Dual Bait
Hybrid Hunter™

Version A

190513
25-0289

Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System

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

Catalog no. K5200-01

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Important Information

Shipping and Storage

The Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System is shipped on dry ice. Upon receipt:

- Remove the glycerol stock of SKY48/pLacGUS and store at -80°C.
- Store the vectors and primers at -20°C.
- Store the Zeocin™ and the X-Gluc at +4°C protected from exposure to light. For long-term storage, store at -20°C protected from exposure to light.

Kit Contents

The following reagents are included in the Dual Bait Hybrid Hunter™ Two-Hybrid System.

Vectors: All vectors are supplied lyophilized. Store at -20°C.

Vector	Amount	Purpose
pHybLex/Zeo	20 µg	Cloning vector for bait protein X
pHybcI/HK	20 µg	Cloning vector for bait protein Y
pYESTrp2	20 µg	Cloning vector for prey protein or cDNA library
pHybLex/Zeo-Fos2	10 µg	Positive control for pHybLex/Zeo bait plasmid
pHybcI/HK-Krev	10 µg	Positive control for pHybcI/HK bait plasmid
pYESTrp-Jun	10 µg	Positive control for prey plasmid
pYESTrp2-RalGDS	10 µg	Positive control for prey plasmid

Primers: All primers are supplied lyophilized. Primers may be used for sequencing or PCR. Additional primers are available separately from Invitrogen (see the next page). Store at -20°C.

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)
cI Forward	5´-GGATAGCGGTCAGGTGTT-3´	2 µg (358 pmoles)

Yeast Strain: Supplied as a 20% glycerol stock in 0.5 ml volume. Store at -80°C.

Strain	Genotype	Phenotype
SKY48/ pLacGUS	MATα *ura3 trp1 his3* 6*lex*Aop-*LEU2* 3*cI*op-*LYS2* *pLacGUS (URA3)*	Ura$^+$, Trp$^-$, His$^-$, Leu$^-$, Lys$^-$

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Important Information, continued

Kit Contents, continued

Other Reagents: Zeocin™ is supplied in liquid form and X-Gluc is supplied as a powder. Enough Zeocin™ is supplied for *E. coli* transformation and one large-scale library transformation. Enough X-Gluc is provided to perform β-glucuronidase activity assays on sixteen 100 mm plates or eight 150 mm plates. Store the Zeocin™ and X-Gluc at +4°C protected from exposure to light. For long-term storage, store the Zeocin™ and the X-Gluc at -20°C protected from exposure to light.

Reagent	Amount	Purpose
Zeocin™	10 x 1.25 ml, 100 mg/ml (1.25 g total)	Selection agent
X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid)	10 mg	Reporter substrate

Additional Products

Many of the reagents in the Dual Bait Hybrid Hunter™ System as well as additional reagents that may be used in conjunction with the Dual Bait Hybrid Hunter™ System are available from Invitrogen. Ordering information is provided below. The quantity of antibody supplied is sufficient for 25 westerns

Item	Amount	Catalog no.
Zeocin™	1 gram	R250-01
	5 grams	R250-05
Kanamycin	5 grams	Q100-18
X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid)	10 mg	R730-10
S.c. EasyComp™ Kit	20 reactions	K5050-01
pHybLex/Zeo	20 µg	V610-20
pHybcI/HK	20 µg	V614-20
pYESTrp2	20 µg	V615-20
pLacGUS Reporter Plasmid	20 µg	V616-20
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
cI Forward Primer	2 µg	N832-02
SKY48 Yeast Strain	0.5 ml	C833-00
SKY48/pLacGUS Yeast Strain	0.5 ml	C832-00
SKY191 Yeast Strain	0.5 ml	C834-00
cI Antibody	50 µl	R991-25
Anti-LexA Antibody	50 µl	R990-25
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-HRP Antibody	50 µl	R961-25

Purchaser Notification

Introduction

The Dual Bait Hybrid Hunter™ Two-Hybrid System ("System") includes technologies licensed under one or more of the following U.S. patents, patent applications and their foreign counterparts:

- No. 4,833,080 entitled "Regulation of Eukaryotic Gene Expression" owned by Harvard University
- Nos. 5,283,173; 5,468,614 and 5,667,973 covering a "System to Detect Protein-Protein Interactions" owned by the State University of New York
- No. 5,599,173 entitled "Beta-glucuronidase and Glucuronide Permease Gene System" owned by Cambia Biosystems, LLC
- No. 60/059,065 entitled "An Improved Yeast Interaction Trap Assay" owned by Fox Chase Cancer Center.

The 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

Invitrogen has a license to sell the System **for research purposes only** under the terms described below. COMMERCIAL ENTITIES MUST OBTAIN A COMMERCIAL LICENSE FROM THE RESEARCH FOUNDATION OF THE STATE UNIVERSITY OF NEW YORK BEFORE USING PRODUCTS (AS DEFINED BELOW) FOR ANY PURPOSE, AS DETAILED BELOW. Note that such a license would cover only one part of the System. In addition, commercial entities will be required to obtain a commercial license from Fox Chase Cancer Center following a one year evaluation period, as detailed on the next page.

Use of Products by any user for any Commercial Purpose (as defined below) requires the user to obtain additional commercial licenses, as detailed on the next page. Commercial licenses for other technologies in the System may or may not be available. Please contact Invitrogen Technical Service and/or the appropriate owner for information.

Before using this product, please read these terms and instructions carefully. If you do not agree to be bound by the terms described here, contact Invitrogen within 10 days for authorization to return the unopened product and to receive a full credit. If you do agree to these terms, please follow the instructions below.

Definition of Commercial Purpose

"Products" means the Dual Bait Hybrid Hunter™ Two-Hybrid System and any materials produced through use of the System. "Commercial Product" means any product intended for sale or commercial use. Commercial Purpose includes:

- any use of the System in a Commercial Product or Service;
- any use of the System in the manufacture of a Commercial Product;
- any sale of the System; and
- any use of the System to facilitate or advance applied research or development of a Commercial Product.

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Purchaser Notification, continued

Two-Hybrid License (Required for Commercial Entities)

Practice of the two-hybrid system is covered by U.S. Patent Nos. 5,283,173; 5,468,614 and 5,667,973 assigned to The Research Foundation of the State University of New York. Purchase of any two-hybrid reagents does not imply or convey a license to practice the two-hybrid system covered by these patents, 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:

Barbara A. Sawitsky
The Research Foundation of SUNY at Stony Brook
Office of Technology Licensing
W5530 Melville Memorial Library
Stony Brook, NY 11794-3368
Phone: 516-632-9077
Fax: 516-632-9839
E-mail: bsawitsky@notes.cc.sunysb.edu

Dual Bait License (One Year Evaluation)

Practice of "Improvements to the Yeast Interaction Trap" is covered by U.S. Patent Application No. 60/059,065 assigned to the Fox Chase Cancer Center (FCCC). Purchase of any Invitrogen Dual Bait Hybrid Hunter™ Two-Hybrid System reagents does not imply or convey a License to practice the "Improvements" covered by this patent application, beyond use of the enclosed kit for non-commercial research. If you are a commercial entity, your right from FCCC to use the System expires after one year**. Any commercial entity that wishes to use the System beyond this one-year period must obtain a commercial license from FCCC.** Commercial entities will be contacted during this one-year period by FCCC regarding their desire to obtain a commercial license. For license information, please contact:

Fox Chase Cancer Center
Office of Business Development
7701 Burholme Avenue
Philadelphia, PA 19111
Phone: 215-728-1113
Fax: 215-728-2594

Use of Prokaryotic Control Elements in Eukaryotes

This product is also licensed under U.S. Patent No. 4,833,080 and corresponding patents in other countries **for research purposes only**. It may not be used for gene expression in plants nor for certain molecular modifications and screening. Licenses for commercial manufacture or research use outside the research kits and reagents market should be directed to:

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Harvard University
University Place, Suite 410 South
124 Mt. Auburn Street
Cambridge, MA 02138
Phone: 617-495-3067
Fax: 617-495-9568

Inquiries regarding commercial use in the research kit and reagent market should be directed to the Licensing Coordinator, Invitrogen Corporation.

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Purchaser Notification, continued

β-glucuronidase and Glucuronide Permease

This product is also licensed under U.S. Patent No. 5,599,173 and corresponding patents in other countries **for research purposes only**. For commercial license information, please contact the Licensing Coordinator, Invitrogen Corporation.

Zeocin™ Selection Marker

Zeocin™ is a trademark of CAYLA. For commercial license information, please contact:

Licensing Coordinator
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
Phone: 760-603-7200
Fax: 760-603-7201

Product Specifications

Introduction

This section describes the criteria used to qualify the components of the Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System.

Vectors

Each vector is qualified by restriction enzyme digestion with specific restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

Vector	Restriction Enzymes
pYESTrp2	*Hin*d III *Bam*H I *Hin*d III/*Pvu* II
pHybLex/Zeo	*Eco*R I *Pst* I *Eco*R V
pHybcI/HK	*Kpn* I *Pvu* II *Eco*R I *Not* I
pHybLex/Zeo-Fos2	*Eco*R I *Not* I *Eco*R V
pYESTrp-Jun	*Hin*d III *Nco* I *Hin*d III/*Pvu* II
pHybcI/HK-Krev	*Eco*R I/*Sac* II *Pst* I *Not* I
pYESTrp2-RalGDS	*Bam*H I/*Eco*R I *Bgl* I *Eco*R I

Primers

Sequencing primers are lot tested by automated DNA sequencing experiments.

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Product Specifications, continued

SKY48/pLacGUS Yeast Strain

SKY48/pLacGUS is tested for growth on YPD and YC-uracil.

SKY48/pLacGUS is also qualified in a functional assay. The strain is transformed with the following vectors as listed below, plated on selective medium, and assayed for growth or no growth as expected. A β-glucuronidase overlay assay is performed with colonies on the YC-UHWK Z200 Gal/Raff plates. Color development must be visible within one hour after addition of the X-Gluc staining solution.

Transformation	Selective Medium	Growth
pHybLex/Zeo-Fos2 pYESTrp-Jun pHybcI/HK-Krev pYESTrp2-RalGDS	YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff	Yes
pYESTrp2		No

Zeocin™

Zeocin™ is lot qualified by demonstration that LB media with 25 µg/ml Zeocin™ prevents growth of the *E. coli* strain, TOP10.

X-Gluc

X-Gluc is lot tested by performing a β-glucuronidase overlay assay on selective medium plates containing colonies of SKY48/pLacGUS transformed with pHybLex/Zeo-Fos2, pYESTrp-Jun, pHybcI/HK-Krev, and pYESTrp2-RalGDS. Color development must be visible within one hour after addition of the X-Gluc staining solution.

Introduction

Overview

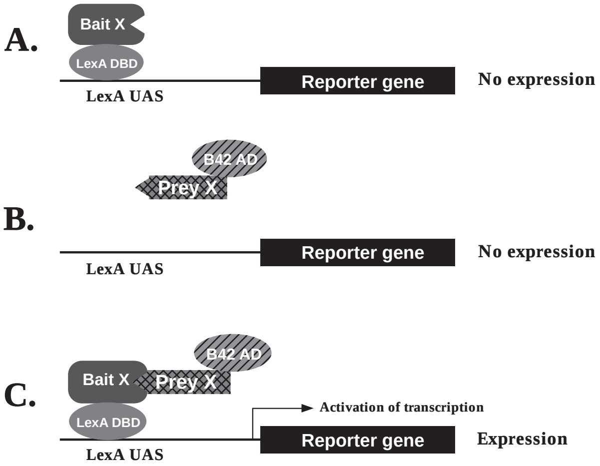
Introduction

The Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System is a modified version of the original Hybrid Hunter™ Two-Hybrid System available from Invitrogen (Catalog no. K5000-01). The Dual Bait Hybrid Hunter™ System, originally developed by Erica Golemis and coworkers (Serebriiskii *et al.*, 1999) allows the *in vivo* detection of molecular interactions between two sets of proteins in the yeast, *Saccharomyces cerevisiae*. The Dual Bait Hybrid Hunter™ System can be used for the following applications:

- To screen a library for novel proteins that specifically interact with either or both of two known bait proteins of interest
- To test complex formation between sets of known proteins or protein domains for which there is a prior reason to expect an interaction
- To determine the specificity of interactions between a prey protein and two different forms (i.e. wild-type and mutant) of the same bait protein

General Description of the Hybrid Hunter™ System

The Hybrid Hunter™ Two-Hybrid System is based on the interactive trap system originally developed by Roger Brent and coworkers (Golemis *et al.*, 1996; Gyuris *et al.*, 1993). All 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/Bait X fusion (Figure A, below) while the second hybrid protein is the B42 AD/Prey X fusion (Figure B, below). Prey X 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 (an auxotrophic marker and *lac*Z). 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 bait X interacts with prey X 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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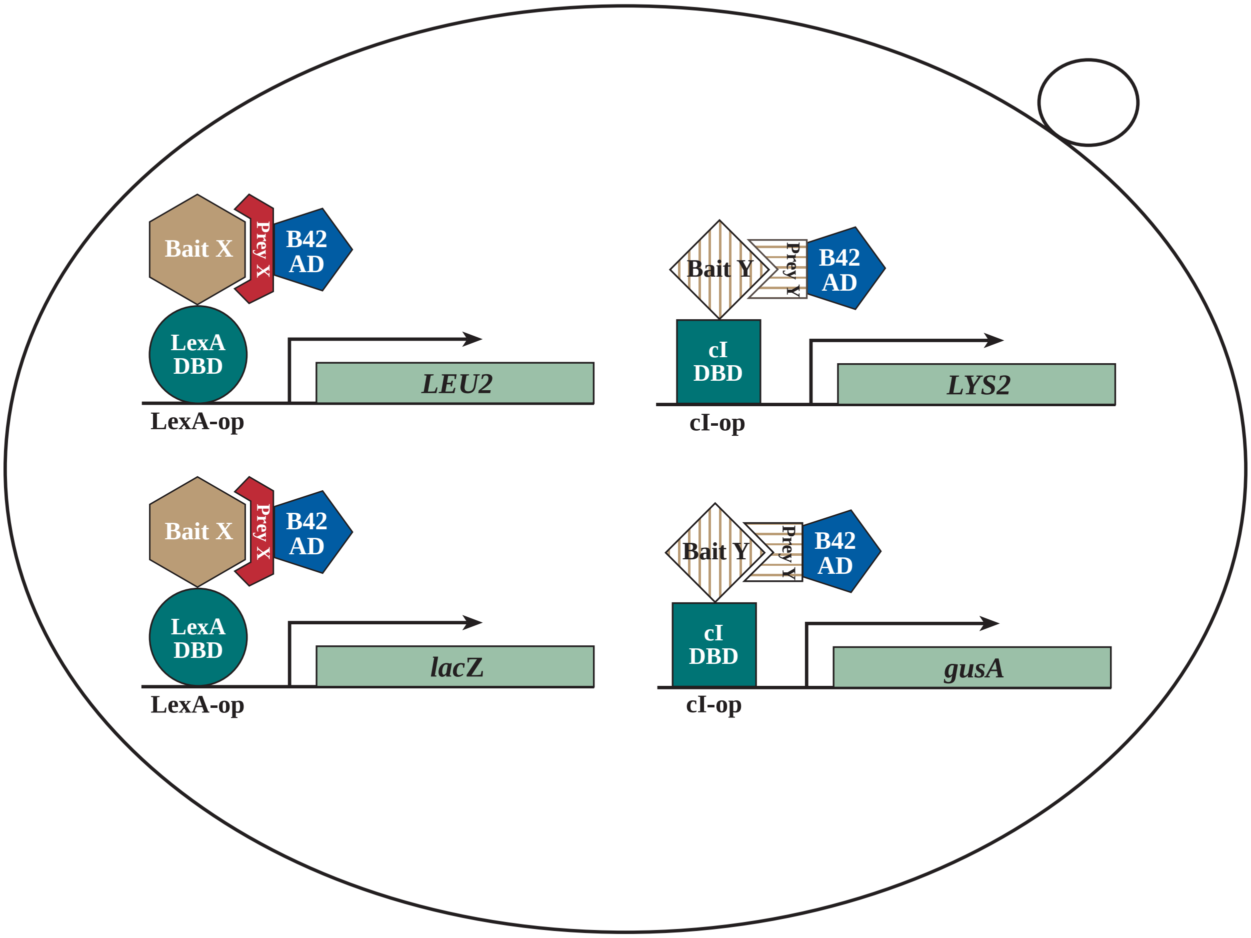
General Description of the Dual Bait Hybrid Hunter™ System

The Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System is a modification of the Hybrid Hunter™ System and is designed to allow simultaneous screening and isolation of interactors to two different bait proteins (Serebriiskii *et al.*, 1999). As with the Hybrid Hunter™ System, the first bait protein consists of a LexA DBD/Bait X fusion while the prey protein consists of a B42 AD/Prey X fusion. Prey X can be replaced with a cDNA library to screen for unknown interactors. An interaction between bait X and prey X brings the B42 AD together with the LexA DBD to reconstitute transcriptional activation and allow expression of two reporter genes, the *LEU2* auxotrophic marker and *lacZ*, via binding to the LexA operator sites (see figure, below). Positive interactions can be detected by selecting for leucine prototrophy and β-galactosidase activity.

A further level of complexity is introduced to the system with the addition of a second bait protein consisting of a fusion between the bacteriophage lambda cI DBD and bait Y. Screening a cDNA library will then allow identification of prey Y proteins that interact with the bait Y. In this case, an interaction between bait Y and prey Y brings the B42 AD together with the cI DBD to reconstitute transcriptional activation and allow expression of two reporter genes, the *LYS2* auxotrophic marker and *gusA*, via binding to the cI operator sites (see figure, below). Positive interactions can be detected by selecting for lysine prototrophy and β-glucuronidase activity.



