the table below for ordering information.

Primer	Sequence	Amount	Catalog no.
pJG4-5 Forward	5´-GATGCCTCCTACCCTTATGATGTGCC-3´	2 μg	N810-01
pJG4-5 Reverse	5´-GGAGACTTGACCAAACCTCTGGCG-3´	2 μg	N811-01

Map of pJG4-5



Comments for pJG4-5
6449 nucleotides

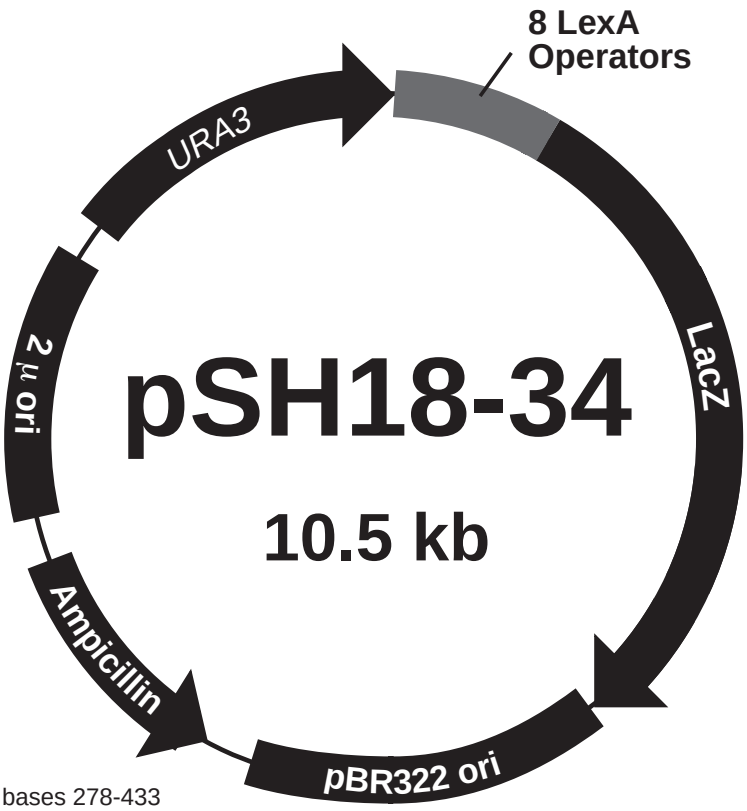
GAL1 promoter: bases 408-522
Initiation ATG: bases 534-536
Nuclear localization signal (NLS): bases 543-569
B42 activation domain: bases 573-810
Hemagglutinin epitope: bases 816-842
pJG4-5 Forward priming site: bases 807-832
Multiple cloning site: bases 849-866
pJG4-5 Reverse priming site: bases 883-906
ADH1 transcription termination region: bases 1039-1196
2 µ origin: bases 2178-2875
TRP gene: bases 3416-4090
Ampicillin resistance gene: bases 4400-5260
ColE1 origin: bases 5405-6078

pSH18-34

Description

pSH18-34 (Catalog no. V611-20) is a LacZ reporter plasmid containing 8 LexA operator (binding) sites upstream of the *lac*Z gene. The plasmid contains the *URA3* selectable marker, the 2 μ origin to allow propagation in yeast, the ampicillin resistance gene, and the pBR322 origin to allow replication in *E. coli*. The complete sequence of pSH18-34 is available for downloading from our web site (www.invitrogen.com) or from Technical Service (see page 51).

Map of pSH18-34



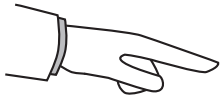
Comments for pSH18-34:
10484 nucleotides

LexA operators (8 LexAops): bases 278-433
LacZ ORF: bases 667-3906
ColE1 (pBR322-derived) origin: bases 5218-5891
Ampicillin resistance gene: bases 6036-6896
2 μ origin: bases 7910-9374
URA3 gene: bases 9374-10481

Small-Scale Library Transformation Using EGY48/pSH18-34

Introduction

A small-scale library transformation protocol for 1 step direct selection or 2 step selection is provided below. The 1 step direct selection is the easiest to perform, but if you are expressing a toxic protein, the 2 step protocol is recommended. A typical library transformation will result in 2 to 3 x 10^6 primary transformants. Assuming a transformation efficiency of 10^5 per μg library DNA, this transformation requires a total of 20 to 30 μg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination.



NOTE

L40 can also be used for a small-scale library transformation as well. Be sure to prepare the correct selective medium and remember that you do not need to induce with galactose.

Materials Needed

Be sure to have the following materials and reagents on hand before starting. Please read the protocols through and decide if you want to do the 1-step or the 2-step protocol. Pay close attention to the number and type of plates required as well as the medium.

