Invitrogen™
life technologies

Instruction Manual

ProQuest™ Two-Hybrid System with Gateway® Technology

CAT. SERIES 10835

Version C
December 23, 2002
53888

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Choosing Products to Build Gateway® Expression Clones

Step 1: Construct or Select an Entry Clone starting from:

PCR Fragment

Design primers with *att*B sites
Amplify PCR product

↓

Clone *att*B-PCR product into pDONR™ Vector with BP Clonase™ Enzyme Mix
PCR Cloning System with pDONR™221 (BP Clonase™ Enzyme Mix included) Cat. No. 12535-019

↓

Analyze / Propagate Entry Clone

Restriction Fragment

Choose Entry Vector
Individual Entry Vectors Cat. Nos. 11813-011; 11816-014; 11817-012; 11818-010; 11819-018; K2400-20, K2420-20

↓

Ligation of restriction fragment into Entry Vector

↓

Analyze / Propagate Entry Clone

Library Screening

Isolate clone from pCMV•SPORT 6; pSPORT-P; or pEXP-AD502 library
See Catalog or web site for list of Gateway® Libraries and Custom Libraries

↓

Transfer insert with BP Clonase™ Enzyme Mix into pDONR™ Vector
BP Clonase™ Enzyme Mix Cat. No. 11789-013
pDONR™201 Cat. No. 11798-014
pDONR™207 Cat. No. 12213-013
pDONR™221 Cat. No. 12536-017

↓

Analyze / Propagate Entry Clone

Step 2: Construct an Expression Clone

A. Choose or Construct your Destination Vector

Destination Vector **OR** Use Your Own Vector

E. coli

Native: pDEST™14
N-GST: pDEST™15
N-His: pDEST™17
C-GST: pDEST™24
***E. coli* Expression System with BL21-AI™ Cells Cat. No. 11824-026 (LR Clonase™ Enzyme Mix included)**

Mammalian

C-V5: pcDNA3.2-DEST™
N-His: pDEST™26
N-GST: pDEST™27
Mammalian Expression System
Cat. No. 11826-013 (LR Clonase™ Enzyme Mix included)

Baculovirus

Native: pDEST™8
N-His: pDEST™10
N-GST: pDEST™20
Baculovirus Expression System*
Cat. No. 11827-011 (LR Clonase™ Enzyme Mix included)

Use Your Own Vector

Convert existing vector by cloning Conversion Cassette into MCS
Gateway™ Vector Conversion System Cat. No. 11828-019

Choose a complete Expression System(s) **OR** purchase **Destination Vector(s) individually**

B. Transfer gene from Entry Clone into Destination Vector with LR Clonase™ Enzyme Mix to make Expression Clone

↓ **LR Clonase™ Enzyme Mix Cat. No. 11791-019**

Analyze / Propagate Expression Clone - Express Protein

*Baculovirus Expression Systems provide components to construct a transfer vector. User must also purchase **MAX Efficiency® DH10Bac™ Competent Cells, Cat. No. 10361-012,** and **Cellfectin® Reagent, Cat. No. 10362-010,** included in Bac-to-Bac® Baculovirus Expression System.

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Notices to Customer

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1.4 Notice for European Customers

S. cerevisiae strains MaV103 and MaV203 are genetically modified and contain three GAL4 inducible reporter genes stably integrated into the genome. *S. cerevisiae* control strains A-E are derivatives of MaV103 each containing two *S. cerevisiae*/*E. coli* shuttle plasmids. The shuttle plasmids contain the following cDNA inserts: Control A, none/none; Control B, human retinoblastoma/human E2F1; Control C, *Drosophila* DP/*Drosophila* E2F; Control D, rat cFos/mouse cJun; and Control E, *GAL*4/none. The shuttle plasmids contain an F1 replication origin as well as an *ori*T site, but lack all *tra* genes. As a condition of sale, this product must only be used in accordance with all applicable local legislation and guidelines including EC directive 90/219/EEC on the contained use of genetically modified organisms.