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Overview, continued

Experimental Outline for a Library Screen

The table below outlines the major steps required to isolate positive clones from a cDNA library using the Dual Bait Hybrid Hunter™ System. The approximate time required for each step is provided. Some of the steps may be performed concurrently. 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 cDNA library in pYESTrp2 using standard techniques	1-2 weeks	12
	Purchase a Hybrid Hunter™ Library in pYESTrp, pYESTrp2, or pJG4-5	2 days	8
	Prepare library plasmid DNA for small-scale transformation	2 days	12
Construct the bait plasmids	Clone the gene of interest in pHybLex/Zeo to create the LexA/bait X fusion protein	1-2 days	13-15
	Clone a second gene of interest in pHybcI/HK to create the cI/bait Y fusion protein	1-2 days	13-15
	Transform the pHybLex/Zeo bait construct into *E. coli*, select transformants, and sequence to confirm that the gene of interest is cloned in frame with the LexA DBD	4 days	16
	Transform the pHybcI/HK bait construct into *E. coli*, select transformants, and sequence to confirm that the gene of interest is cloned in frame with the cI DBD	4 days	16
Transform the bait plasmids into yeast to create the bait strain and test for expression	Prepare competent SKY48/pLacGUS using a small-scale preparation and transform the bait plasmids into the strain	3-5 days	18-19
	Test transformants for expression of bait proteins by immunoblot analysis	2 days	20-21
Test the bait plasmids for non-specific activation	Test for leucine or lysine prototrophy Test for β-galactosidase or β-glucuronidase activity	5 days	22-25
Perform a library screen for proteins that interact with the bait plasmids	Use a small-scale protocol to transform the bait strain with the library and use a two-step protocol to select for Leu$^+$ or Lys$^+$ transformants	1-2 weeks	27-31
	Test positive transformants for β-galactosidase or β-glucuronidase activity	2-3 days	32
	Retrieve prey plasmids encoding putative interactors and classify by restriction analysis	1 week	34-37
	Re-confirm positive bait/prey interactions, if desired	1 week	26
	Sequence selected prey plasmids to identify the interacting protein(s)	1 week	37

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Overview, continued

Experimental Outline for Testing Known Proteins for Interaction

The table below outlines the major steps required to clone and test known proteins for a potential interaction (i.e. wild-type and mutant versions of a bait protein for an interaction with a known prey protein). The approximate time required for each step is provided. Some steps may be performed concurrently. For more information on each step, please see the indicated page.

Step	Action	Time Required	Page
Construct the prey plasmid	Clone the gene for a known protein into pYESTrp2	2 days	9-10
	Transform the pYESTrp2 construct into *E. coli*, select transformants, and sequence to confirm that the gene is cloned in frame with the V5-NLS-B42 peptide	4 days	11
Construct the bait plasmids	Clone the gene of interest in pHybLex/Zeo to create the LexA/bait X fusion protein	2 days	13-14
	Clone a second gene of interest in pHybcI/HK to create the cI/bait Y fusion protein	2 days	13-15
	Transform the pHybLex/Zeo bait construct into *E. coli*, select transformants, and sequence to confirm that the gene of interest is cloned in frame with the LexA DBD	4 days	16
	Transform the pHybcI/HK bait construct into *E. coli*, select transformants, and sequence to confirm that the gene of interest is cloned in frame with the cI DBD	4 days	16
Transform the two bait plasmids and the prey plasmid into yeast	Use a small-scale transformation protocol to transform the bait plasmids and the prey plasmid into SKY48/pLacGUS, and use a two-step protocol to select for leucine or lysine prototrophy	5-7 days	26-31
	Test positive transformants for β-galactosidase or β-glucuronidase activity	2-3 days	24

Important

The Dual Bait Hybrid Hunter™ Two-Hybrid System manual is designed to help you isolate positive clones in the simplest, most direct fashion. References for more sophisticated uses of two-hybrid systems are available (see page 41 for more information).

We recommend that the user be familiar with basic yeast molecular biology and microbiological techniques. A number of general references are 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 grow and manipulate yeast.

Current Protocols in Protein Science (1998) *Interaction Trap/Two-Hybrid System to Identify Interacting Proteins*, pp. 19.2.1-19.2.40.

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

Propagation and Maintenance of Plasmids

Introduction

The following section contains guidelines for maintaining and propagating the vectors in the Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System.

E. coli Strain

Many *E. coli* strains are suitable for the propagation of the Dual Bait Hybrid Hunter™ vectors including TOP10 (Catalog no. C610-00), TOP10F´ (Catalog no. C615-00), DH5α, JM109 (Catalog no. C666-00), or equivalent. We recommend that you propagate the Dual Bait Hybrid Hunter™ vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
One Shot™ TOP10 (chemically competent cells)	21 x 50 µl	C4040-03
Electrocomp™ TOP10 (electrocompetent cells)	5 x 80 µl	C664-55

Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of Plasmids

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

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

   Resuspend pHybLex/Zeo-Fos2, pYESTrp-Jun, pHybcI/HK-Krev, and pYESTrp2-RalGDS in 10 µl sterile water.

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

Vector	Medium	Antibiotic
pYESTrp pYESTrp-Jun pYESTrp2-RalGDS	LB	50 µg/ml ampicillin
pHybcI/HK pHybcI/HK-Krev	LB	50 µg/ml kanamycin
pHybLex/Zeo pHybLex/Zeo-Fos2	Low Salt LB (see page 49 for a recipe)	25 µg/ml Zeocin™

4. Store the stock solution at -20°C when finished.
5. Prepare a glycerol stock of each strain containing plasmid for long-term storage (see the next page for a protocol).

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imMedia™ Agars

For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin, kanamycin, or Zeocin™, imMedia™ Agars are available from Invitrogen. Ordering information is provided below. For more information, please see our Web site (www.invitrogen.com) or call Technical Service (see page 70).

Item	Pouches[*]	Catalog no.
imMedia™ Amp Agar	20	Q601-20
imMedia™ Kan Agar	20	Q611-20
imMedia™ Zeo Agar	20	Q621-20

[*] Each pouch contains enough reagents to prepare 8-10 standard-sized agar plates

Preparing a Glycerol Stock

Once you have transformed the Dual Bait Hybrid Hunter™ plasmids into a suitable *E. coli* strain, 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.

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

Choosing a Library

Introduction

As noted earlier, you can use the Dual Bait Hybrid Hunter™ Yeast Two-Hybrid System for the following applications:

- Screening a cDNA library for potential interactors with your two bait proteins
- Testing known proteins for a potential interaction
- Testing the specificity of an interaction between known proteins

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 Dual Bait Hybrid Hunter™ System, **or**
- Purchase a Premade Hybrid Hunter™ library (see the next page)

To test known proteins for a potential interaction or to test for the specificity of an interaction, you may:

- Clone the gene of a known protein into the pYESTrp2 prey vector (see pages 9-10).

Using Your Own Library

It is possible to use different two-hybrid libraries in conjunction with the Dual Bait Hybrid Hunter™ System, but your choices may be limited by auxotrophic markers and compatibility with a particular yeast host strain. In principle, you can use any acidic activating domain with the LexA DNA binding domain or the cI DNA binding domain (e.g. VP16, Gal4) (Brent and Ptashne, 1985) in a two-hybrid screen. You may also use any library that is compatible with the SKY48/pLacGUS yeast host strain.

Please note that if you use SKY48/pLacGUS as the host strain for your own library, the library plasmids will need to contain a *TRP1* marker for auxotrophic selection.

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 12 for a few general guidelines.

continued on next page

Choosing a Library, continued

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 (see page 70) for more information or visit our Web site (www.invitrogen.com). For more information about the pJG4-5 vector, please see page 63 in the **Appendix**.

Source	Plasmid	Primary Clones	Size Selection	Catalog no.
Cell Lines				
HeLa cells (Human cervical carcinoma)	pYESTrp	3.66×10^6	0.3 to 1.2 kb	A201-01
HeLa cells (Human cervical carcinoma)	pJG4-5	9.6×10^6	0.5 to 2 kb	A211-01
BeWo cells (Human fetal placental choriocarcinoma)	pYESTrp	5.35×10^6	0.3 to 0.8 kb	A208-01
BeWo cells (Human fetal placental choriocarcinoma)	pYESTrp	5.5×10^6	0.3 to 1.2 kb	A208-02
Jurkat cells (Human T cell leukemia)	pYESTrp	3.2×10^6	0.3 to 1.2 kb	A209-01
A20 cells (Mouse B cell lymphoma)	pYESTrp	3.11×10^6	0.3 to 1.2 kb	A210-01
Human Adult Tissue				
Bladder	pYESTrp2	17.6×10^6	0.4 to 1.2 kb	A225-01
Brain	pYESTrp2	10.8×10^6	0.4 to 1.2 kb	A204-01
Breast	pYESTrp2	9.00×10^6	0.4 to 1.2 kb	A217-01
Breast Tumor	pYESTrp2	8.84×10^6	0.4 to 1.2 kb	A216-01
Colon Tumor	pYESTrp2	7.98×10^6	0.4 to 1.2 kb	A222-01
Kidney	pYESTrp2	6.96×10^6	0.4 to 1.2 kb	A223-01
Liver	pYESTrp	2.21×10^6	0.3 to 1.2 kb	A203-01
Lung	pYESTrp2	5.95×10^6	0.4 to 1.2 kb	A213-01
Lung Tumor	pYESTrp2	1.85×10^6	0.4 to 1.2 kb	A215-01
Ovary	pYESTrp	4.54×10^6	0.3 to 1.2 kb	A206-01
Placenta	pYESTrp	4.75×10^6	0.3 to 1.2 kb	A207-01
Prostate	pYESTrp2	5.46×10^6	0.4 to 1.2 kb	A218-01
Spleen	pYESTrp2	11.4×10^6	0.4 to 1.2 kb	A214-01
Testes	pYESTrp	6.4×10^6	0.3 to 1.2 kb	A205-01
Human Fetal Tissue				
Fetal Liver	pYESTrp	2.37×10^6	0.3 to 1.2 kb	A202-01

Cloning into pYESTrp2

Introduction

The prey vector, pYESTrp2 (5823 bp), can be used to make two-hybrid cDNA libraries or to clone genes encoding known proteins. Use the diagram on the next page to help you design a strategy to clone a cDNA library or your gene of interest into pYESTrp2. General considerations for cloning into pYESTrp2 are listed below. A map and a description of the features of pYESTrp2 can be found in the **Appendix**, pages 52-53.

General Molecular Biology Techniques

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).

Cloning Considerations

When designing your cloning strategy, remember that 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.

NOTE

The N-terminal peptide contains a V5 epitope to allow detection of your expressed prey fusion protein by immunoblot (western analysis). Anti-V5 antibodies are available from Invitrogen to facilitate detection (see page 11 for more information).

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Cloning into pYESTrp2, continued

Multiple Cloning Site of pYESTrp2

Below is the multiple cloning site for pYESTrp2. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete nucleotide sequence of pYESTrp2 is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).** The map and a description of the features of the vector may be found in the **Appendix**, pages 52-53.

```
      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

3621  CTTCAAATGA ACGAATCAAA TTAACAACCA TAGGATGATA ATGCGATTAG TTTTTTAGCC

                                                                 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 CTCACC ATG GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC
                        Met Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu
                                                     SV40 NLS
3978  GAT TCT ACA CAA GCT ATG GGT GCT CCT CCA AAA AAG AAG AGA AAG GTA GCT
      Asp Ser Thr Gln Ala Met Gly Ala Pro Pro Lys Lys Lys Arg Lys Val Ala

4029  GGT ATC AAT AAA GAT ATC GAG GAG TGC AAT GCC ATC ATT GAG CAG TTT ATC
      Gly Ile Asn Lys Asp Ile Glu Glu Cys Asn Ala Ile Ile Glu Gln Phe Ile

4080  GAC TAC CTG CGC ACC GGA CAG GAG ATG CCG ATG GAA ATG GCG GAT CAG GCG
      Asp Tyr Leu Arg Thr Gly Gln Glu Met Pro Met Glu Met Ala Asp Gln Ala
                                 B42 activation domain
4131  ATT AAC GTG GTG CCG GGC ATG ACG CCG AAA ACC ATT CTT CAC GCC GGG CCG
      Ile Asn Val Val Pro Gly Met Thr Pro Lys Thr Ile Leu His Ala Gly Pro

4182  CCG ATC CAG CCT GAC TGG CTG AAA TCG AAT GGT TTT CAT GAA ATT GAA GCG
      Pro Ile Gln Pro Asp Trp Leu Lys Ser Asn Gly Phe His Glu Ile Glu Ala
           pYESTrp Forward priming site
                                                           Hind III
4233  GAT GTT AAC GAT ACC AGC CTC TTG CTG AGT GGA GAT GCC TCC AAG CTT GGT
      Asp Val Asn Asp Thr Ser Leu Leu Leu Ser Gly Asp Ala Ser Lys Leu Gly

      Kpn I   Sac I BamH I                               BstX I* EcoR I
4284  ACC GAG CTC GGA TCC ACT AGT AAC GGC CGC CAG TGT GCT GGA ATT CTG CAG
      Thr Glu Leu Gly Ser Thr Ser Asn Gly Arg Gln Cys Ala Gly Ile Leu Gln

                   BstX I* Not I   Xho I         Sph I
4335  ATA TCC ATC ACA CTG GCG GCC GCT CGA GGC ATG CAT CTA GAG GGC CGC ATC
      Ile Ser Ile Thr Leu Ala Ala Ala Arg Gly Met His Leu Glu Gly Arg Ile
                         pYESTrp Reverse priming site
4386  ATG TAA TTAGTTA TGTCACGCTT ACATTCACGC CCTCCCCCCA
      Met ***
```

*Please note that there are two *Bst*X I sites in the polylinker.

---

continued on next page

Cloning into pYESTrp2, continued

E. coli Transformation

Transform your ligation mixture into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10, TOP10F´) and select on LB agar plates containing 50 to 100 µg/ml ampicillin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the pYESTrp Forward and pYESTrp Reverse primers provided in the kit to confirm that your gene is cloned in the proper orientation for expression and that it is fused in frame with the B42 activation domain. See the diagram on the previous page for the sequences and location of the priming sites. The primers are also available separately from Invitrogen in 2 µg aliquots (see page v for ordering information).

Expression of Prey Fusion Protein

If you want to test your pYESTrp2 construct for expression of the prey fusion protein prior to performing your interactor hunt, you may use a small-scale yeast transformation protocol to transform your prey plasmid into the SKY48/pLacGUS yeast strain. For more information about the SKY48/pLacGUS strain, please see page 17. The pYESTrp2 plasmid contains the *TRP1* gene to allow selection of yeast transformants by tryptophan prototrophy. Follow the guidelines provided on pages 18-19 to transform your pYESTrp2 construct into SKY48/pLacGUS. Select transformants on YC-W medium (see page 43 for a recipe). To detect expression of your prey fusion protein by western blot, please see page 20 for a protocol to prepare cell lysates from your Trp$^+$ transformants. Information about available antibodies to detect your prey fusion protein is provided below.

Antibodies for Detection

The prey fusion protein contains the N-terminal V5 epitope to allow detection of the expressed prey protein by western blot analysis. Anti-V5 antibodies are available from Invitrogen. Ordering information is provided below. The quantity provided is sufficient for 25 westerns. For the sequence of the V5 epitope, please refer to the diagram on page 10.

Antibody	Quantity	Catalog no.
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-HRP Antibody	50 µl	R961-25

NOTE

Please note that the N-terminal peptide containing the V5 epitope, nuclear localization signal, and B42 activation domain will add approximately 12 kDa to the size of your prey protein.

continued on next page

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 (Ausubel *et al.*, 1994) for the details of cDNA library construction.

- Isolate mRNA from the source of interest.
- Prepare first strand cDNA using random primers, Oligo dT(*Not* I) primer (Catalog no. N430-01), or an Oligo dT (*Xho* I) primer.

  Alternatively, the Copy™ Kit (Catalog no. L1311-03) is available from Invitrogen for efficient production of double-stranded blunt-ended cDNA for either bidirectional or unidirectional cloning. Call Technical Service (see page 70) for more information.
- After second strand synthesis, be sure the ends are blunt prior to adding *Bst*X I/*Eco*R I adaptors. BstX I/EcoR I adaptors (Catalog no. N418-18) are available from Invitrogen.
- 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 the number of primary recombinants. You may wish to amplify the library prior to large-scale isolation of plasmid DNA for the library screen.

Plasmid Preparation

You will need 30 µg of library plasmid DNA to perform a small-scale library transformation. If you wish to perform a large-scale library transformation, you will need 500 µg of library plasmid DNA. To isolate plasmid DNA, follow the procedure below. Other methods are suitable. 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 µg/ml ampicillin) with sufficient bacterial library stock to ensure 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.

Constructing the Bait Plasmids

Introduction

The Dual Bait Hybrid Hunter™ System contains two bait plasmids, pHybLex/Zeo and pHybcI/HK. You may clone a different bait protein into each plasmid. Cloning into pHybLex/Zeo will create a LexA DBD fusion protein, while cloning into pHybcI/HK will create a cI DBD fusion protein. The pHybLex/Zeo plasmid contains the Zeocin™ resistance gene to allow selection of yeast transformants with Zeocin™. The pHybcI/HK plasmid contains the *HIS3* gene to allow selection of transformants by histidine prototrophy. This section provides information on cloning the genes for the two bait proteins of interest into the pHybLex/Zeo and pHybcI/HK plasmids. For more information about the LexA and cI repressors, please see below.