- EGY48/pSH18-34 + bait plasmid
- YC-U Z200 medium and plates
- 30°C incubator and shaking incubator
- Centrifuge
- Sterile water
- 50 ml conical centrifuge tubes
- 1X TE/1X LiAc
- Library DNA (30 μg)
- 50 μg carrier DNA (sheared salmon sperm or yeast tRNA)
- 1.5 ml sterile microcentrifuge tubes
- 40% PEG-3350/1X LiAc/1X TE
- DMSO
- 42°C heat block
- 100 mm and/or 150 mm YC-WU Z200 plates
- YC-WU Z200 Gal/Raff dropout medium (**Note**: EGY48/pSH18-34 appears to be more sensitive to Zeocin™ than L40)
- 150 mm YC-WU Z200 Gal/Raff plates
- 100 mm YC-WUL Z200 plates
- 100 mm YC-WUL Z200 Gal/Raff plates
- 24 x 24 cm plates or 150 mm plates
- Sterile glass microscope glass
- Sterile TE buffer
- Glycerol solution

continued on next page

Small-Scale Library Transformation Using EGY48/pSH18-34, continued

Small-Scale Library Transformation

1. Inoculate 20 ml of YC-U Z200 with EGY48/pSH18-34 containing your bait plasmid (pHybLex/Zeo). Grow overnight at 30°C.
2. In the morning, dilute culture into 300 ml YC-U Z200 to 2 x 10^6 cells/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains 2 x 10^7 cells/ml (OD_{600} = ~0.50).
3. Centrifuge 5 minutes at 1000 to 1500 x g in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to a 50 ml conical tube.
4. Centrifuge 5 min. at 1000 to 1500 x g. Decant supernatant and resuspend cells in 1.5 ml 1X TE/1X LiAc.
5. Add 1 μg library DNA and 50 μg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5 ml microcentrifuge tubes. Add 50 μl of the resuspended yeast solution from Step 4 to each tube.

   Note: The total volume of library and salmon sperm DNA added should be <20 μl and preferably <10 μl.
6. Add 300 μl of sterile 40% PEG-3350/1X LiAc/1X TE to each tube, and invert to mix thoroughly. Incubate 30 minutes at 30°C.
7. Add DMSO to 10% (~40 μl per tube) and invert to mix. Heat shock 10 minutes in 42°C heating block. Proceed to Step 8 for the One-Step Direct Selection or Step 12 for the Two-Step Selection.

One-Step Direct Selection

8. Pool yeast from 28 tubes (11.2 ml) and dilute 1:10 with YC-WU Z200 Gal/Raff dropout medium (112 ml).
9. Incubate with shaking 4 hour at 30°C to induce the *GAL* promoter on the library. Grow to an OD_{600} = ~0.5 to obtain a concentration of 10^7 cells/ml.
10. Plate 100 μl on as many 100 mm YC-WUL Z200 Gal/Raff plates as are necessary for full representation of transformants.
11. For the two remaining tubes, plate 360 μl from each tube onto a separate 150 mm YC-WU Z200 plate. Use the remaining 40 μl from each tube to make a series of 1:10 dilutions in sterile water. Plate dilutions on 100 mm YC-WU Z200 plates. Incubate all plates 2 to 3 days at 30°C until colonies appear. Proceed to Step 24, page 49.

    Note: The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

Two-Step Selection

12. Take the 28 tubes from Step 7 and plate the complete contents of one tube per 150 mm YC-WU Z200 plates and incubate at 30°C for 1 to 2 days.
13. For the two remaining tubes, plate 360 μl from each tube onto separate 150 mm YC-WU Z200 plates. Use the remaining 40 μl from each tube to make a series of 1:10 dilutions in sterile water. Plate dilutions on 100 mm, YC-WU Z200 plates. Incubate all plates 2 to 3 days at 30°C until colonies appear. Proceed to Step 14, next page.

    Note: The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

continued on next page

Collect Primary Transformant Cells

Conventional replica plating does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells is plated for each primary transformant.

14. Cool all of the 150 mm plates containing transformants from Step 12 for several hours at +4°C to harden agar.
15. Wearing gloves and using a sterile glass microscope slide, gently scrape yeast cells off the plate. Be careful not to damage the agar. Pool cells from the 30 plates into one or two sterile 50 ml conical tubes.

    Note: This is the step where contamination is most likely to occur. Be careful.
16. Wash cells by resuspending the transferred cells into an equal volume of sterile TE buffer or water. Centrifuge ~5 minutes at 1000 to 1500 x g, room temperature, and discard supernatant. Repeat wash.
17. Resuspend pellet in 1 volume glycerol solution, mix well, and store up to 1 year in 1 ml aliquots at -80°C. Proceed to Step 18, below.