Overview

2

2.1 Basis of the Two-Hybrid System

The two-hybrid system is an *in vivo* yeast-based system that identifies the interaction between two proteins (X and Y) by reconstituting an active transcription factor (1). The active transcription factors are formed as a dimer between two fusion proteins, one of which contains a **D**NA-**B**inding Domain (DB) fused to the first protein of interest (DB-X; also known as the "bait") and the other, an **A**ctivation **D**omain (AD) fused to the second protein of interest (AD-Y; also known as the "prey" or "target protein"). DB-X:AD-Y interaction reconstitutes a functional transcription factor that activates chromosomally-integrated reporter genes driven by promoters containing the relevant DB binding sites (1) (Figure 1)**.** When a selectable marker such as *HIS3* is used as a reporter gene, two-hybrid-dependent transcription activation can be monitored by growth of cells on plates lacking histidine (2-4), thereby providing a means to detect protein:protein interactions genetically. This method can be used to test whether two known proteins interact with each other or detect an unknown protein encoded by a cDNA (or genomic) library that interacts with a protein of interest.

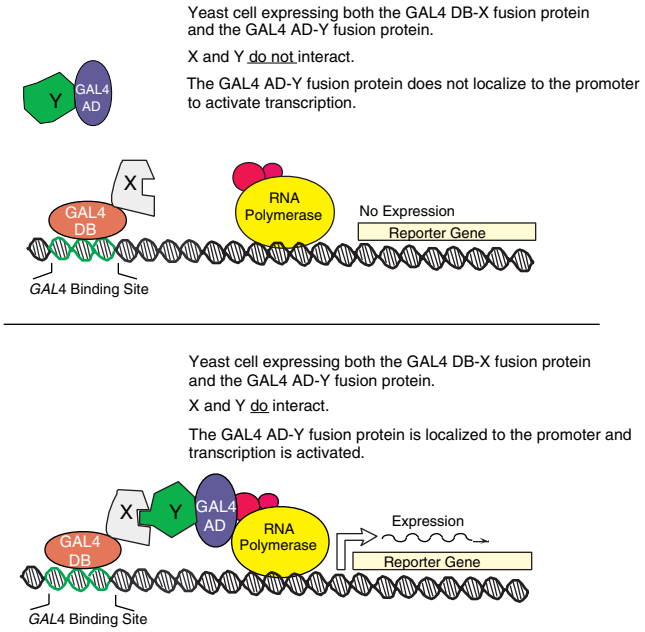


Figure 1. Basis of the Two-Hybrid System.

Early two-hybrid systems suffered from false positives - candidate proteins identified as interacting but which do not truly interact or are biologically irrelevant. False positives can result from: proteins containing regions with surfaces having low affinities for many different proteins, (*e.g.,* large hydrophobic surfaces); proteins that normally interact with a large number of proteins (*e.g.,* heat shock proteins); proteins containing regions functioning as activation domains; proteins affecting chromatin structure; or proteins having low or nonspecific affinities for the promoter regions (or proteins bound there) that drive the expression of reporter genes (5,6).

Overview

The ProQuest™ Two-Hybrid System with Gateway® Technology has been designed to decrease false positives and extend the two-hybrid system to allow rapid characterization of the interacting proteins (4,7-10). The primary modifications include:

- low-copy-number (ARS/CEN) vectors,
- three reporter genes with independent promoters,
- a positive/negative selection reporter gene (*URA*3),
- an extended panel of yeast control strains, and
- incorporation of the Gateway® Technology.

2.2 Gateway® Technology

Gateway® Technology is a novel universal system for cloning and subcloning DNA sequences, facilitating gene functional analysis, and protein expression (Figure 2). Once in this versatile operating system, DNA segments are transferred between vectors using site-specific recombination. This powerful system can easily transfer a DNA sequence into multiple vectors in parallel reactions, while maintaining orientation and reading frame.