A Brief Note About the LexA Repressor

Your first bait of interest will be fused to the *E. coli* LexA repressor (or LexA DNA binding protein) (Horii *et al.*, 1981; Markham *et al.*, 1981) in pHybLex/Zeo. The LexA repressor has been shown to bind to LexA operator sequences in the promoters of LexA-responsive genes to activate transcription of those genes (Brent and Ptashne, 1981). The LexA sequence in pHybLex/Zeo encodes the complete 202 amino acid LexA DNA binding protein (Horii *et al.*, 1981; Markham *et al.*, 1981) which also includes a dimerization domain. For more information about the LexA protein, please refer to published references (Brent, 1982; Schnarr *et al.*, 1985).

A Brief Note About the cI Repressor

Your second bait of interest will be fused to the bacteriophage lambda cI repressor (or cI DNA binding protein) (Ptashne, 1978) in pHybcI/HK. The cI repressor has been shown to bind to cI operator sequences in the promoters of cI-responsive genes to activate transcription of those genes (Ptashne, 1978). The DNA fragment encoding the complete cI DNA binding protein in pHybcI/HK is derived from nucleotides 37230-37940 of the bacteriophage lambda genome (LAMCG nt 37230-37940). For more information and references about the cI repressor, the LAMCG sequence can be accessed through Genbank (Accession No. J02459) on the World Wide Web at:

www.ncbi.nlm.nih.gov/entrez/nucleotide.html

Bait Protein Criteria

The first step in construction of the pHybLex/Zeo and pHybcI/HK bait plasmids is to decide whether to fuse full length proteins or a particular domain of those proteins in frame with the LexA or cI DBD. Screens employing full length bait proteins fused to the LexA or the cI DBD tend to have a lower background of false positives. However, if full length proteins activate transcription of the reporter genes, then domains or fragments of the proteins should be fused to the LexA or cI DBD and tested as possible baits.

NOTE

Neither pHybLex/Zeo nor pHybcI/HK contain a nuclear localization signal. Both LexA and cI fusions appear to be produced in sufficient amounts to allow entry of bait fusion proteins into the nucleus by mass action. Avoid using bait proteins containing extensive transmembrane domains or signal sequences that would cause the proteins to be directed to locations other than the nucleus.

Cloning into pHybLex/Zeo or pHybcI/HK

To ensure proper expression of your bait proteins from pHybLex/Zeo or pHybcI/HK, you must clone the bait genes in frame with the LexA DBD or the cI DBD. See the diagrams on pages 14-15 to develop a cloning strategy.

continued on next page

Multiple Cloning Site of pHybLex/Zeo

Below is the multiple cloning site for pHybLex/Zeo. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete nucleotide sequence of pHybLex/Zeo is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).** A map and a description of the features of the vector may be found in the **Appendix**, pages 54-55.

```
                                    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 CAA GCT AAT TCC GGG CGA ATT TCT TAT GAT TTA TGA TTT
      Thr Cys Ser Gln Ala Asn Ser Gly Arg Ile Ser Tyr Asp Leu ***
                                          pHybLex/Zeo Reverse priming site
                                      |                           |
1131  TTATTATTAA ATAAGTTATA AAAAAAATAA GTGTATACAA ATTTTAAAGT GACTCTTAGG
```

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Multiple Cloning Site of pHybcI/HK

Below is the multiple cloning site for pHybcI/HK. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pHybcI/HK is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).** See pages 56-57 for a map and a description of the features of the vector.

```
                                    cI repressor ORF
 966  ATG AGC ACA AAA AAG AAA CCA TTA ACA CAA GAG CAG CTT GAG GAC GCA
      Met Ser Thr Lys Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala

1014  CGT CGC CTT AAA GCA ATT TAT GAA AAA AAG AAA AAT GAA CTT GGC TTA
      Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys Asn Glu Leu Gly Leu

1062  TCC CAG GAA TCT GTC GCA GAC AAG ATG GGG ATG GGG CAG TCA GGC GTT
      Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val

1110  GGT GCT TTA TTT AAT GGC ATC AAT GCA TTA AAT GCT TAT AAC GCC GCA
      Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala

1158  TTG CTT GCA AAA ATT CTC AAA GTT AGC GTT GAA GAA TTT AGC CCT TCA
      Leu Leu Ala Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser

1206  ATC GCC AGA GAA ATC TAC GAG ATG TAT GAA GCG GTT AGT ATG CAG CCG
      Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro

1254  TCA CTT AGA AGT GAG TAT GAG TAC CCT GTT TTT TCT CAT GTT CAG GCA
      Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala

1302  GGG ATG TTC TCA CCT GAG CTT AGA ACC TTT ACC AAA GGT GAT GCG GAG
      Gly Met Phe Ser Pro Glu Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu

1350  AGA TGG GTA AGC ACA ACC AAA AAA GCC AGT GAT TCT GCA TTC TGG CTT
      Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu

1398  GAG GTT GAA GGT AAT TCC ATG ACC GCA CCA ACA GGC TCC AAG CCA AGC
      Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser

1446  TTT CCT GAC GGA ATG TTA ATT CTC GTT GAC CCT GAG CAG GCT GTT GAG
      Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu

1494  CCA GGT GAT TTC TGC ATA GCC AGA CTT GGG GGT GAT GAG TTT ACC TTC
      Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
                                          cI Forward priming site
1542  AAG AAA CTG ATC AGG GAT AGC GGT CAG GTG TTT TTA CAA CCA CTA AAC
      Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn

1590  CCA CAG TAC CCA ATG ATC CCA TGC AAT GAG AGT TGT TCC GTT GTG GGG
      Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
                                                                  EcoR I
1638  AAA GTT ATC GCT AGT CAG TGG CCT GAA GAG ACG TTT GGG AATT TGGAATTCGA
      Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
        Sac I                      Apa I      Not I/Sac II  Xho I   Sal I
1691  GCTCAGATCT CAGCTGGGCC CGGTACCGCG GCCGCTCGAG TCGACCTGCA GCCAAGCTAA

                                         ADH1 transcription termination signal
1751  TTCCGGGCGA ATTTCTTATG ATTTATGATT TTTATTATTA AATAAGTTAT AAAAAAAATA

            pHybLex/Zeo Reverse priming site
1811  AGTGTATACA AATTTTAAAG TGACTCTTAG GTTTTAAAAC GAAAATTCTT
```

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Constructing the Bait Plasmids, continued

E. coli Transformation

Once you have completed your ligation reactions, transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10, TOP10F´) and select for transformants as follows:

- Select pHybLex/Zeo transformants on Low Salt LB agar plates containing 25 µg/ml Zeocin™ (see page 49 for a recipe for Low Salt LB medium containing Zeocin™)
- Select pHybcI/HK transformants on LB agar plates containing 50 µg/ml kanamycin

For each transformation, choose 10-20 clones and analyze for the presence and orientation of your insert.

RECOMMENDATION

We recommend that you sequence your constructs with the appropriate primers, provided in the kit, to confirm that your gene(s) are fused in frame with either the LexA DBD (for pHybLex/Zeo) or the cI DBD (for pHybcI/HK). Use the following primers to sequence your constructs:

- For pHybLex/Zeo constructs, use the pHybLex/Zeo Forward and Reverse primers
- For pHybcI/HK constructs, use the cI Forward and the pHybLex/Zeo Reverse primers

Please refer to the diagrams on pages 14 and 15 for the sequences and location of the priming sites for each vector. The primers are also available separately from Invitrogen in 2 µg aliquots (see page v for ordering information).

The Next Step

Once you have confirmed that your bait proteins are fused correctly in frame with the LexA DBD or the cI DBD, respectively, you are ready to transform your bait constructs into the yeast strain SKY48/pLacGUS. Proceed to generate the bait strain and to confirm expression of the two bait fusion proteins (see pages 18-21).

SKY48/pLacGUS Host Strain

Introduction

The Dual Bait Hybrid Hunter™ System provides SKY48/pLacGUS as the host strain for your dual bait two-hybrid hunt. The SKY48 yeast strain (Serebriiskii *et al.*, 1999), derived from the EGY48 strain (Estojak *et al.*, 1995) has been transformed with the pLacGUS reporter plasmid (see below). See page iv for the genotype of SKY48/pLacGUS.

Features of SKY48/pLacGUS

The SKY48/pLacGUS yeast strain exhibits the following features:

- The strain contains the pLacGUS reporter plasmid already transformed. Please note that the pLacGUS plasmid contains a *URA3* gene for selection in yeast, therefore, the SKY48/pLacGUS strain is no longer auxotrophic for uracil.
- *GAL1* promoters are inducible by galactose and repressed by glucose. This helps to eliminate false positives and allows detection of potentially toxic interactors.
- The strain is wild-type for *GAL4*, a regulator of *GAL1* expression.
- The strain will express cDNA libraries that have been cloned into pYESTrp, pYESTrp2, and pJG4-5.
- The strain contains two auxotrophic markers - *LEU2* and *LYS2*, whose expression is controlled by 6 LexA operators and 3 cI operators, respectively.

pLacGUS Reporter Plasmid

The pLacGUS reporter plasmid contains two reporter genes, *lacZ* and *gusA*, whose expression is controlled by upstream activating sequences. Expression of the *lacZ* gene is controlled by 8 *LexA* operator sites (lexA-op), while expression of the *gusA* gene is controlled by 3 *cI* operator sites (cI-op). The plasmid also contains the *URA3* gene for selection in yeast. The complete sequence of pLacGUS is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 70). For a map of pLacGUS, please refer to the **Appendix**, page 62. If you wish to generate your own yeast reporter strain, the pLacGUS plasmid is available separately from Invitrogen (see page v for ordering information).

Initiating SKY48/pLacGUS Cultures

To initiate cultures from frozen yeast stocks, streak a small amount of the frozen glycerol stock on a YPD plate. Once growth is established, you may check the phenotype of the strain by streaking cells on a minimal plate supplemented with the appropriate amino acids. SKY48/pLacGUS will not grow in minimal medium that is deficient in tryptophan, histidine, leucine, or lysine. Be sure to make a glycerol stock of the strain. If you plan to use the strain directly from plates, make sure that the plates are less than 4 days old.

Other Yeast Host Strains

If you wish to transform the SKY48 yeast strain with your own reporter plasmid, the SKY48 strain is available separately from Invitrogen. If you wish to use a yeast host strain that allows more stringent selection of interactors and exhibits lower background with baits, the SKY191 yeast strain is also available from Invitrogen. The SKY191 strain is similar to SKY48, but contains only 2 *LexA* operators to direct transcription of the *LEU2* reporter gene. Using both strains in the Dual Bait Hybrid Hunter™ System will allow you to differentiate between strong and weak interactors with your protein of interest.

Strain	Genotype	Phenotype	Catalog no.
SKY48	MATα *ura3 trp1 his3* 6*lex*Aop-*LEU2* 3*cI*-op-*LYS2*	Ura$^-$, Trp$^-$, His$^-$, Leu$^-$, Lys$^-$	C833-00
SKY191	MATα *ura3 trp1 his3* 2*lex*Aop-*LEU2* 3*cI*-op-*LYS2*	Ura$^-$, Trp$^-$, His$^-$, Leu$^-$, Lys$^-$	C834-00

Yeast Transformation with the Bait Plasmids

Introduction

In this section, you will use a small-scale yeast transformation protocol to transform your pHybLex/Zeo and pHybcI/HK bait plasmids into SKY48/pLacGUS to create the bait strain. We generally cotransform SKY48/pLacGUS with pHybLex/Zeo and pHybcI/HK and screen for both plasmids simultaneously. If you have trouble obtaining transformants, you may transform one bait plasmid into SKY48/pLacGUS, select for transformants, and then use the resulting bait strain as the host for the second bait plasmid.

Once you have generated a bait strain containing both bait plasmids, we recommend that you test for proper expression of the LexA fusion and the cI fusion and for any non-specific activation of reporter constructs (see pages 22-25) before proceeding with your interactor hunt.

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.

Reagents for Yeast Transformation

The *S. c.* EasyComp™ Kit (Catalog no. K5050-01) provides a quick and easy method to prepare 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 transformation protocol is included in the **Appendix**, page 64. 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

The pHybLex/Zeo plasmid contains the Zeocin™ resistance gene to allow selection of transformants in medium containing Zeocin™. Please note that when selecting for pHybLex/Zeo transformants, you will not be performing auxotrophic selection. To select for yeast transformants containing the pHybLex/Zeo bait plasmid, use 200 µg/ml Zeocin™ in plates and medium. Please see pages 47-48 for instructions on how to prepare and handle Zeocin™.

RECOMMENDATION

Two control bait plasmids, pHybLex/Zeo-Fos2 and pHybcI/HK-Krev, are supplied in the Dual Bait Hybrid Hunter™ System. When transforming your two bait plasmids into SKY48/pLacGUS to create your bait strain, we recommend that you also create a control bait strain expressing pHybLex/Zeo-Fos2 and pHybcI/HK-Krev. This control bait strain may then be used as the host for the control prey plasmids. Creating the control bait strain will allow you to assay for positive bait/prey interactions in conjunction with your own interactor hunt.

For convenience, you may transform the four control plasmids (two control bait plasmids and two control prey plasmids) into SKY48/pLacGUS to generate a control strain that may be assayed directly for positive bait/prey interactions. For more information about the control plasmids, please refer to pages 50 and 58-61.

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Yeast Transformation with Bait Plasmids, continued

Transformation of Bait Plasmids

General guidelines are provided below to transform your bait plasmids into competent SKY48/pLacGUS to generate the bait strain.

1. Using one of the methods described on the previous page (or one of your own choosing), transform the pHybLex/Zeo and pHybcI/HK bait plasmids into competent SKY48/pLacGUS.
2. Select transformants on YC-UH Z200 plates (see **Appendix**, page 43 for a recipe)
3. Grow for 2 to 3 days at 30°C.
4. Select several His^+ Zeo^R transformants to characterize for expression of the LexA and cI bait fusion proteins by western blot analysis (see the next page).

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

Materials Required

To assay for expression of your bait fusion proteins by immunoblot (western blot) analysis, 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
- Selective medium and plates (see **Transformation of Bait Plasmids**, below)
- Cracking buffer (see page 45 for recipe), prewarmed to 60°C
- Acid washed glass beads (Sigma G-8772, 425-600 microns)
- Reagents for SDS-PAGE and immunoblotting
- Antibodies to your bait proteins or Anti-LexA Antibody and cI Antibody (see the next page)

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Expression of Bait Fusion Proteins

Use the protocol below to prepare cell lysates from your His$^+$, Zeocin™-resistant transformants (putative bait strain) and untransformed SKY48/pLacGUS for western blot analysis. Test several transformants in case of heterogeneity in LexA and cI fusion expression levels.

1. Inoculate 10 ml of YC-UH Z200 with a single colony of your bait strain (previous page) and inoculate 10 ml of YC-U with SKY48/pLacGUS 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 in Step 1 by centrifuging at 2500 rpm for 5 minutes at room temperature. Decant the medium.
4. Transfer the cell pellets 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 at room temperature and transfer supernatant to a new tube.
10. Add SDS-PAGE sample buffer and boil sample for 5 minutes. Use 30 to 50 µl for immunoblot analysis. Detect LexA and cI fusions using antibodies to your proteins of interest or antibodies available from Invitrogen (see below).

Antibodies for Detection

To detect expression of your LexA and cI bait fusion proteins by western blot, you will need to have antibodies to your proteins of interest. Alternatively, the Anti-LexA Antibody and the cI Antibody are available from Invitrogen to detect LexA and cI fusion proteins, respectively. The amount of antibody supplied is sufficient for 25 westerns. Ordering information is provided below.

Item	Quantity	Catalog no.
Anti-LexA Antibody	50 µl	R990-25
cI Antibody	50 µl	R991-25

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Yeast Transformation with Bait Plasmids, continued

What You Should See

The calculated molecular weight (MW) of the LexA and cI protein expressed from pHybLex/Zeo and pHybcI/HK, respectively, is listed below. The calculated molecular weight of each protein includes additional amino acids encoded by the multiple cloning sites. The table also lists the observed migration of each protein on an SDS polyacrylamide gel.

Protein	Calculated MW	Observed MW
LexA	26 kDa	32 kDa
cI	29 kDa	36 kDa

The results from your immunoblotting experiment should show that the bait strain expresses intact LexA and cI fusion proteins whereas the untransformed SKY48/pLacGUS strain should not show any cross-reactivity to either LexA or cI. Please note that the background may be increased when using crude lysates.

Once you have confirmed that your bait strain expresses the LexA and cI fusions, we recommend that you perform functional tests to ensure that both baits do not exhibit non-specific activation of reporter genes before performing your interactor hunt. Please see the next section for more details.

NOTE

We have found that expression levels of bait proteins from pHybLex/Zeo and pHybcI/HK are generally comparable.

Testing the Bait Plasmids

Introduction

In this section you will test the LexA and the cI baits for non-specific activation. Well-behaved baits (i.e., a protein fused to LexA or cI) **should not:**

- Non-specifically transactivate the reporter constructs in the SKY48/pLacGUS strain.
- Interact with either the nuclear localization signal (NLS) or with the acidic activation domain in the empty prey plasmid (e.g. pYESTrp2).

The bait plasmids are transformed alone into SKY48/pLacGUS or together with pYESTrp2 as described below. The resulting strains are tested for leucine or lysine prototrophy as well as β-galactosidase or β-glucuronidase activity. The transformed strains **should not** grow in the absence of leucine or lysine **OR** exhibit detectable β-galactosidase or β-glucuronidase activity.