Determine Replating Efficiency

18. Remove an aliquot of frozen transformed yeast (Step 17, above) and dilute 1:10 with YC-WU Z200 Gal/Raff dropout medium. Incubate with shaking for 4 hours at 30°C to induce the *GAL* promoter to express the library.

    Note: Raffinose (Raff) is not required for growth, but it helps the cells to grow better without diminishing transcription from the *GAL1* promoter.
19. Make serial dilutions of the yeast cells using the YC-WU Z200 Gal/Raff dropout medium. Plate on 100 mm YC-WU Z200 Gal/Raff dropout plates and incubate 2 to 4 days at 30°C until colonies are visible.
20. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

    In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = ~2.0×10^7 yeast cells. In general, if the harvest is done carefully, viability will be greater than 90%. Some researchers perform this step simultaneously with plating out on Leu selective medium (Steps 21 through 22).

continued on next page

Screen for Interacting Proteins

Because not all cells that contain interacting proteins plate at 100% efficiency on -Leu medium, it is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by three to ten individual yeast cells. This will in some cases lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogenous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

It is easiest to visually scan for Leu^+ colonies using cells plated at ~10^6 cfu per 100 mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which 3 x 10^6 colonies are obtained, plate ~2 x 10^7 cells on a total of 20 selective plates.

21. Thaw the appropriate quantity of transformed yeast based on the plating efficiency (see calculation, previous page), dilute, and incubate as in Step 18.
22. Dilute cultures in YC-WUL Z200 medium as necessary to obtain a concentration of 10^7 cells/ml (OD_{600} = ~0.5).
23. Plate 100 μl of each on as many YC-WU Z200 Gal/Raff 100 mm plates as are necessary for full representation of transformants. Incubate 2 to 3 days at 30°C until colonies appear.
24. Carefully pick appropriate colonies and streak on a new YC-WUL Z200 Gal/Raff master dropout plate. Incubate 2 to 7 days at 30°C until colonies appear.

    A good strategy is to pick a master plate with colonies obtained on Day 2, a second master plate (or set of plates) with new colonies appearing on Day 3, and a third with colonies obtained on Day 4. Colonies from Day 2 and 3 master plates should generally be characterized further. If many apparent positives are obtained, it may be worth making master plates of the much larger number of colonies likely to be obtained at Day 4 (and after).

    If no colonies appear within a week, those arising at later time points are likely to be an artifact. Contamination that has occurred at an earlier step (e.g., during plate scraping) is generally reflected by the growth of a very large number of colonies (>500/plate) within 24 to 48 hours after plating on selective medium.

Preparation of Denatured Salmon Sperm DNA

Introduction

A convenient protocol is provided to make denatured salmon sperm DNA (Schiestl and Gietz, 1989). You may also purchase denatured salmon sperm DNA from Sigma (Catalog no. D9156). Alternatively, some researchers have found that using yeast total RNA (Sigma, Catalog no. R9001) as a carrier results in a cleaner transformation although there are less total colonies.

Before Starting

Prepare or have on hand the following reagents.

- Salmon Sperm DNA (Sigma, Catalog no. D-1626)
- TE
- Sonicator
- 50 ml conical centrifuge tubes
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- Chloroform
- Low-speed centrifuge
- 95% ethanol (-20°C)
- 250 ml centrifuge bottle
- Boiling water bath

Procedure

1. Take a 250 ml flask and dissolve 1 g salmon sperm DNA into 100 ml of TE (10 mg/ml). Pipet up and down with a 10 ml pipet to dissolve completely.
2. Incubate overnight at +4°C.
3. Using a sonicator with a large probe, sonicate the DNA twice for 30 seconds at 3/4 power. The resulting DNA will have an average size of 7 kb.
3. Distribute the sonicated DNA between four 50 ml conical centrifuge tubes (25 ml per tube).
4. Extract with 25 ml of TE-saturated phenol. Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
5. Extract with 25 ml of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
6. Extract with 25 ml of chloroform. Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a 250 ml centrifuge tube.
7. Add 125 ml ice-cold (-20°C) 95% ethanol (2.5 volume) and 5 ml 3 M sodium acetate, pH 6.0 (1/10 volume).
8. Pellet the DNA at 12,000 x g for 15 minutes at +4°C.
9. Wash the DNA once with 200 ml 70% ethanol and centrifuge as described in Step 8.
10. Partially dry DNA in a speed-vac by covering tubes with parafilm and poking holes in it. Dry for ~20 min.
11. Transfer DNA to a 250 ml sterile flask and dissolve DNA in 100 ml sterile TE (10 mg/ml).
12. Denature by boiling in a water bath for 20 minutes. Immediately place on ice, aliquot in 1 ml samples, and freeze at -20˚C.

Technical Service

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