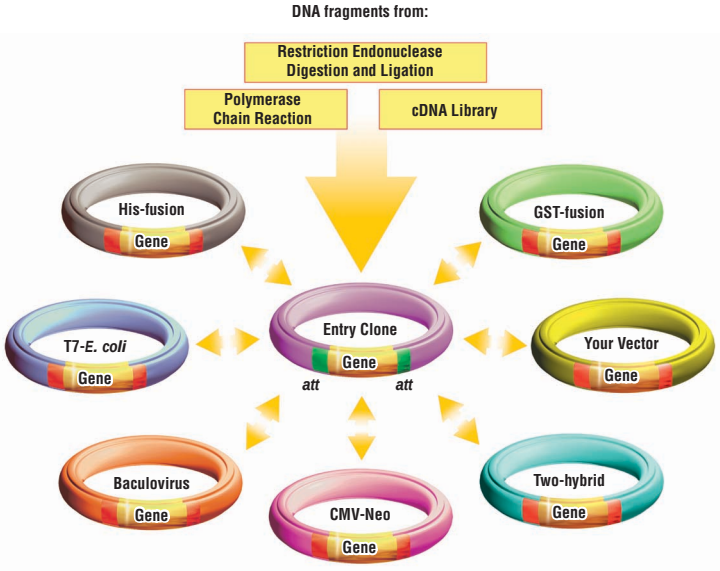


Figure 2. The Power of Gateway® Technology. The gene of interest can be moved into an Entry Vector via PCR, restriction endonuclease digestion and ligation, or site-specific recombination from a cDNA library constructed in a Gateway®-compatible vector. A gene in the Entry Clone can then be transferred simultaneously into Destination Vectors. This is done by combining the Entry Clone with a Gateway® Destination Vector and Clonase™ Enzyme Mix in a single tube, incubating for 1 h, transforming *E. coli*, and plating.

The Gateway® Cloning System uses phage lambda-based site-specific recombination instead of restriction endonucleases and ligase. This recombination system is used by lambda during the switch between the lytic and lysogenic pathways (11). The key DNA recombination sequences (*att* sites) and proteins that mediate the recombination reactions are the foundation of Gateway® Technology (12).

Two reactions constitute the Gateway® Technology. The LR Reaction is a recombination reaction between an Entry Clone and a Destination (pDEST™) Vector, mediated by a cocktail of recombination proteins, to create an Expression

Clone. It is used to move the sequence of interest to one or more Destination Vectors in parallel reactions. The BP Reaction is a recombination reaction between an Expression Clone (or an *att*B-flanked PCR product) and a Donor (pDONR™) Vector to create an Entry Clone.

For more detailed information on Gateway® Technology, please see the Invitrogen web site at www.invitrogen.com.

2.3 The ProQuest™ Two-Hybrid System with Gateway® Technology

The ProQuest™ Two-Hybrid System is based on modifications by Chevray and Nathans (13) and Vidal, Boeke, and Harlow (4,7,8). Incorporation of Gateway® into the ProQuest™ System accelerates the cloning of genes into the ProQuest™ two-hybrid vectors. The genes of interest are PCR amplified using Gateway® primers. Through recombination reactions, the genes of interest are transferred into either