Important

Remember that SKY48/pLacGUS contains the pLacGUS reporter plasmid with a wild-type *URA3* gene, therefore, the strain should grow and should be maintained in 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).

Media Requirements

The table below describes the media used for testing the bait plasmids in SKY48/pLacGUS. Please refer to the protocol on the next page to determine which media you will need. Please see page 42 for a description of these media and page 43 for a recipe.

Experiment	Plasmids	Selective Medium
Selection	Bait Plasmids	YC-UH Z200
	Bait Plasmids + pYESTrp2	YC-UHW Z200
Assay for Prototrophy (leucine or lysine)	Bait Plasmids	YC-UHL Z200 or YC-UHK Z200
	Bait Plasmids + pYESTrp2	YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff
Assay for β-Galactosidase Activity	Bait Plasmids	YC-UH Z200
	Bait Plasmids + pYESTrp2	YC-UHWL Z200 Gal/Raff
Assay for β-glucuronidase Activity	Bait Plasmids	YC-UH Z200
	Bait Plasmids + pYESTrp2	YC-UHWK Z200 Gal/Raff

NOTE

When transforming the pYESTrp2 plasmid into your bait strain, we recommend that you use a two-step selection protocol (see below) to assay for positive bait/prey interactions.

1. Plate transformants on YC-UHW Z200 medium to select for the bait and prey plasmids.
2. Patch transformants on YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff plates to assay for positive bait/prey interactions by leucine or lysine prototrophy.

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Testing the Bait Plasmids, continued

Materials Required

Be sure to have the following reagents and equipment on hand before proceeding. Please see page 43 for specific media recipes.

- 30°C incubator and shaking incubator
- Reagents for yeast transformation
- Centrifuges
- Bait plasmids
- pYESTrp2
- β-galactosidase activity reagents (see the next page)
- β-glucuronidase activity reagents (see the next page)

Controls

In addition to your two bait constructs and pYESTrp2, you may wish to transform the following plasmids into SKY48/pLacGUS as controls.

Plasmid	Control
pHybLex/Zeo (no insert)	Positive control for low activation of the reporter constructs*
pHybcI/HK (no insert)	Positive control for low activation of the reporter constructs*

*The activity of unfused LexA expressed from pHybLex/Zeo and unfused cI expressed from pHybcI/HK is high enough to weakly activate the *LEU2*, *LYS2*, *lac*Z, and *gusA* reporters. Fusions to the LexA or cI DBD generally decrease this background activation. When SKY48/pLacGUS containing the parental pHybLex/Zeo and pHybcI/HK vectors is patched onto Leu$^-$ or Lys$^-$ plates, only a few colonies should be apparent as opposed to a solid streak observed when genuine bait/prey interactions are detected.

In addition to determining the degree of non-specific reporter activation by the bait plasmids, we recommend that you test the interaction of the baits 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.

Transforming and Testing Bait Plasmids

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 and β-glucuronidase activity. Please refer to the table on the previous page for the correct medium.

1. Transform the bait plasmids alone and together with pYESTrp2 into SKY48/pLacGUS using a small-scale transformation protocol.

   Note: You may also use the bait strain you constructed on page 19. However, you must make competent cells of the bait strain before transforming with pYESTrp2. You may wish to keep frozen, competent SKY48/pLacGUS (prepared using the *S.c.* EasyComp™ Kit) on hand for testing the bait plasmids.
2. Select transformants on the appropriate selective medium (see previous page).
3. Incubate plates at 30°C for 3 days or until single colonies appear.
4. To assay for leucine or lysine prototrophy, patch individual transformants from Step 3 onto the appropriate selective medium.
5. To assay for β-galactosidase or β-glucuronidase activity, patch individual colonies from Step 3 and arrange in a grid pattern on the appropriate selective plates.
6. Incubate all plates at 30°C for 2 to 3 days until colonies form.

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β-Galactosidase and β-Glucuronidase Overlay Assay

We recommend using an overlay assay to detect β-galactosidase or β-glucuronidase reporter activity (Duttweiler, 1996). You may use a filter assay, but the overlay assay is generally easier to perform. A protocol to perform a β-galactosidase or β-glucuronidase overlay assay is provided below. **Note:** For your convenience, X-Gluc is supplied in the Dual Bait Hybrid Hunter™ System.

Materials to Have on Hand

- Low Melt Agarose (LMA; Invitrogen, Catalog no. R420-100)
- 100 mM Potassium Phosphate Buffer, pH 7.0 (see page 46 for a recipe)
- X-Gluc (25 mg/ml in DMF; see page 46 for a recipe)
- X-Gal (25 mg/ml in DMF; see page 45 for a recipe)
- Dimethylformamide (DMF)
- Patched plate(s) containing your positive transformants

Procedure

1. Add 1 g of Low Melt Agarose (LMA) to 100 ml of 100 mM Potassium Phosphate Buffer, pH 7.0. Dissolve the low melt agarose by heating for 3-5 minutes in the microwave. Do not overheat the agarose as the solution will boil over.
2. Allow the agarose solution to cool to 65°C.
3. Prepare X-Gluc/DMF or X-Gal/DMF solution by adding the following amount of X-Gluc or X-Gal solution to DMF:

   For X-Gluc, add 100 µl of freshly prepared 25 mg/ml X-Gluc to 8 ml DMF.
   For X-Gal, add 800 µl of freshly prepared 25 mg/ml X-Gal to 8 ml DMF.
4. Mix the 8 ml of X-Gluc/DMF or X-Gal/DMF solution from Step 3 with 12 ml of the dissolved LMA solution to make an X-Gluc/LMA or X-Gal/LMA solution with a final concentration of 40% DMF, 0.6% LMA. The total volume will be 20 mls.

   Note: The amount of X-Gluc/LMA or X-Gal/LMA solution prepared is sufficient to perform overlay assays on 4 small plates (100 mm) or 2 large plates (150 mm). If you need more X-Gluc/LMA or X-Gal/LMA solution, scale up the procedure to fit your needs.
5. Incubate the X-Gluc/LMA or X-Gal/LMA solution at 65°C for 5 minutes.
6. Carefully overlay the patched plates with the following amount of X-Gluc/LMA or X-Gal/LMA solution:

   For 100 mm plates, use 5 ml per plate
   For 150 mm plates, use 10 ml per plate
7. Let the plates sit at room temperature for 5-10 minutes until the X-Gluc/LMA or X-Gal/LMA solution solidifies. Do not disturb the plates during the solidification process. To prevent exposure to light, keep the plates covered with aluminum foil.
8. Incubate the plates in the dark for up to 1 hour at 30°C, but monitor the color development regularly by eye during this time (see the next page). **Do not incubate plates for longer than 1 hour.**

   Colonies that are positive for β-glucuronidase or β-galactosidase activity will turn blue.

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Important

Monitor the color development on the overlay plates carefully within the first hour. We suggest that you check the degree of color development every 15 minutes. This is particularly important for the β-glucuronidase assay as X-Gluc degrades easily and long incubations can lead to high background that might be interpreted as a false positive result.

In general, the intensity and length of time that it takes for the color signal to develop should provide an indication of the strength of your positive bait/prey interaction. For a strong positive interaction between your bait and prey, you may see intense blue color develop within 15 minutes. For a weak positive interaction, the blue color may take up to an hour to develop.

Analysis

A well-behaved bait expressed from pHybLex/Zeo should **NOT** allow the yeast to grow in the absence of leucine or express detectable β-galactosidase activity **UNLESS** there is an interactor present. If the bait strain becomes Leu^+ $LacZ^+$ upon transformation of the pHybLex/Zeo bait plasmid, please see the **Troubleshooting** section, page 38.

Similarly, a well-behaved bait expressed from pHybcI/HK should **NOT** allow the yeast to grow in the absence of lysine or express detectable β-glucuronidase activity **UNLESS** there is an interactor present. If the bait strain becomes Lys^+ GUS^+ upon transformation of the pHybcI/HK bait plasmid, please see the **Troubleshooting** section, page 38.

The Next Step

Once you have confirmed that your bait is behaving properly, keep a fresh plate and make a glycerol stock of SKY48/pLacGUS containing the two bait plasmids (bait strain). Proceed to the interactor hunt (see the next page).

Interactor Hunt Overview

Introduction

Once you have both bait plasmids transformed into SKY48/pLacGUS (bait strain) and have tested for non-specific activation, you are ready to perform one of the following two applications:

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

2) test for an interaction with a known protein or proteins.

You may use your own library or one of the libraries available in pYESTrp, pYESTrp2, or pJG4-5 (see page 8). **Please note that the pHybLex/Zeo and pHybcI/HK bait plasmids can be used with any library that uses the *TRP1* marker for auxotrophic selection.** Alternatively, you may clone a prey protein of interest into pYESTrp2 for coexpression with your baits in SKY48/pLacGUS (see pages 9-10).

Experimental Outline

The table below provides a simplified outline of how an interactor hunt is performed using SKY48/pLacGUS.

Step	Action
1	Transform the bait strain with a library in pYESTrp, pYESTrp2 or pJG4-5 (or other compatible vector). **Note:** The library is expressed by induction with galactose (see Step 4)
2	Plate transformants in YC-UHW Z200 medium to select for the bait and prey plasmids.
3	Harvest and pool transformants.
4	Plate transformants in YC-UHWK Z200 Gal/Raff or YC-UHWL Z200 Gal/Raff medium to assay for leucine or lysine prototrophy. Cells that contain an interactor with the LexA fusion will grow in the absence of leucine, while cells that contain an interactor with the cI fusion will grow in the absence of lysine.
5	Test positive transformants for β-galactosidase or β-glucuronidase activity.

General Resources

The protocols that you will use to perform your Dual Bait interactor hunt are generally similar to those used for any interactor hunt. For general reference information, please refer to *Current Protocols in Protein Science*, Unit 19 (Coligan *et al.*, 1995) or *Current Protocols in Molecular Biology*, Unit 20 (Ausubel *et al.*, 1994). Please note that the selective medium used in the Dual Bait interactor hunt will vary.

RECOMMENDATION

If you have generated a control strain containing the four control plasmids (see page 18), we recommend that you include this strain in your experiment. Plating the control strain in the appropriate selective medium in conjunction with your interactor hunt may help you to troubleshoot your experiment. The control strain should exhibit leucine or lysine prototrophy and β-galactosidase or β-glucuronidase activity when plated on the appropriate selective medium. For more information, please see the section on **Dual Bait Control Transformations** in the **Appendix**, pages 50-51.

Interactor Hunt

Introduction

The SKY48/pLacGUS strain allows expression of library genes to be induced by galactose. This is particularly important if expression of potential interactors is toxic to yeast. In the SKY48/pLacGUS strain, the upstream activating sequences (UAS) of the chromosomal *LEU2* gene are replaced with LexA operator sites and the UAS of the chromosomal *LYS2* gene are replaced with cI operator sites. In addition, the *lacZ* gene and the *gusA* gene in the pLacGUS reporter plasmid are controlled by LexA operator sites and cI operator sites, respectively. In the interactor hunt, positive interactions between a prey protein and the LexA fusion protein (bait X) can be detected by leucine prototrophy and β-galactosidase activity. Positive interactions between a prey protein and the cI fusion protein (bait Y) can be detected by lysine prototrophy and β-glucuronidase activity.

Using SKY48/pLacGUS

In an interactor hunt, the strain SKY48/pLacGUS containing the bait plasmids is transformed with a prey library made in the vector pYESTrp, pYESTrp2, or pJG4-5. Expression of prey genes (fused to the B42 activation domain) in these vectors is controlled by the inducible *GAL1* promoter of yeast. Thus, expression of prey proteins can be induced by plating transformants on medium containing galactose (and raffinose, if desired). Yeast cells containing prey proteins that interact with the LexA fusion bait or the cI fusion bait will form colonies within 2 to 5 days on medium lacking leucine or lysine. Putative positive interactors may then be screened further by assaying for β-galactosidase or β-glucuronidase activity.

Small-Scale vs. Large-Scale Library Transformation

You may use a small-scale or a large-scale protocol to transform your prey library into the SKY48/pLacGUS bait strain. If this is the first time that you have performed library transformation in yeast, we recommend that you perform small-scale library transformation. The transformation efficiency of a small-scale protocol is generally lower than for large-scale transformation, but you will use fewer reagents. We have found that performing a small-scale library screen generally yields a sufficient number of positive interactors with a given bait. A protocol for small-scale library transformation is provided on the next page. A protocol for large-scale library transformation is provided in the **Appendix**, pages 65-68.

Selection of Library Transformants

Once you have transformed your prey library into the bait strain, you may use a one-step or two-step protocol to select for positive bait/prey interactions. We recommend using a two-step selection protocol as it allows the bait and library proteins to be expressed before selecting for an interaction.

Positive Controls

As mentioned previously, we recommend that you include the control plasmids in your experiment to help you to evaluate your library screening results. For more information about the control plasmids, pHybLex/Zeo-Fos2, pHybcI/HK-Krev, pYESTrp-Jun, or pYESTrp2-RalGDS, please refer to the **Appendix**, pages 58-61.

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Interactor Hunt, continued

Materials Needed

We suggest that you read the protocols through before beginning. Pay close attention to the number and type of plates required as well as the medium. Be sure to have the following materials and reagents on hand before starting.

A typical small-scale 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 30 µg library DNA and 1.5 mg of carrier DNA. Performing transformations in small aliquots helps reduce the likelihood of contamination.

- SKY48/pLacGUS + bait plasmids (bait strain)
- YC-UH Z200 medium and plates
- 30°C incubator and shaking incubator
- Centrifuge
- Sterile water
- 50 ml conical centrifuge tubes
- 1X LiAc/1X TE (see recipe on page 44)
- 1X LiAc/40% PEG-3350/1X TE (see recipe on page 45)
- Library DNA (30 µg)
- 1.5 mg carrier DNA (sheared salmon sperm or yeast tRNA)
- 1.5 ml sterile microcentrifuge tubes
- DMSO
- 42°C heat block
- 100 mm and/or 150 mm YC-UHW Z200 plates
- YC-UHW Z200 Gal/Raff liquid medium
- 150 mm YC-UHW Z200 Gal/Raff plates
- 150 mm YC-UHWL Z200 Gal/Raff plates
- 150 mm YC-UHWK Z200 Gal/Raff plates
- Sterile cell scraper
- Sterile TE buffer
- Glycerol solution (see recipe on page 46)

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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Small-Scale Library Transformation

1. Inoculate 20 ml of YC-UH Z200 with SKY48/pLacGUS containing your bait plasmids (pHybLex/Zeo and pHybcI/HK). Grow overnight at 30°C.
2. The next day, dilute culture into 300 ml YC-UH Z200 to 2 x 10^6 cells/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture reaches 2 x 10^7 cells/ml (OD_{600} = 1).
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 minutes at 1000 to 1500 x g. Decant supernatant and resuspend cells in 1.5 ml 1X LiAc/1X TE.
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 1X LiAC/40% PEG-3350/1X 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 **Two-Step Selection**.

Two-Step Selection

8. Take 28 of the 30 tubes from Step 7 and plate the complete contents of one tube per 150 mm YC-UHW Z200 plates and incubate at 30°C for 1 to 2 days.
9. For the two remaining tubes, plate 360 µl from each tube onto separate 150 mm YC-UHW 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-UHW Z200 plates. Incubate all plates 2 to 3 days at 30°C until colonies appear. Proceed to Step 10, next page.

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

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Collect Primary Transformants

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.

10. Cool all of the 150 mm plates containing transformants from Step 8 for several hours at +4°C to harden agar and dry the plates.
11. Wearing gloves and using a sterile cell scraper, 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.
12. Wash cells by resuspending the transferred cells into an equal volume of sterile TE buffer or water. Centrifuge at 1000 to 1500 x g for ~5 minutes at room temperature, and discard supernatant. Repeat wash.
13. 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 14, below.

Determine Replating Efficiency

14. Remove an aliquot of frozen transformed yeast (Step 13, above) and dilute 1:10 with YC-UHW Z200 Gal/Raff medium. Incubate with shaking for 4 hours at 30°C to induce the *GAL1* promoter to express the library.

    Note: Raffinose (Raff) is not required for growth, but it helps the cells to grow faster without diminishing transcription from the *GAL1* promoter.
15. Make serial dilutions of the culture using the YC-UHW Z200 Gal/Raff medium. Plate on 150 mm YC-UHW Z200 Gal/Raff plates and incubate 2 to 4 days at 30°C until colonies are visible.
16. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

    If the harvest is done carefully, viability will generally be greater than 90%. Some researchers perform this step simultaneously with plating out on leucine or lysine-deficient selective medium (Steps 17-18, next page).

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Interactor Hunt, continued

Screening for Interacting Proteins

Because not all cells that contain interacting proteins plate at 100% efficiency on leucine or lysine-deficient 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$^+$ or Lys$^+$ colonies using cells plated at ~10^6 cfu per 150 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 ~1 x 10^7 cells on a total of 10 selective plates.

17. Thaw the appropriate quantity of transformed yeast based on the plating efficiency (calculated on previous page), dilute 1:10 with YC-UHW Z200 Gal/Raff medium, and incubate as in Step 14.
18. Centrifuge at 1000 to 1500 x g for 5 minutes at room temperature and resuspend the pellet in 1 ml of YC-UHW Z200 Gal/Raff medium.
19. Plate 50 µl each on 10 YC-UHWL Z200 Gal/Raff plates and 10 YC-UHWK Z200 Gal/Raff plates. Incubate 2 to 3 days at 30°C until colonies appear.

    Carefully pick appropriate Leu$^+$ or Lys$^+$ colonies and patch on new YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff master plates. Incubate 2 to 7 days at 30°C until colonies appear.