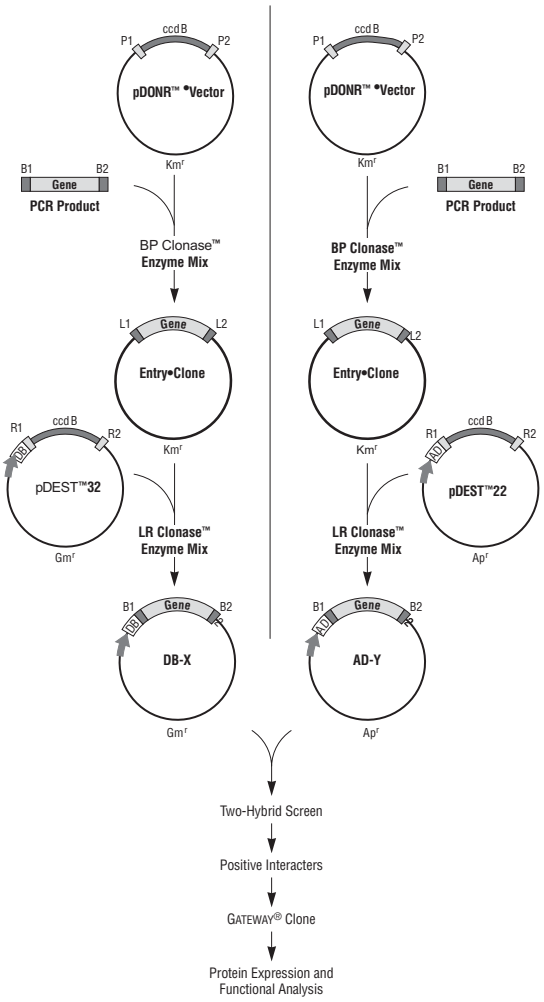


Figure 3. Testing the Interaction of a Known Protein X with a Second Known Protein Y. The genes of interest are PCR amplified, using Gateway® *att*B primers. Gateway® recombination reactions with the PCR products and pDONR™ vectors generate Entry Clones. The genes in the Entry Clones are transferred into the Destination Vectors pDEST™32 or pDEST™22, generating DB-X and AD-Y vectors. The protein-protein interactions are tested in a two-hybrid screen. Once proteins of interest are identified, they can be transferred into various expression systems using Gateway® recombination reactions.

the DB-X (bait) or AD-Y (prey) vector. Perform the two-hybrid screen. Once the two-hybrid screen is complete, the genes of interest can be transferred into multiple vectors for further testing or processing (Figure 3).

Similarly, the ProQuest™ System can be used to screen cDNA or genomic libraries (see section 3.4.4 for information on library construction) for interactions with a

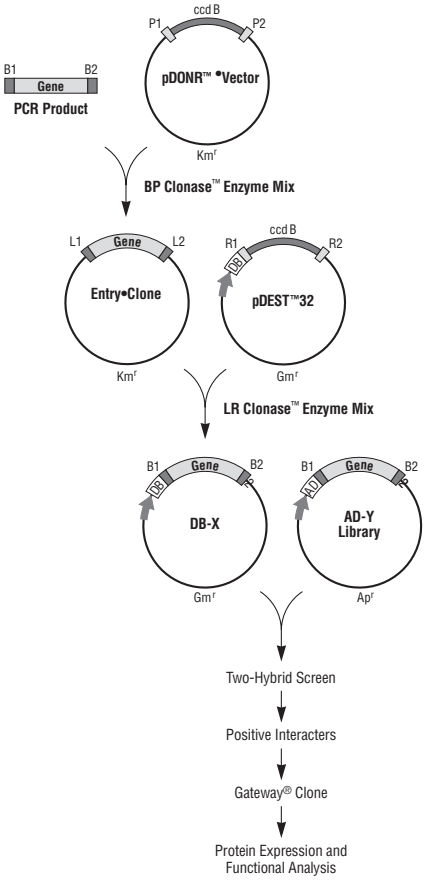


Figure 4. Screening a Library with a Known Protein X. The known gene is PCR amplified using Gateway® *att*B primers. A Gateway® recombination reaction with the PCR product and pDONR™ Vector generates an Entry Clone. The gene in the Entry Clone is transferred into pDEST™32 generating an in-frame fusion with the GAL4DNA binding domain (DB-X). The system is used to screen for interactions between the protein of interest in DB-X and library in pEXP-AD502. Once proteins of interest have been identified, they can be transferred into various expression systems using Gateway® recombination reactions.

known protein. Gateway® recombination reactions are used to generate a bait vector (DB-X) with the gene of interest in frame with the GAL4 DNA binding domain (Figure 4). Once positive interactors are identified by a two-hybrid screen with the library, the interactors can be easily transferred into a variety of expression vectors for protein expression and functional analysis.

2.3.1 Low-Copy-Number DB and AD Vectors (ARS/CEN)

Overexpression of the DB-X and AD-Y hybrid proteins can increase the nonspecific interaction between the fusion proteins resulting in higher numbers of false

positives. Additionally, many proteins can be toxic when overexpressed and potentially interesting interacting proteins might not be recovered (false negatives). In yeast, protein expression can be maintained at a relatively low level by the use of low-copy-number (ARS/CEN) expression vectors. ARS/CEN-based vectors further provide more consistent plasmid copy numbers (versus the high variability of two micron-based vectors), leading to increased reproducibility of the reporter gene expression levels (14). The consistent expression of fusion proteins at levels closer to physiological conditions is particularly valuable when characterizing mutations (by allowing detection of subtle differences between different alleles), peptides, and other proteins or compounds that alter or disrupt known protein:protein interactions.

The maps of the vectors are shown in section 5.

pDEST™32 is the DNA Binding Domain (DB) Gateway® Destination Vector derived from pDBLeu (see section 5.12). This vector is used to clone the known gene of interest in frame with the sequence encoding the DNA Binding Domain of the GAL4 protein (generating DB-X).

Features of this vector include:

- the sequence encoding the GAL4 Activation Domain (amino acid 1-147),
- the *ARS*4/*CEN*6 sequence for replication and low-copy-number maintenance in yeast,
- the LEU2 gene for selection in yeast on medium lacking leucine,
- the constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene (*ADH*1) expressing the GAL4 DB,
- the dominant $CYH2^S$ allele that confers sensitivity to cycloheximide in yeast (for plasmid shuffling),
- a pUC-based replication origin and gentamicin resistance gene (Gm^r) for replication and maintenance in *E. coli* , and
- two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance gene (Cm^r) and a *ccd*B gene. Following the LR Clonase™ reaction, the Cm^r and *ccd*B genes are replaced by the gene of interest and the *att*R sites are converted to *att*B sites. As a result, the gene of interest is now fused in frame with DB flanked by *att*B sites in the Destination Vector backbone.

pDEST™22 is an Activation Domain (AD) Gateway® Destination Vector. This vector is used to clone the second known gene of interest in frame with the sequence encoding the Transcription Activation Domain of the GAL4 protein (generating AD-Y).

Features of this vector include:

- the sequence encoding the GAL4 Activation Domain (amino acid 768-881),
- the *ARS*4/*CEN*6 sequence for replication and maintenance at low-copy-number in yeast,
- the *TRP1* gene for selection in yeast on medium lacking tryptophan,
- the constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene (*ADH*1) expressing the GAL4 AD,
- a pUC-based replication origin and ampicillin resistance gene (Ap^r) for replication and maintenance in *E. coli*), and
- two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance gene and a *ccd*B gene. Following the LR Clonase™ reaction, the Cm^r and *ccd*B genes are replaced by the gene of interest and the *att*R sites are converted to *att*B sites. As a result, the gene of interest is now fused in frame with AD flanked by *att*B sites in the Destination Vector backbone.

pEXP-AD502 is an Activation Domain (AD) Gateway® Expression Vector. This plasmid is used to construct a cDNA or genomic library for identifying proteins (AD-Y) that interact with the fusion protein (DB-X).