RECOMMENDATION

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.

NOTE

Leu$^+$ and Lys$^+$ colonies grow poorly if the density on the plates is too high. The optimal plating volume depends on the recovery time of yeast in YC-UHW Z200 and on the transformation efficiency. You should expect to see from 20-100 colonies/plate depending on the efficiency of transformation.

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Interactor Hunt, continued

Analysis of LexA/Bait X Interactors

The Leu^{+} colonies that grow on YC-UHWL Z200 Gal/Raff selective plates represent colonies that were transformed with a prey plasmid that encodes a potential interactor with your LexA fusion protein. We recommend that you screen as many Leu^{+} colonies as possible for β-galactosidase activity.

1. Patch Leu^{+} colonies to two YC-UHWL Z200 Gal/Raff plates (e.g. a master plate and a duplicate plate for the β-galactosidase overlay assay) and one YC-UHWK Z200 Gal/Raff plate (to test for specificity of your interaction and to eliminate potential false positives). Arrange the colonies in a grid-like pattern using the grid provided on the next page.
2. Grow at 30°C for 1 to 2 days. Analyze the duplicate YC-UHWL Z200 Gal/Raff plate for β-galactosidase activity using the overlay assay (see page 24).

   If you have isolated genuine interactors, you should obtain several colonies that are Leu^{+} and exhibit β-galactosidase activity (LacZ^{+}). You are now ready to analyze your positive clones (see page 34-37).

Analysis of cI/Bait Y Interactors

The Lys^{+} colonies that grow on YC-UHWK Z200 Gal/Raff selective plates represent colonies that were transformed with a prey plasmid that encodes a potential interactor with your cI fusion protein. We recommend that you screen as many Lys^{+} colonies as possible for β-glucuronidase activity.

1. Patch Lys^{+} colonies to two YC-UHWK Z200 Gal/Raff plates (e.g. a master plate and a duplicate plate for the β-glucuronidase overlay assay) and one YC-UHWL Z200 Gal/Raff plate (to test for specificity of your interaction and to eliminate potential false positives). Arrange the colonies in a grid-like pattern using the grid provided on the next page.
2. Grow at 30°C for 1 to 2 days. Analyze the duplicate YC-UHWK Z200 Gal/Raff plate for β-glucuronidase activity using the overlay assay (see page 24).

   If you have isolated genuine interactors, you should obtain several colonies that are Lys^{+} and exhibit β-glucuronidase activity (GUS^{+}). You are now ready to analyze your positive clones (see page 34-37).

NOTE

If your two bait proteins are not related, then you should be able to eliminate false positives and identify specific interactors for each bait protein. For example, prey proteins that specifically interact with the LexA bait protein will grow on YC-UHWL Z200 Gal/Raff plates. If the Leu^{+} colonies are patched to a YC-UHWK Z200 Gal/Raff plate, then true interactors should not grow in medium deficient in lysine. Those colonies that are also Lys^{+} are most likely false positives.

However, if your two bait proteins are closely related, then the same prey may interact with both bait proteins. In this case, Leu^{+} colonies might also be Lys^{+}.

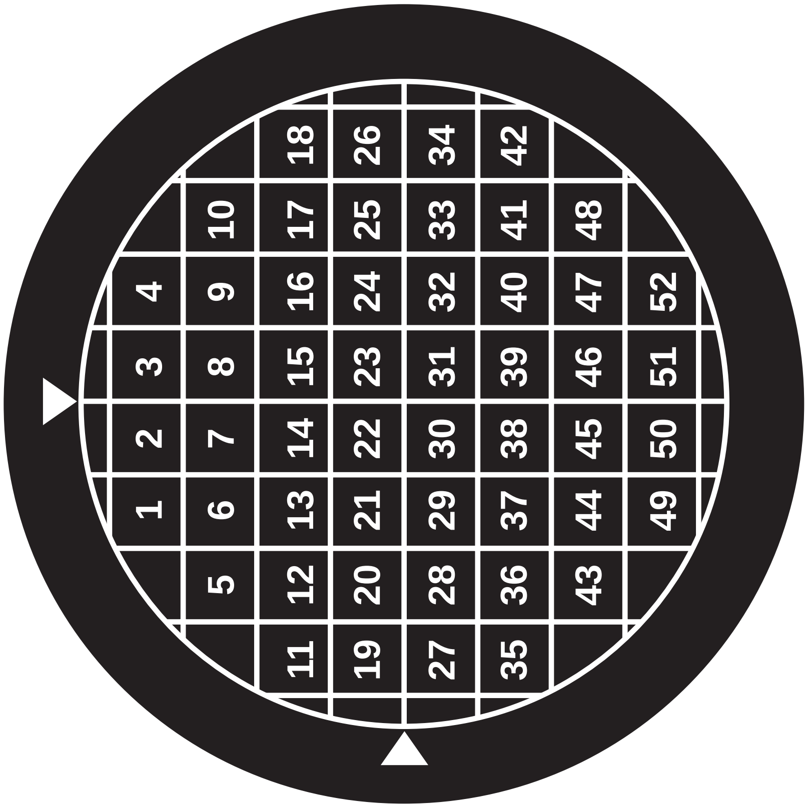
Sensitivity of LexA vs. cI-based Reporters

Serebriiskii *et al.* (1999) have demonstrated that cI and LexA-based reporter genes exhibit a similar sensitivity range to transcriptional activation as a result of positive bait/prey interactions. This allows the Dual Bait Hybrid Hunter™ System to be used to compare the specificity of an interaction between a prey and two related bait proteins.

continued on next page

Colony Grid Pattern

The grid below is provided for your convenience. Other grids are suitable.



Retrieving Putative Interactors

Introduction

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

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

We routinely use PCR to amplify the prey gene of interest and TOPO® Cloning to clone the gene into a PCR cloning vector. The other methods allow you to obtain the original prey plasmid and use it for additional analyses, but the quality and quantity of DNA isolated from yeast is poor. The DNA must pass through *E. coli* to generate sufficient plasmid for additional characterizations. Protocols for each of these methods are provided below.

PCR Cloning of Interactor

PCR cloning is the recommended method of choice for quick retrieval of interactors. You can perform PCR directly from the yeast colony to determine the size of the insert in pYESTrp2, and obtain the cDNA insert by cloning the PCR product into a PCR cloning vector. You will then have a source of DNA for further analyses.

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

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

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

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

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	10 minutes	1X
Denaturation	94°C	1 minute	25X
Annealing	56°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	1X

4. Analyze 10 µl of the amplified DNA on a 1% agarose gel.
5. Ligate 2 µl into a TOPO TA Cloning® vector or other PCR cloning vector. Sequence your clone using Universal M13 primers. For efficient PCR cloning and sequencing of *Taq*-amplified PCR products, the TOPO TA Cloning® Kit for Sequencing (Catalog no. K4575-01) is available from Invitrogen. Call Technical Service for details (page 70).

continued on next page

Isolation of Plasmid DNA from Yeast

Plasmid DNA can be extracted from double positive yeast colonies (Leu$^+$ LacZ$^+$ or Lys$^+$ GUS$^+$) 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. For an alternative protocol, please see the next page. Be sure you have the following reagents and equipment on hand before starting.

Materials Required:

YC-W medium (see recipe on page 43)
30°C shaking incubator
Clinical centrifuge
Yeast Lysis buffer (see recipe on page 46)
Acid washed glass beads (Sigma G-8772, 425-600 microns)
Phenol/Chloroform
80% and 100% Ethanol
1X TE (see recipe on page 44)

Protocol:

1. Inoculate 5 ml of YC-W with a single double positive colony 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 1X TE. Proceed to transformation (see next page).
9. Transform competent *E. coli* with 5 µl of the DNA suspension and plate out the whole transformation on LB plates containing 50 µg/ml ampicillin.
10. Select 10-20 ampicillin-resistant transformants and isolate plasmid DNA. Analyze by agarose gel electrophoresis. Each transformant should only contain one plasmid. Identify those plasmids with a size corresponding to the pYESTrp2 prey plasmid (5.8 kb plus the size of your insert).

    Note: Although each transformant will contain only one plasmid, some will contain one of the bait plasmids or the reporter plasmid.
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 Forward and Reverse primers) to identify the interactor.

continued on next page

Isolation of Plasmid DNA from Yeast Using the S.N.A.P.™ MiniPrep Kit

We have successfully used the S.N.A.P.™ MiniPrep Kit available from Invitrogen (Catalog no. K1900-01) to isolate plasmid DNA from yeast. The yield and quality of plasmid DNA is generally higher than that obtained using the lysis method detailed on the previous page. A protocol using the S.N.A.P.™ MiniPrep Kit to isolate yeast plasmid DNA is provided below.

Materials Needed:

S.N.A.P.™ MiniPrep Kit (includes Resuspension Buffer, RNase A, Precipitation Salts, Binding Buffer, Wash Buffer)
1X TE
β-mercaptoethanol
3 mg/ml Zymolyase in water (Seikagaku, Catalog no. 120493-1)
1% SDS

Protocol:

1. Inoculate 5 ml of YC-W with a single double positive colony and incubate overnight at 30°C with shaking. The culture should be in stationary phase (OD_{600} = 1-2) before proceeding further.
2. Pellet cells at 2500 rpm for 5 minutes in a clinical centrifuge.
3. Resuspend the cell pellet in 1 ml of 1X TE and re-pellet cells.
4. Resuspend the cell pellet in 1 ml of 1X TE. Add 1 µl of β-mercaptoethanol and 1.5 µl of zymolyase. Incubate at 30°C for 1 hour.
5. Centrifuge at 1000 x g for 4 minutes at room temperature to gently pellet the cells.
6. Remove the supernatant and resuspend the cell pellet in 150 µl of Resuspension Buffer containing RNase A.
7. Add 150 µl of 1% SDS. Mix gently by inversion. Incubate at 65°C for 10 minutes.
8. Place on ice for 3 minutes.
9. Add 150 µl of ice cold Precipitation Salts. Mix by inverting.
10. Centrifuge at 14,000 x g for 10 minutes.
11. Remove supernatant to a new microcentrifuge tube and add 600 µl of Binding Buffer. Mix by inverting 5-6 times. Apply the entire solution onto the S.N.A.P.™ MiniPrep Column/Collection Tube.
12. Centrifuge the S.N.A.P.™ MiniPrep Column/Collection Tube at room temperature at 1000-3000 x g for 30 seconds. Discard the column flow-through.
13. Add 900 µl of Wash Buffer. Centrifuge as in Step 12.
14. Discard the column flow-through. Centrifuge the S.N.A.P.™ MiniPrep Column/ Collection Tube at room temperature for 2 minutes at maximum speed to dry the resin.
15. To elute the plasmid DNA, place the S.N.A.P.™ MiniPrep Column into a new sterile microcentrifuge tube and add 70 µl of 1X TE or sterile water directly to the resin.
16. Incubate for 2 minutes at room temperature.
17. Centrifuge the S.N.A.P.™ MiniPrep Column/Collection Tube at room temperature for 2 minutes at maximum speed. The plasmid DNA is now eluted from the column. Remove and discard the column. Proceed to transformation.
18. Transform competent *E. coli* with 10 µl of the DNA suspension and plate out the whole transformation on LB plates containing 50 to 100 µg/ml ampicillin.
19. Isolate plasmid DNA from *E. coli* transformants and analyze as described in Steps 10-12 of **Isolation of Plasmid DNA from Yeast**, previous page.

continued on next page

Plasmid Segregation

To eliminate bait plasmids prior to plasmid rescue, you can let yeast do the work by plating on selective medium. Prepare the following reagents:

YC-W medium and plates
YC-HW Z200 plates

1. For each double positive colony, inoculate 5 ml of YC-W with a Leu^+ LacZ^+ **or** Lys^+ GUS^+ transformant. Grow for 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-HW Z200 and then to YC-W plates.
5. Incubate 1 to 2 days at 30˚C.
6. Identify colonies that are sensitive to histidine and Zeocin™ (i.e., colonies that grow on YC-W but not on YC-HW Z200). These colonies have segregated the bait plasmids.
7. Isolate plasmid DNA from yeast using the protocol on the previous page, transform into *E. coli,* isolate plasmid DNA, and sequence.

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:

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 SKY48/pLacGUS is Ura$^+$ His$^-$, Trp$^-$, Leu$^-$, Lys$^-$. This strain is unable to grow in medium deficient in histidine, tryptophan, leucine and lysine. Confirm the phenotype of this strain by streaking on YPD (or YC), YC-U, YC-H, YC-W, YC-L, and YC-K. SKY48/pLacGUS should only grow on YPD or YC-U.

Testing Reporter Function

Two sets of control plasmids, pHybLex/Zeo-Fos2 and pYESTrp-Jun, and pHybcI/HK-Krev and pYESTrp2-RalGDS, are provided to test reporter function. Transform the four plasmids into SKY48/pLacGUS and select on YC-UHW Z200. To assay for reporter function, plate the cells on the appropriate selective media as listed below.

For more information about the control plasmids, please refer to pages 58-61 in the **Appendix**.

Control Vectors	Reporter	Selective Medium
pHybLex/Zeo-Fos2 pYESTrp-Jun	*LEU2* *lacZ*	YC-UHWL Z200 Gal/Raff
pHybcI/HK-Krev pYESTrp2-RalGDS	*LYS2* *gusA*	YC-UHWK Z200 Gal/Raff

Troubleshooting the Bait Plasmids

Transformation of the bait plasmids alone results in a Leu$^+$ LacZ$^+$ (for the pHybLex/Zeo construct) or a Lys$^+$ GUS$^+$ (for the pHybcI/HK construct) 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 or cI.** You may have to truncate the proteins of interest or use specific domains to avoid activating transcription when there are no interactors present. Identifying bait fusion constructs that do not exhibit non-specific activation will require less effort than analyzing clones arising from a screen with a high background of false positives.
- **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 plasmids fail to grow. If SKY48/pLacGUS fails to grow or grows poorly on selective medium after transformation with the bait plasmids, then one or both of the baits may be toxic to the cell. Truncating one or both of the proteins of interest may alleviate toxicity.

Unusually large or small colonies, rapid growth, or strange colony morphology. If you observe any of these three phenotypes with the bait strain in comparison to a strain containing the empty bait vector or the control vector, it is probably due to bait over-expression in the nucleus. In most cases, you should proceed with the experiment. Unexpected behavior may give some clues as to function, particularly if working with unknown proteins.

continued on next page

Troubleshooting the Library Screen

Very few leucine or lysine prototrophs. Make sure your transformation is working by performing a 2-step selection. Use the small-scale transformation protocol described on pages 28-31 and select on YC-UHW Z200 medium (see page 43) before screening for leucine or lysine prototrophy.

Excessive background growth on library screening medium. Check to make sure you used the correct selective medium for screening. You should be using YC-UHWL Z200 Gal/Raff medium if you are screening for interactors with the pHybLex/Zeo bait protein and YC-UHWK Z200 Gal/Raff medium if you are screening for interactors for the pHybcI/HK bait protein. The bait strain lacking the prey library should not exhibit any growth on either selective medium.

Low transformation efficiency. The transformation efficiency should be between 10^3 and 10^4 cfu/µg for a large-scale library transformation. This number is determined in Step 18 of the **Large-Scale Library Transformation** protocol on page 67. To improve the transformation efficiency, we recommend that you:

- Use clean plasmid DNA. Ethanol precipitate the DNA to ensure a clean preparation.
- Use sufficient carrier DNA.
- Perform a small-scale transformation first before performing your large-scale library transformation. Performing a small-scale transformation first will allow you to conserve reagents and evaluate your results.
- Plate transformants on YC-UHW Z200 first, then replica-plate to YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff medium to select for leucine or lysine prototrophs.

False Negative Results

False negative results occur when there is a failure to detect interactions between two proteins that normally interact *in 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 Plasmids**, page 38).
- 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 are 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

Technical Assistance, continued

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 pages 35-37, then you can perform the following retransformations into SKY48/pLacGUS to test for non-specific and specific interactions between your bait and prey.

Transformation	Selective Medium	Testing	What You Should See
pHybLex/Zeo bait alone or pHybcI/HK bait alone	YC-UL Z200 or YC-UHK	Non-specific activation by bait	No growth on selective medium.
pYESTrp2 prey plasmid alone	YC-UW Gal/Raff	Non-specific activation by prey	No growth on selective medium.
pYESTrp2 prey plasmid with pHybLex/Zeo or pHybcI/HK	YC-UWL Z200 Gal/Raff or YC-UHWK Gal/Raff	Non-specific binding of prey to LexA DBD or cI DBD	No growth on selective medium.
pYESTrp2 prey plasmid with pHybLex/Zeo bait construct **or** pHybcI/HK bait construct	YC-UWL Z200 Gal/Raff or YC-UHWK Gal/Raff	Specific interaction between one bait and prey	Growth on one selective medium, but not the other
pYESTrp2 prey plasmid with pHybLex/Zeo bait construct **and** pHybcI/HK bait construct	YC-UHWL Z200 Gal/Raff and YC-UHWK Z200 Gal/Raff	Specific interaction between one bait and prey and to rule out non-specific interactions	Growth on one selective medium, but not the other. If the two baits are closely related, you may see growth on both selective media.
pYESTrp2 prey plasmid with pHybLex/Zeo bait construct **and** pHybcI/HK-Krev control bait	YC-UHWLK Z200 Gal/Raff	Specific interaction between bait and prey	No growth on selective medium. If your bait protein is related to Krev or Fos, your prey may interact with the control bait and you will see growth on selective medium.
pYESTrp2 prey plasmid with pHybcI/HK bait construct **and** pHybLex/Zeo-Fos2 control bait			

Other analyses may be performed to test for false positives and the stringency of the interaction between your bait and prey, but will involve additional cloning steps. Other possible tests are listed below:

- Clone your bait protein into both pHybLex/Zeo and pHybcI/HK. After transformation with the pYESTrp2 library plasmid, select for leucine and lysine prototrophy and β-galactosidase and β-glucuronidase activity. True positives should exhibit activation of all four reporters.
- Switch the bait and prey proteins by cloning the bait protein into the pYESTrp2 plasmid and the prey protein into the pHybLex/Zeo or pHybcI/HK plasmid. After transformation and selection, true postives should still exhibit leucine or lysine prototrophy and β-galactosidase or β-glucuronidase activity.
- If you have two related bait proteins (i.e. wild-type and mutant forms), you may clone the baits into pHybLex/Zeo and pHybcI/HK and test for the specificity of the interaction between your prey and each bait protein by assaying for leucine and lysine prototrophy and β-galactosidase and β-glucuronidase activity.