Features of this vector include:

- the sequence encoding the GAL4 Activation Domain (amino acid 768-881) fused to the nuclear localization signal from SV40 Large T antigen,
- the *ARS*4/*CEN*6 sequence for replication and low-copy-number maintenance in yeast,
- the *TRP1* gene for selection in yeast on medium lacking tryptophan,
- the constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene (*ADH*1) expressing the GAL4 AD,
- a pUC-based replication origin and ampicillin resistance gene (Ap^r) for replication and maintenance in *E. coli*, and
- two recombination sites, *att*B1 and *att*B2, flanking a multiple cloning site, including *Sal* I and *Not* I sites for generation of cDNA libraries using the SuperScript™ Plasmid System with Gateway® Technology for cDNA Synthesis and Plasmid Cloning.

pDBLeu is the DNA Binding Domain (DB) cloning vector derived from pPC97 (13). This vector has all the features of pDEST™32 except there are no *att*R sites for Gateway® compatibility. It contains a multiple cloning site with blunt end-generating restriction sites (*Sma* I, *Stu* I, *Msc* I) in each of the three reading frames allowing any blunt-end, filled-in or polished-end DNA fragment to be inserted in frame using DNA ligase. The selection antibiotic is kanamycin (Km^r) instead of gentamicin Gm^r.

2.3.2 Three Reporter Genes

A single copy of each of three reporter genes (*HIS3*, *URA*3 and *lacZ*) are stably integrated at different loci in the yeast genome. The promoter regions of *URA*3, *HIS*3, and *lacZ* are unrelated (except for the presence of GAL4 binding sites). Details of these promoters have been published (1,2,7,8) and are summarized in Figure 5. A major class of false positives has been described (6) whereby either DB-X enhances induction or it is required for induction of the reporter genes by AD-Y, but DB-X and AD-Y do not interact. This reporter gene induction is promoter context dependent [*e.g.*, the fusion protein(s) recognize promoter sequences or other proteins bound to the promoter]. In the ProQuest™ Two-Hybrid System, these false positives are reduced because three independent transcription events (from distinct promoters) must occur at independent chromosomal loci.

Induction of the *HIS3* and *URA3* reporter genes allow two-hybrid-dependent transcription activation to be monitored by cell growth on plates lacking histidine or uracil, respectively. Induction of the *lacZ* gene results in a blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Moreover, two-hybrid-dependent induction of *URA3* results in conversion of the compound 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Hence, cells containing interacting proteins grow when plated on medium lacking uracil, but growth is inhibited when plated on medium containing 5FOA (Figure 5).

This system therefore reduces false positives by:

- providing four phenotypes [His^+ ($3AT^R$), β-gal, Ura^+ and $5FOA^S$] for assessing true interactors,
- including a third unrelated promoter that facilitates discrimination of artifactual reporter gene activation due to “illegitimate” binding of AD-Y fusion proteins or to spontaneous revertants, and
- using low-copy-number (ARS/CEN) vectors that reduce expression levels and toxicity.

Growth inhibition resulting from induction of the *URA*3 reporter gene on 5FOA-containing medium also provides a way to rapidly characterize protein:protein interactions. Mutations, peptides, proteins or substances that inhibit or modulate the protein:protein interaction can be selected by identifying cells that are able to grow on the correct selective plates containing 5FOA (SC-Leu-Trp+5FOA) (4,7,8,15).