Resources for Two-Hybrid Technology

Introduction

The Dual Bait Hybrid Hunter™ Two-Hybrid System is a modified version of the two-hybrid (interactive trap) system. You may find that you need additional resources about the two-hybrid technology. A number of resources are listed below ranging from informative web sites to additional applications to review articles.

Web Sites

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

Roger Brent Laboratory: http://xanadu.mgh.harvard.edu/
Erica Golemis Laboratory: http://www.fccc.edu:80/research/labs/golemis/

Review Articles

Selected reviews are provided 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.

Fields, S., and Sternglanz, R. (1994). The Two-Hybrid System: An Assay For Protein-Protein Interactions. Trends Genet. *10*, 286-292.

Golemis, E. A., Gyuris, J., and Brent, R. (1996) Interaction Trap/Two-Hybrid System to Identify Interacting Proteins. In *Current Protocols in Molecular Biology*, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, eds. (New York: Greene Publishing Associates and Wiley-Interscience), pp. 20.1.1.-20.1.28.

Golemis, E.A., and Serebriiskii, I. (1998) Two-Hybrid Systems/Interaction Trap. In *Cells: A Laboratory Manual*, D.L. Spector, R. Goldman, and L. Leinwand, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Golemis, E.A., Serebriiskii, I., Finley, R.L., Jr., Kolonin, M.G., Gyuris, J., and Brent, R. (1998) Interaction Trap/Two-Hybrid System to Identify Interacting Proteins. In *Current Protocols in Protein Science*, J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P.T. Wingfield, eds. (New York: John Wiley), pp. 19.2.1-19.2.40.

Guarente, L. (1983). Strategies for the Identification of Interacting Proteins. Proc. Natl. Acad. Sci. USA *90*, 1639-1641.

Luban, J., and Goff, S. P. (1995). The Yeast Two-Hybrid System for Studying Protein-Protein Interactions. Curr. Opinion Biotechnol. *6*, 59-64.

Two-Hybrid Affinity Data

A 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 correlate linearly with affinity measured *in vitro*.

Appendix

Recipes

Media Table

The table below describes the different media used to characterize the bait plasmids and prey plasmid in SKY48/pLacGUS.

Medium	Use with.....	Selectable Marker	Purpose
YPD	SKY48/pLacGUS	None	Complex medium for general, non-selective growth.
YC-U	SKY48/pLacGUS	*URA3*	Minimal defined medium for selection of pLacGUS. Does not contain uracil.
YC-UH	SKY48/pLacGUS + pHybcI/HK	*URA3* *HIS3*	Selection of pLacGUS and pHybcI/HK. Does not contain uracil or histidine.
YC-UH Z200	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo	*URA3* *HIS3* Zeocin™	Selection of pLacGUS, pHybcI/HK, and pHybLex/Zeo. Does not contain uracil or histidine. Contains Zeocin™.
YC-UHW Z200	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo + pYESTrp2	*URA3* *HIS3* Zeocin™ *TRP1*	Selection of pLacGUS, pHybcI/HK, pHybLex/Zeo, and pYESTrp2. Does not contain uracil, histidine, or tryptophan. Contains Zeocin™.
YC-UHL Z200	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo	*URA3* *HIS3* Zeocin™ *LEU2*	Testing activation by LexA bait. Does not contain uracil, histidine, or leucine. Contains Zeocin™.
YC-UHK Z200	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo + pYESTrp2	*URA3* *HIS3* Zeocin™ *TRP1* *LYS2*	Testing activation by cI bait. Does not contain uracil, histidine, or lysine. Contains Zeocin™.
YC-UHWL Z200 Gal/Raff	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo + pYESTrp2	*URA3* *HIS3* Zeocin™ *TRP1* *LEU2*	Selection of Leu$^+$ transformants. Does not contain uracil, histidine, tryptophan, or leucine. Contains Zeocin™, galactose, and raffinose.
YC-UHWK Z200 Gal/Raff	SKY48/pLacGUS + pHybcI/HK + pHybLex/Zeo + pYESTrp2	*URA3* *HIS3* Zeocin™ *TRP1* *LYS2*	Selection of Lys$^+$ transformants. Does not contain uracil, histidine, tryptophan, or lysine. Contains Zeocin™, galactose, and raffinose.

continued on next page

Recipes, continued

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 that they need.

	0.1 g each	**0.05 g each**
1.2 g Yeast Nitrogen Base	adenine	aspartic acid
5 g Ammonium sulfate	arginine	histidine (H)
10 g Succinic acid	cysteine	isoleucine
6 g NaOH	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 200 µg/ml (2 ml per liter).

   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 Dual Bait Hybrid Hunter™ System. Other recipes may be suitable, but should be tested with the host strain, plasmid markers, and reporters.

Recipes, continued

YPD
± Zeocin™

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (D-glucose)
± 200 µg/ml Zeocin™

1. Dissolve the following in 900 ml of water:
   10 g yeast extract
   20 g of peptone
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 2.0 ml of 100 mg/ml Zeocin™ just prior to use.

Store medium at room temperature. Store medium containing Zeocin™ at room temperature protected from exposure to light. 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.

1X LiAc/1X TE

100 mM Lithium Acetate, pH 7.5
10 mM Tris, pH 7.5
1 mM EDTA

1. For 100 ml, mix together 10 ml of 10X LiAc and 10 ml of 10X TE. Add deionized water to 100 ml.
2. Filter-sterilize and store at room temperature.

continued on next page

Recipes, continued

1X LiAc/0.5X TE

100 mM Lithium Acetate, pH 7.5
5 mM Tris, pH 7.5
0.5 mM EDTA

1. For 100 ml, mix together 10 ml of 10X LiAc and 5 ml of 10X TE.
2. Add deionized water to 100 ml.
3. Filter-sterilize and store at room temperature.

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.

X-Gal

X-Gal
Dimethylformamide (DMF)

1. Prepare X-Gal solution fresh immediately before use.
2. To make a 25 mg/ml stock solution, dissolve 25 mg in 1 ml DMF.
3. Store at -20°C protected from exposure to light until use.

continued on next page

Recipes, continued

X-Gluc

5-bromo-4-chloro-3-indolyl-β-D-gluruonic acid (X-Gluc)
Dimethylformamide (DMF)

1. Prepare X-Gluc solution fresh immediately before use.
2. To make a 25 mg/ml stock solution, dissolve 10 mg in 0.4 ml DMF. Vortex for 2 minutes to solubilize.
3. Store at -20°C protected from exposure to light until use.

100 mM Potassium Phosphate, pH 7.0

Before beginning, have the following reagents on hand.

Potassium phosphate, monobasic (KH_2PO_4; Sigma P5379)
Potassium phosphate, dibasic (K_2HPO_4; Sigma P3786)

1. Prepare 100 ml of 0.1 M KH_2PO_4 by dissolving 1.36 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
2. Prepare 100 ml of 0.1 M K_2HPO_4 by dissolving 1.74 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
3. For 100 ml of 100 mM potassium phosphate, pH 7.0, mix together 39 ml of 0.1 M KH_2PO_4 and 61 ml of 0.1 M K_2HPO_4.
4. Filter-sterilize and store at room temperature.

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.

Zeocin™

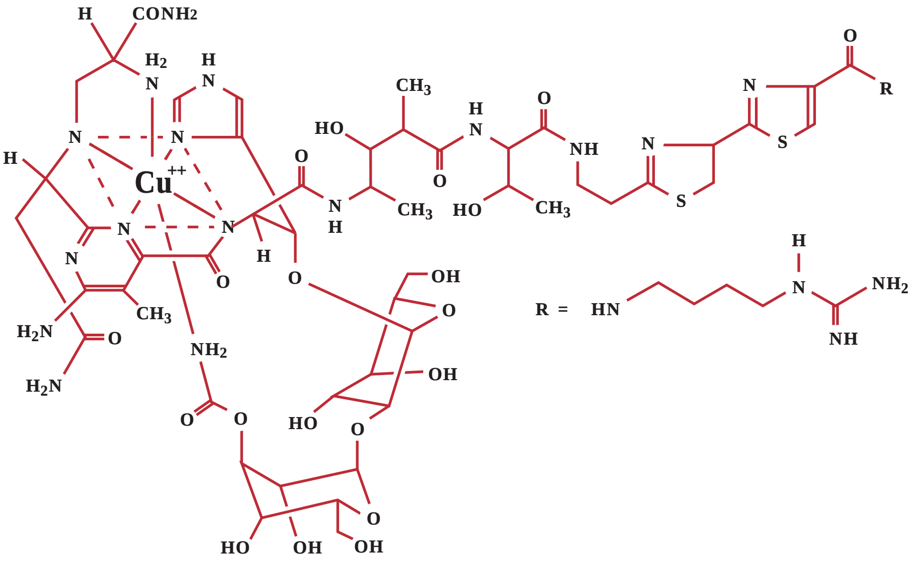
Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in the SKY48/pLacGUS yeast strain and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25 µg/ml in **Low Salt LB** medium* (see page 49 for a recipe)
Saccharomyces cerevisiae (SKY48/pLacGUS)	200 µg/ml in YPD or other selective medium

*Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).

continued on next page

Zeocin™, continued

Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see **Low Salt LB Medium,** next page).
- Zeocin™ is fully active when used in YPD or YC yeast medium as defined in the recipes on pages 43-44. No pH adjustment is necessary.
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark at +4°C. Medium containing Zeocin™ may be stored for up to one month.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Preparing and Storing Zeocin™

Zeocin™ is included in the Dual Bait Hybrid Hunter™ System, but may also be obtained separately from Invitrogen (see page v for ordering information). For your convenience, the drug is prepared in autoclaved, deionized water in 1.25 ml aliquots at a concentration of 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C.

Zeocin™ Selection of *E. coli* Transformants

Introduction

The pHybLex/Zeo and pHybLex/Zeo-Fos2 plasmids contain the Zeocin™ resistance gene for selection of transformants in *E. coli* and in yeast. When selecting for transformants in *E. coli*, please note that for maximal activity of Zeocin™, the salt concentration of LB medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt 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

Any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5αF´IQ, SURE, SURE2) encodes the *ble* (bleomycin resistance) gene. These strains will confer resistance to Zeocin™. We recommend that you choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10, TOP10F´).

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 (Catalog no. Q100-01) or in bulk (Catalog no. Q100-02). Alternatively, imMedia™ Zeo Liquid medium (Catalog no. Q620-20) is available from Invitrogen. Please contact Technical Service (see page 70) for more information.

Transformation

Transform pHybLex/Zeo containing your insert into TOP10 or TOP10F´ (or another appropriate *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 16).

NOTE

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

Dual Bait Control Transformations

Introduction

The Dual Bait Hybrid Hunter™ System supplies four control plasmids to allow detection of bait/prey interactions using LexA and cI fusion bait proteins. We recommend including the control plasmids in your experiment to help you to evaluate your results. Performing the control transformations involves introducing and assaying for positive and negative bait/prey interactions between varying sets of bait and prey plasmids in SKY48/pLacGUS.

Control Plasmids

The control plasmids included in the Dual Bait Hybrid Hunter™ System are pHybLex/Zeo-Fos2, pHybcI/HK-Krev, pYESTrp-Jun, and pYESTrp2-RalGDS. A map of each vector may be found in the **Appendix**, pages 58-61. The LexA-Fos bait protein expressed from pHybLex/Zeo-Fos2 interacts with the B42-Jun prey protein expressed from pYESTrp-Jun, while the cI-Krev bait protein expressed from pHybcI/HK-Krev interacts with the B42-RalGDS prey protein expressed from pYESTrp2-RalGDS. A positive Fos-Jun interaction is detected by growth of transformants in leucine-deficient medium and β-galactosidase activity. A positive Krev-RalGDS interaction is detected by growth of transformants in lysine-deficient medium and β-glucuronidase activity. For more information about the Fos-Jun and Krev-RalGDS interactions, see below and the next page.

Control Transformations

When performing your interactor hunt, we recommend that you include the following set of control transformations in parallel. Successful transformation of the control plasmids will allow you to detect the phenotypes listed below:

Bait Plasmid	Prey Plasmid	Phenotype	Reporter Activity
pHybLex/Zeo-Fos2	pYESTrp-Jun	Ura$^+$, Trp$^+$, Zeo^R, Leu$^+$	LacZ$^+$
pHybcI/HK-Krev	pYESTrp2-RalGDS	Ura$^+$, Trp$^+$, His$^+$, Lys$^+$	GUS$^+$
pHybLex/Zeo-Fos2	pYESTrp2-RalGDS	Ura$^+$, Trp$^+$, Zeo^R, Leu$^-$, Lys$^-$	LacZ$^-$, GUS$^-$
pHybcI/HK-Krev	pYESTrp-Jun	Ura$^+$, Trp$^+$, His$^+$, Leu$^-$, Lys$^-$	LacZ$^-$, GUS$^-$
pHybLex/Zeo-Fos2 pHybcI/HK-Krev	pYESTrp-Jun pYESTrp2-RalGDS	Ura$^+$, Trp$^+$, His$^+$, Zeo^R, Leu$^+$, Lys$^+$	LacZ$^+$, GUS$^+$

A Brief Note about the Fos-Jun Interaction

Fos and Jun are DNA binding proteins that were originally identified for their ability to act as nuclear oncoproteins (Sassone-Corsi *et al.*, 1988), and were subsequently identified as transcription factors. The Fos and Jun proteins have been shown to interact to form a heterodimer via a structure known as the leucine zipper (Gentz *et al.*, 1989; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988). The portions of Fos and Jun that are found in the pHybLex/Zeo-Fos2 bait plasmid and the pYESTrp-Jun prey plasmid comprise the region that forms the leucine zipper. When expressed from the two plasmids, the LexA-Fos and B42-Jun fusion proteins interact to activate expression of the *LEU2* and *lacZ* reporter genes.

continued on next page

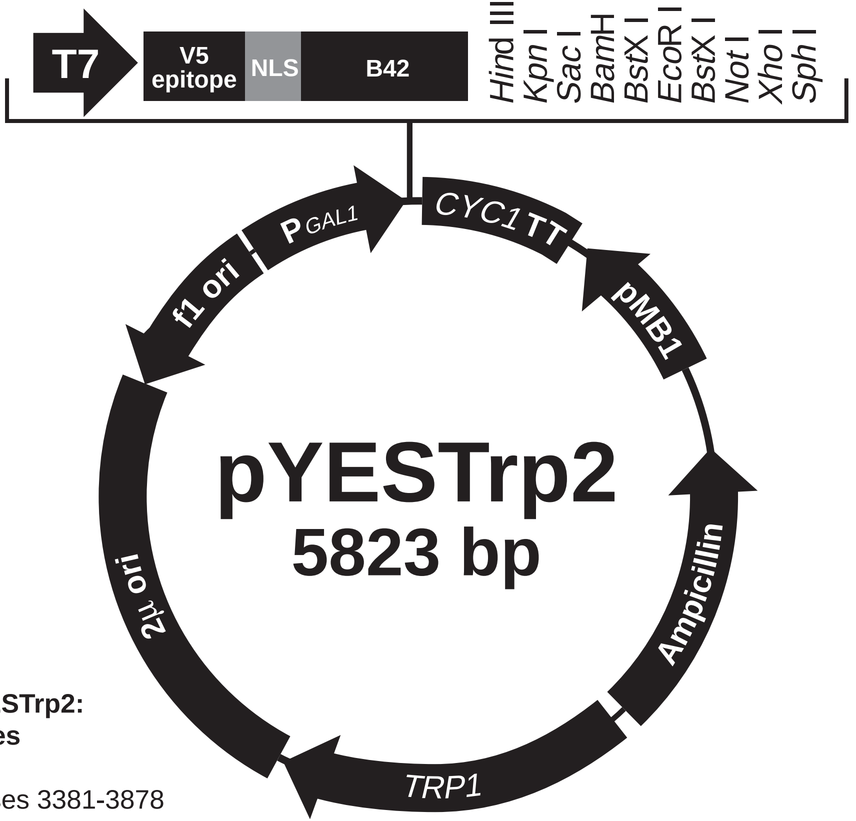
A Brief Note about the Krev-RalGDS Interaction

Krev1 (also known as Rap1A) is a member of the *ras* gene family and has been shown to possess a high degree of structural homology to the H-ras protein (Kitayama *et al.*, 1989). The Ral guanine dissociation stimulator protein (RalGDS) was originally identified in a yeast two-hybrid screen as an interactor with the H-ras protein (Hofer *et al.*, 1994). The interaction of RalGDS with H-ras occurs through a region of the protein known as the Ras-binding domain. Subsequent studies have shown that RalGDS is also able to interact with Krev1 via the Ras-binding domain (Herrmann *et al.*, 1996; Serebriiskii *et al.*, 1999). The DNA fragment encoding the mature Krev1 peptide is found in the pHybcI/HK-Krev control plasmid while a fragment encoding the Ras-binding domain of RalGDS is found in the pYESTrp2-RalGDS plasmid. When expressed from the two plasmids, the cI-Krev and B42-RalGDS fusion proteins interact to activate expression of the *LYS2* and *gusA* reporter genes.

pYESTrp2 Vector

Map of pYESTrp2

The figure below summarizes the features of the pYESTrp2 vector. **The complete nucleotide sequence for pYESTrp2 is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 70).**



Comments for pYESTrp2:
5823 nucleotides

GAL1 promoter: bases 3381-3878
T7 promoter/priming site: bases 3902-3921
Initiation ATG: bases 3937-3939
V5 epitope: bases 3940-3981
Nuclear localization signal (NLS): bases 3997-4023
B42 activation domain: bases 4027-4264
pYESTrp Forward priming site: bases 4228-4246
Multiple cloning site: bases 4270-4363
pYESTrp Reverse priming site: bases 4395-4413
CYC1 transcription termination region: bases 4378-4626
pMB1 (pUC-derived) origin: bases 4808-5481 (complementary strand)
bla promoter: bases 664-762 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5626-663 (complementary strand)
TRP1 promoter: bases 871-972
TRP1 gene: bases 973-1647
2μ origin: bases 2051-2885
f1 origin: bases 2954-3326 (complementary strand)

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Features of pYESTrp2

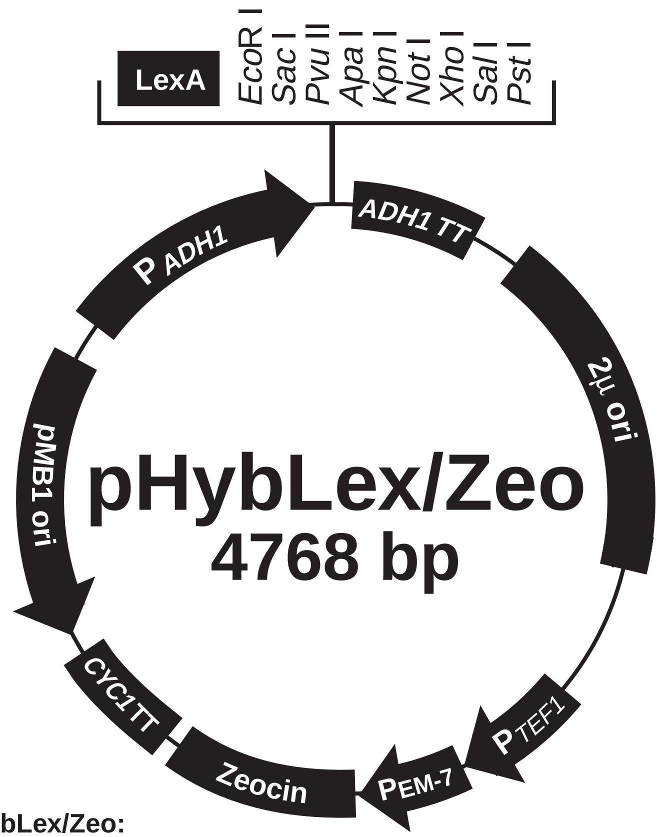
pYESTrp2 is a 5823 bp prey vector that can be used to make two-hybrid cDNA libraries or to clone genes encoding known proteins. The table below describes the features of pYESTrp2. All elements have been functionally tested.

Feature	Benefit
GAL1 promoter	Expression of genes cloned into pYESTrp2. Expression is inducible in SKY48/pLacGUS
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 and cI fusions
B42 activation domain (AD) ORF	Transcriptional activation domain that allows expression of reporter genes when brought into proximity with the LexA DNA binding domain (DBD) or cI DBD by two interacting proteins (Ma and Ptashne, 1987)
pYESTrp Forward priming site	Allows sequencing through the insert
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
pYESTrp Reverse priming site	Allows sequencing through the insert
CYC1 transcription termination signal	Permits efficient termination and stabilization of mRNA
pMB1 (pUC-derived) origin	Maintenance and high-copy replication in *E. coli*
bla promoter	Allows expression of the *bla* resistance gene
Ampicillin (*bla*) resistance gene	Selection of transformants in *E. coli*
TRP1 promoter	Allows expression of the *TRP1* gene
TRP1 gene	Auxotrophic selection of the plasmid in Trp^- yeast hosts (e.g. SKY48/pLacGUS) (Tschumper and Carbon, 1980)
2µ origin	Maintenance and high-copy replication in yeast
f1 origin	Rescue of single-stranded DNA

pHybLex/Zeo Vector

Map of pHybLex/Zeo

The figure below summarizes the features of the pHybLex/Zeo vector. **The complete nucleotide sequence for pHybLex/Zeo is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 70).**



Comments for pHybLex/Zeo:
4768 nucleotides

ADH1 promoter: bases 1-399
LexA ORF: 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
ADH1 transcription termination region: bases 1144-1301
2µ origin: 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
pMB1 (pUC-derived) origin: bases 4041-4714 (complementary strand)

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Features of pHybLex/Zeo

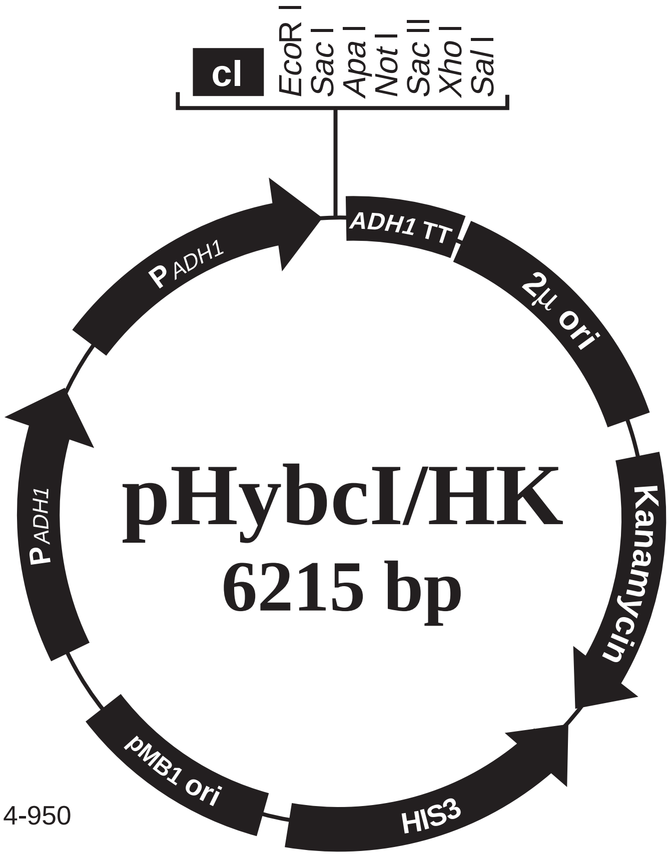
pHybLex/Zeo is a 4768 bp vector that expresses your bait protein as a fusion to the LexA DNA binding domain. The table below summarizes the features of pHybLex/Zeo. All features have been functionally tested.

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)
pHybLex/Zeo Forward priming site	Allows sequencing of the insert
Multiple cloning site with 9 unique restriction sites	Allows insertion of your gene into the expression vector
ADH1 transcription termination (TT)	Provides efficient transcription termination and stabilization of the mRNA
pHybLex/Zeo Reverse priming site	Allows sequencing of the insert
2µ 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) to allow selection of Zeocin™-resistant transformants in *E. coli* and yeast
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
pMB1 (pUC-derived) origin	Allows high-copy replication and maintenance of the plasmid in *E. coli*

pHybcI/HK Vector

Map of pHybcI/HK

pHybcI/HK is a 6215 bp vector that allows expression of a protein of interest as a fusion to the bacteriophage lambda cI repressor. The figure below summarizes the features of the pHybcI/HK vector. **The complete nucleotide sequence for pHybcI/HK is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).**



Comments for pHybcI/HK
6215 nucleotides

ADH1 promoter (2 copies): bases 4-950
cI repressor: bases 966-1676
cI Forward priming site: bases 1556-1573
Multiple cloning site: bases 1683-1735
ADH1 transcription termination signal: bases 1795-1952
pHybLex/Zeo Reverse priming site: bases 1812-1836
2μ origin: bases 2082-2959
Kanamycin promoter: bases 3502-3531
Kanamycin resistance gene: bases 3531-4325
HIS3 promoter: bases 5201-5397 (complementary strand)
HIS3 gene: bases 4538-5200 (complementary strand)
pMB1 (pUC-derived) origin: bases 5487-6160 (complementary strand)

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Features of pHybcI/HK

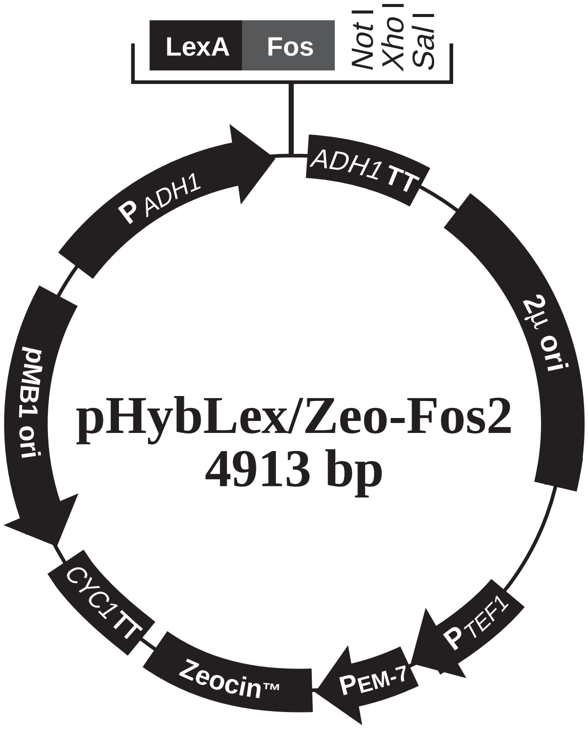
The table below describes the relevant features of the pHybcI/HK vector. All features have been functionally tested and the vector fully sequenced.

Feature	Purpose
Alcohol dehydrogenase (*ADH1*) promoter (2 copies)	Strong, constitutive promoter for expression of lambda cI fusions
cI repressor ORF (DNA binding protein)	Complete bacteriophage lambda *cI* repressor protein (710 bp) (Nilsson *et al.*, 1983) for generation of bait fusion proteins
cI Forward priming site	Permits sequencing of the insert
Multiple cloning site with 7 unique restriction sites	Allows insertion of your gene into the expression vector
ADH1 transcription termination (TT)	Permits efficient transcription termination and stabilization of the mRNA
pHybLex/Zeo Reverse priming site	Permits sequencing of the insert
2μ origin	Maintenance and high-copy replication in yeast
Kanamycin promoter	Allows expression of the kanamycin resistance gene
Kanamycin resistance gene	Selection of transformants in *E. coli*
HIS3 promoter	Allows expression of the *HIS3* gene (Struhl, 1982)
HIS3 gene	Selection of yeast transformants in His$^-$ yeast hosts (Struhl, 1985)
pMB1 (pUC-derived) origin	Maintenance and high-copy replication in *E. coli*

pHybLex/Zeo-Fos 2 Vector

Map of pHybLex/Zeo-Fos2

The figure below summarizes the features of the pHybLex/Zeo-Fos2 control vector. A 300 bp fragment encoding the Fos leucine zipper region 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. The LexA/Fos fusion protein is still active by functional testing. For more information about Fos, please refer to page 50 in the **Appendix**. **The complete nucleotide sequence for pHybLex/Zeo-Fos2 is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).**



Comments for pHybLex/Zeo-Fos2
4913 nucleotides

ADH1 promoter: bases 1-399
LexA ORF: bases 420-952
Fos leucine zipper region: bases 966-1222
pHybLex/Zeo Reverse priming site: bases 1309-1333
ADH1 transcription termination signal: bases 1289-1446
2μ origin of replication: bases 1622-2456
TEF1 promoter: bases 3003-3411
EM-7 promoter: bases 3415-3482
Zeocin™ resistance gene: bases 3483-3857
CYC1 transcription termination signal: bases 3858-4175
pMB1 (pUC-derived) origin: bases 4186-4859 (complementary strand)

Note: Fos is cloned between the first *Bgl* II site 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 active by functional testing.

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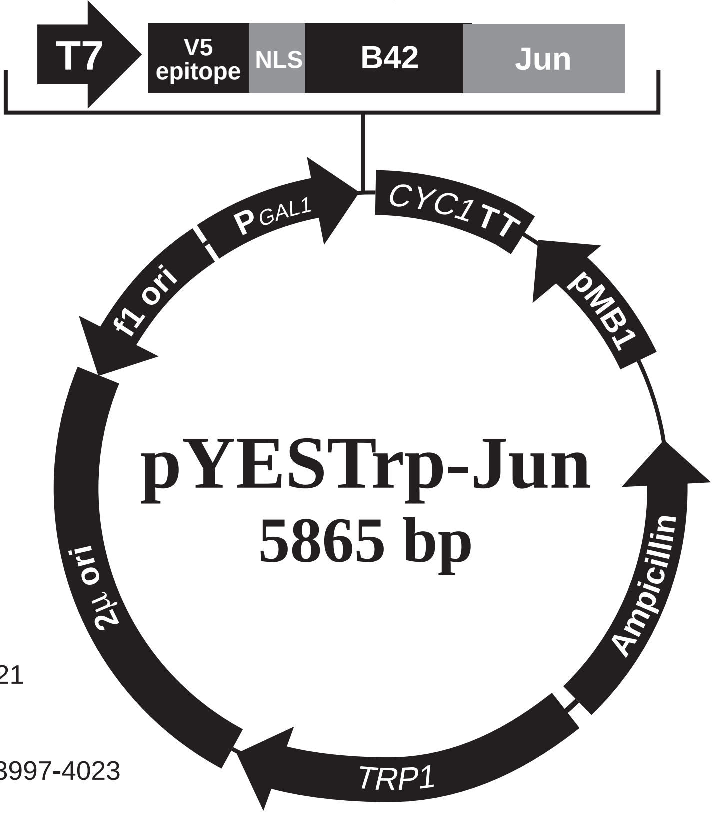
pYESTrp-Jun Vector

Map of pYESTrp-Jun

The figure below summarizes the features of the pYESTrp-Jun control vector. A 135 bp DNA fragment encoding the leucine zipper region of the Jun protein was cloned into the *Hin*d III/Sph I sites of pYESTrp to generate pYESTrp-Jun. For more information about Jun, please refer to page 50 in the **Appendix**. **The compete nucleotide sequence for pYESTrp-Jun is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).**



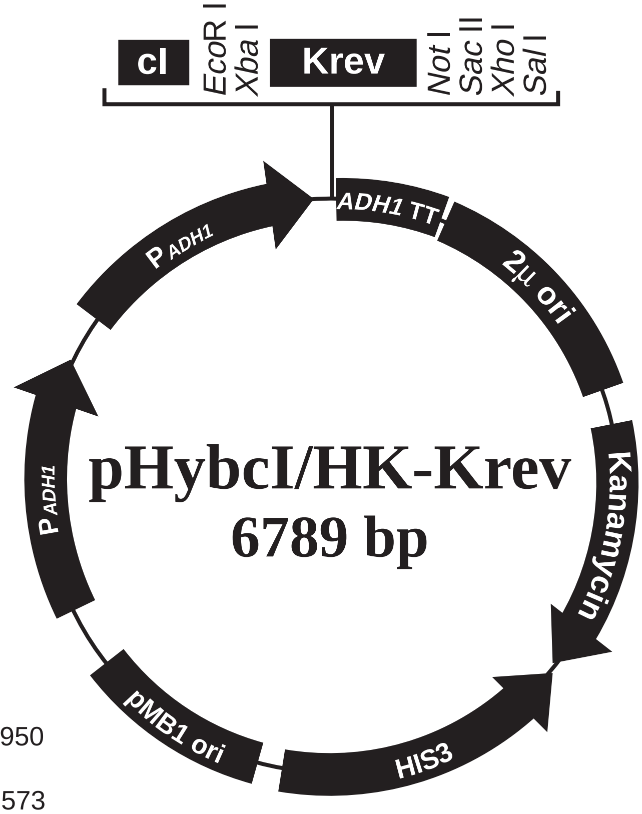
Comments for pYESTrp-Jun:
5865 nucleotides

GAL1 promoter: bases 3381-3878
T7 promoter/priming site: bases 3902-3921
Initiation ATG: bases 3937-3939
V5 epitope: bases 3940-3981
Nuclear localization signal (NLS): bases 3997-4023
B42 activation domain: bases 4027-4264
pYESTrp Forward priming site: bases 4228-4246
Jun leucine zipper region: bases 4270-4405
pYESTrp Reverse priming site: bases 4437-4455
CYC1 transcription termination region: bases 4420-4668
pMB1 (pUC-derived) origin: bases 4850-5523 (complementary strand)
bla promoter: bases 664-762 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5668-663 (complementary strand)
TRP1 promoter: bases 871-972
TRP1 ORF: bases 973-1647
2μ origin: bases 2051-2885
f1 origin: bases 2954-3326 (complementary strand)

pHybcI/HK-Krev Vector

Map of pHybcI/HK-Krev

The figure below summarizes the features of the pHybcI/HK-Krev control vector. A 554 bp DNA fragment encoding the mature Krev1 peptide (Kitayama *et al.*, 1989) was cloned in frame with the cI protein in pHybcI/HK to generate pHybcI/HK-Krev. For more information about Krev1, please refer to page 51 in the **Appendix**. **The complete nucleotide sequence for pHybcI/HK-Krev is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 70).**



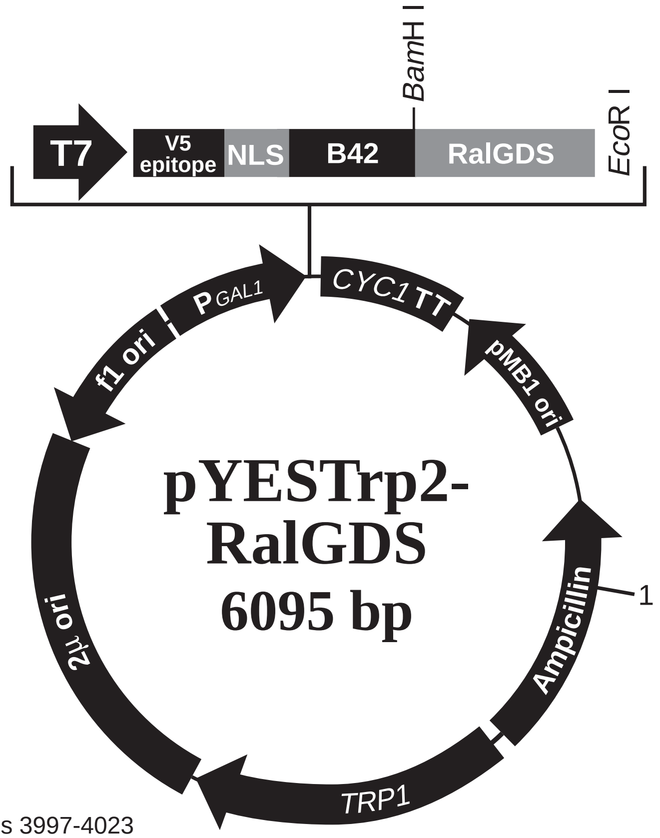
Comments for pHybcI/HK-Krev
6789 nucleotides

ADH1 promoter (2X copies): bases 4-950
cI repressor: bases 966-1676
cI Forward priming site: bases 1556-1573
Krev1 gene: bases 1725-2279
ADH1 transcription termination signal: bases 2369-2526
pHybLex/Zeo Reverse priming site: bases 2386-2410
2μ origin: bases 2656-3533
Kanamycin promoter: bases 4075-4104
Kanamycin resistance gene: bases 4105-4899
HIS3 promoter: bases 5775-5970 (complementary strand)
HIS3 gene: bases 5112-5774 (complementary strand)
pMB1 (pUC-derived) origin: bases 6061-6734 (complementary strand)

pYESTrp2-RalGDS Vector

Map of pYESTrp2-RalGDS

The figure below summarizes the features of the pYESTrp2-RalGDS control vector. A 296 bp DNA fragment containing the Ras-binding domain of the Ral guanine nucleotide dissociation stimulator (RalGDS) (Hofer *et al.*, 1994) has been cloned in frame with the B42 activation domain in pYESTrp2 to generate pYESTrp2-RalGDS. The RalGDS fragment (corresponding to amino acids 767-848 of the RalGDS protein) has been shown to interact with Krev1 in the yeast two-hybrid assay (Serebriiskii *et al.*, 1999). For more information about RalGDS, please refer to page 51 in the **Appendix**. **The complete nucleotide sequence of pYESTrp2-RalGDS is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 70).**



Comments for pYESTrp2-RalGDS
6095 nucleotides

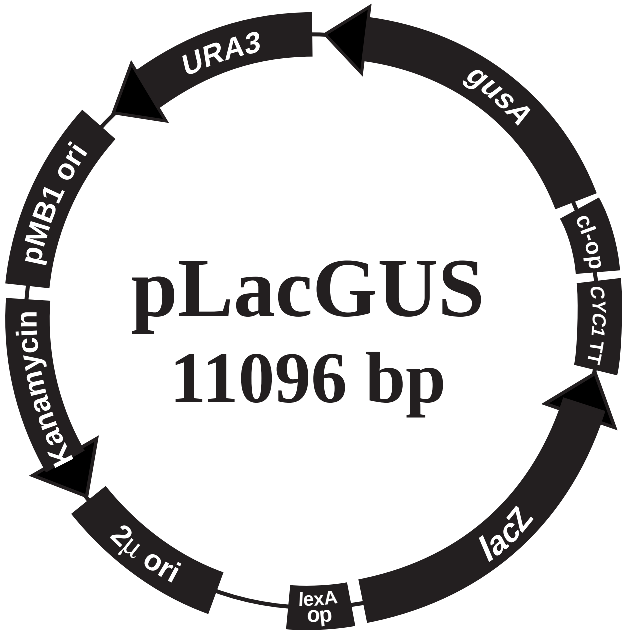
GAL1 promoter: bases 3427-3878
T7 promoter: bases 3902-3921
Initiation ATG: bases 3937-3939
V5 epitope: bases 3940-3981
Nuclear localization signal (NLS): bases 3997-4023
B42 activation domain: bases 4027-4263
pYESTrp Forward priming site: bases 4228-4246
RalGDS: bases 4291-4587
pYESTrp Reverse priming site: bases 4667-4685
CYC1 transcription termination signal: bases 4650-4898
pMB1 (pUC-derived) origin: bases 5080-5753 (complementary strand)
bla promoter: bases 664-762 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5898-663 (complementary strand)
TRP1 promoter: bases 871-972
TRP1 gene: bases 973-1647
2μ origin: bases 1694-2886
f1 origin: bases 2954-3409 (complementary strand)

---

pLacGUS Vector

Map of pLacGUS

pLacGUS (Catalog no. V616-20) is a LacZ and GUSA reporter plasmid containing 8 LexA operator binding sites upstream of the *lacZ* gene and 3 cI operator binding sites upstream of the *E. coli* β-glucuronidase (*gusA*) gene (Jefferson *et al.*, 1986; Schlaman *et al.*, 1994). pLacGUS was transformed into the SKY48 yeast strain to generate the SKY48/pLacGUS strain supplied in the Dual Bait Hybrid Hunter™ System. **The complete nucleotide sequence of pLacGUS is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 70).**



Comments for pLacGUS
11096 nucleotides

β-glucuronidase A (*gusA*) gene: bases 68-1879 (complementary strand)
cI operators (3X cI-ops): bases 2123-2190
CYC1 transcription termination signal: bases 2463-2569
LacZ gene: bases 2716-5955 (complementary strand)
LexA operators (8X lexA-ops): bases 6190-6345 (complementary strand)
2µ origin: bases 7176-8053 (complementary strand)
Kanamycin promoter: bases 8951-9088 (complementary strand)
Kanamycin resistance gene: bases 8156-8950 (complementary strand)
pMB1 (pUC-derived) origin: bases 9336-10008
URA3 gene: bases 10009-11096 (complementary strand)

LexA and cI Operators

The LexA operator sequences used to control expression of the *lacZ* reporter gene in pLacGUS and the *LEU2* auxotrophic marker in SKY48/pLacGUS are derived from the *E. coli recA* promoter as originally described in Estojak et al. (1995).

The cI operator sequences used to control expression of the *gusA* reporter gene in pLacGUS and the *LYS2* auxotrophic marker in SKY48/pLacGUS are derived from bacteriophage lambda. In each case, a 68 bp fragment of the bacteriophage lambda genome (LAMCG nt 37950-38018) containing 3 naturally occuring cI operators was used. The LAMCG sequence can be accessed through Genbank (Accession No. J02459) on the World Wide Web at www.ncbi.nlm.nih.gov/entrez/nucleotide.html.

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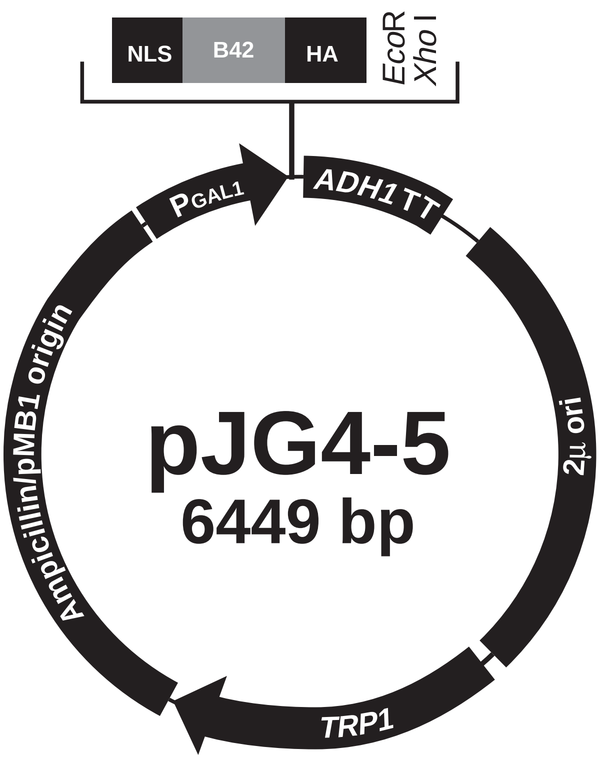
pJG4-5

Description

pJG4-5 is the library plasmid developed by Roger Brent and coworkers (Gyuris *et al.*, 1993). Many libraries designed for two-hybrid screening have been 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, 2µ 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
2µ origin: bases 2178-2875
TRP1 gene: bases 3416-4090
Ampicillin resistance gene: bases 4400-5260
pMB1 origin: bases 5405-6078

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.

- YPD (see recipe on page 44)
- 1X TE (see recipe on page 44)
- 1X LiAc/0.5X TE (see recipe on page 45)
- 1X LiAc/40% PEG-3350/1X TE (see recipe on page 45)
- Denatured sheared salmon sperm DNA (see recipe on page 69)
- Plasmid DNA to be transformed
- DMSO
- Selective plates

Protocol

1. Inoculate 10 ml of YPD with a colony of SKY48/pLacGUS 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 YPD 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 1X TE and plate on an appropriate selective plate.

Large-Scale Library Transformation Using SKY48/pLacGUS

Introduction

A large-scale protocol to transform your prey library into the SKY48/pLacGUS bait strain is provided below.

Materials Needed

We suggest that you read the protocols through before beginning. Pay close attention to the number and type of plates required as well as the medium. Be sure to have the following materials and reagents on hand before starting. For a recipe for various YC selective media, please see the **Appendix**, page 43.

- SKY48/pLacGUS containing pHybLex/Zeo and pHybcI/HK bait constructs
- YC-UH Z200 medium and plates
- 30°C incubator and shaking incubator
- YPD
- Centrifuge
- Sterile 1X TE buffer (see recipe, page 44)
- 50 ml conical centrifuge tubes
- Denatured salmon sperm DNA (see recipe, page 69)
- 1X LiAc/0.5X TE (see recipe, page 45)
- 1X LiAc/40% PEG-3350/1X TE (see recipe, page 45)
- DMSO
- YP (contains no glucose)
- YC-UHW medium (optional)
- YC-UHW Z200 medium and 150 mm plates
- 42°C water bath
- YC-UHWL Z200 Gal/Raff medium and 150 mm plates
- YC-UHWK Z200 Gal/Raff medium and 150 mm plates
- Glycerol solution (see recipe, page 46)
- pYESTrp2 library DNA or other cDNA library

continued on next page

Large-Scale Library Transformation Using SKY48/pLacGUS, continued

Large-Scale Library Transformation

The 2-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 6 days with an additional 2 to 5 days for colonies to appear.

Before Starting

- Prepare (page 69) or purchase denatured salmon sperm DNA or use yeast carrier tRNA.
- Prepare 500 μg of 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.
- YC-UHW medium (optional)
- 26 150 mm YC-UHW Z200 medium and plates.
- 10 150 mm YC-UHWL Z200 Gal/Raff plates.
- 10 150 mm YC-UHWK Z200 Gal/Raff plates.

Preparation of Bait Strain for Transformation

For the large-scale library transformation of the SKY48/pLacGUS 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). Other methods are suitable. The bait strain is prepared for transformation as follows:

1. Inoculate the SKY48/pLacGUS bait strain into 5 ml of YC-UH Z200. Grow overnight at 30°C.
2. Prepare a culture flask containing 100 ml of YC-UH 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

   $$\frac{(0.012\ \text{OD/ml})\ (100\ \text{ml})}{3\ \text{OD/ml}} = 0.40\ \text{ml}$$

Note: The bait strains may exhibit an increased doubling time of 2-3 hours when grown in selective medium. You may want to check the doubling time of your bait strain and adjust your OD_{600} calculations accordingly.

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. Re-pellet the cells.
7. Resuspend cell 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, continued

Transformation of Bait Strain with Library DNA

8. Mix together 1 ml of 10 mg/ml denatured salmon sperm DNA and 500 µg library DNA.
9. Add DNA mixture to cell suspension from Step 7, previous page.
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. Resuspend and wash the cell pellet in 500 ml YPD. Re-pellet the cells.
16. Resuspend the pellet in 1 liter YPD and incubate at 30°C for 1 hour with constant shaking. Proceed to next section.

Plating and Harvesting Primary Transformation

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

17. Remove 1 ml of cells from the culture in Step 16, above, and pellet the cells.
18. Resuspend the cells in 1 ml YC-UHW Z200 and plate 10 and 1 µl aliquots ($1/10^5$ and $1/10^6$ of total) on YC-UHW 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, above.
20. Resuspend and wash pellet in 500 ml YC-UHW. Centrifuge and resuspend pellet in 1 liter of prewarmed YC-UHW Z200. **Note:** You may also wash the yeast pellet in YC-UHW Z200 medium.
21. Incubate, with shaking, at 30°C for 16 hours.
22. Pellet cells and wash with 500 ml YC-UHW (or YC-UHW Z200).
23. Pellet cells and resuspend the final pellet in 10 ml YC-UHW Z200.
24. Remove 5 ml of cells and plate 250 µl each on 20 150 mm YC-UHW Z200 plates. Incubate at 30°C for 2 to 3 days until colonies appear. Save the remaining 5 ml at +4°C as a backup in the event that you wish to plate more cells. Yeast are stable for at least one week when stored in this fashion.
25. Cool all of the 150 mm plates containing transformants from Step 24 for several hours at +4°C to harden agar and dry the plates.
26. Wearing gloves and using a sterile cell scraper, gently scrape yeast cells off the plate. Be careful not to damage the agar. Pool cells from the 20 plates into a sterile 50 ml conical tube containing 5 ml of 1X TE.
27. Centrifuge at 1000 to 1500 x g for 5 minutes at room temperature to pellet the cells.
28. Wash the cells by resuspending the pellet in 10 ml of 1X TE. Centrifuge at 1000 to 1500 x g for 5 minutes at room temperature. Estimate the volume of the cell pellet.
29. Resuspend the pellet in 1 volume of glycerol solution, mix well, and store up to 1 year in 1 ml aliquots at -80°C. Proceed to Step 30, next page.

continued on next page

Large-Scale Library Transformation Using SKY48/pLacGUS, continued

Large-Scale Library Transformation, continued

Determine Replating Efficiency

30. Remove an aliquot of frozen transformed yeast (Step 29, previous page) and dilute 1:10 with YC-UHW Z200 Gal/Raff medium. Incubate with shaking for 4 hours at 30°C to induce the *GAL1* promoter to express the library.
31. Make serial dilutions of the culture using the YC-UHW Z200 Gal/Raff medium. Plate on YC-UHW Z200 Gal/Raff plates and incubate 2 to 4 days at 30°C until colonies are visible.
32. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

Screening for Interacting Proteins

33. Thaw the appropriate quantity of transformed yeast based on the plating efficiency (calculated above), dilute 2 aliquots 1:10 with YC-UHW Z200 Gal/Raff medium, and incubate as in Step 30.
34. Centrifuge for 5 minutes at 1000 to 1500 x g and resuspend the two pellets in 1 ml each of YC-UHW Z200 Gal/Raff medium.
35. Plate 100 µl each on 10 YC-UHWL Z200 Gal/Raff plates and 10 YC-UHWK Z200 Gal/Raff plates. Incubate for 2 to 3 days at 30°C until colonies appear.

    Carefully pick appropriate Leu$^+$ or Lys$^+$ colonies and patch on new YC-UHWL Z200 Gal/Raff or YC-UHWK Z200 Gal/Raff master plates. Incubate 2 to 7 days at 30°C until colonies appear.

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 fewer total colonies.

Before Starting

Prepare or have on hand the following reagents.

- Salmon Sperm DNA (Sigma, Catalog no. D-1626)
- 1X 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 1X TE (10 mg/ml). Pipet up and down with a 10 ml pipet to dissolve completely.
2. Incubate overnight at +4°C on a rotating wheel.
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.
4. Distribute the sonicated DNA between four 50 ml conical centrifuge tubes (25 ml per tube).
5. 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.
6. 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.
7. 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.
8. 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).
9. Pellet the DNA at 12,000 x g for 15 minutes at +4°C.
10. Wash the DNA once with 200 ml 70% ethanol and centrifuge as described in Step 8.
11. Partially dry DNA in a speed-vac by covering tubes with parafilm and poking holes in it. Dry for ~20 min.
12. Transfer DNA to a 250 ml sterile flask and dissolve DNA in 100 ml sterile 1X TE (10 mg/ml).
13. Denature by boiling in a water bath for 20 minutes. Immediately place on ice, aliquot in 1 ml samples, and freeze at -20˚C.

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Indonesia	00180316570620	Netherlands	08000220091
Italy	167870990	Singapore	8001100987
Japan	006633800185	Spain	900931263
Korea	0308116570820	Turkey	00800136570900
Malaysia	1800808714	United Kingdom	0800967491

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