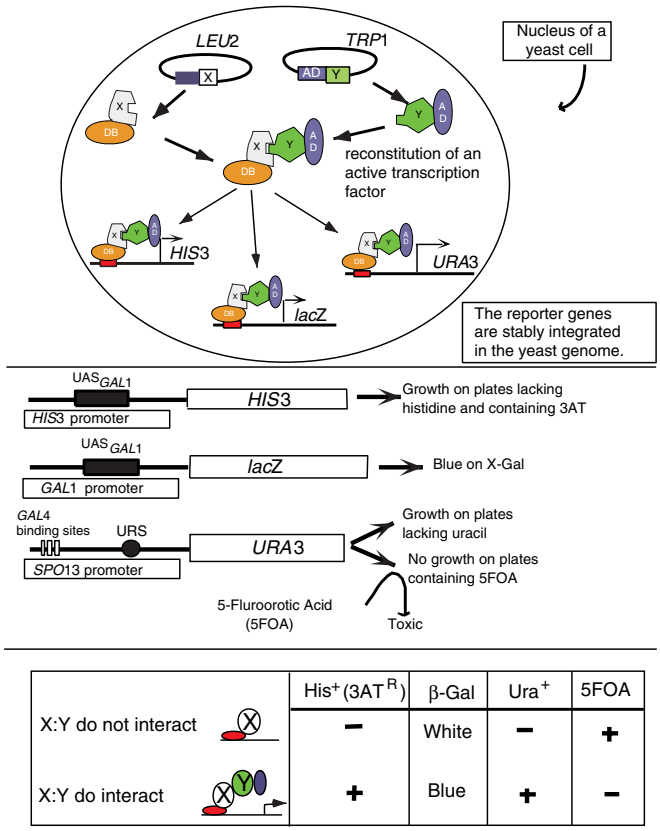


Figure 5. Screening with Three Reporter Genes. *Top:* MaV203 cells containing DB-X and AD-Y plasmids encode fusion proteins DB-X and AD-Y, respectively. The interaction of X:Y reconstitutes an active transcription factor that binds to the *GAL*4 DNA binding sequences present in the promoter regions of the 3 chromosomally-integrated reporter genes and activates transcription. *Center:* Structure of the promoter regions expressing each of the reporter genes and the expected growth/color results. *Bottom*: Expected growth or color results when tested for induction of the reporter genes for interacting and non-interacting DB-X and AD-Y.

2.3.3 Yeast Strains and an Extended Panel of Yeast Controls

NOTE: The following yeast strains are unique to the ProQuest™ Two-Hybrid System. Other strains used for two-hybrid analysis **cannot** be substituted.

Yeast Strains

The yeast strain provided in the ProQuest™ System is MaV203 (*MAT*α) (4,8).

The genotype of MaV203 is as follows:

MaV203 (*MAT*α, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::*lacZ*, *HIS*3$_{\text{UAS GAL1}}$::*HIS*3@*LYS*2, *can*1^{R}, *cyh*2^{R}) (7)

The control strains are derivatives of MaV103. They have the same genotype as MaV203 except they are *MAT***a** (8).

The strains used in this system contain:

- a set of non-reverting auxotrophic mutations: *leu2* and *trp1* to allow selection for the DB-X and AD-Y fusion vectors, and *his3* for growth dependent upon induction of the reporter gene *GAL1::HIS3*,
- deletions of the *GAL4* and *GAL80* genes encoding GAL4 and its repressor GAL80, respectively. In the absence of GAL80, galactose is not required for activation of GAL4-inducible promoters,

Overview

NOTE: While the genotype of MaV203 is *ade2*$^-$, the strain provided in this kit is phenotypically *ADE2*$^+$. The genetic basis of the phenotypic reversion is under investigation. In contrast, the yeast control strains (derivatives of MaV103) remain *ade2*$^-$ (except for Yeast Control Strain D) and will turn red upon starvation for adenine or amino acids.

Table 1. Yeast Control Strains A - E.

Control Strain	Resident Plasmids	cDNA Insert	Interaction Strength	Reference
Control A	pPC97	no insert	none	(13)
	pPC86	no insert		
Control B	pPC97-RB	human RB Acc# M28419 amino acids 302-928	weak	(7,8)
	pPC86-E2F1	human E2F1 Acc# M96577 amino acids 342-437		
Control C	pPC97-*CYH2*^{S}-dDP	*Drosophila* DP Acc# X79708 amino acids 1-377	moderately strong (also a control for plasmid shuffling)	(16)
	pPC86-dE2F	*Drosophila* E2F Acc# U10184 amino acids 225-433		
Control D	pPC97-Fos	rat cFos Acc# X06769 amino acids 132-211	strong	(13)
	pPC86-Jun	mouse cJun Acc# X12761 amino acids 250-325		
Control E	pCL1 (encoding full length GAL4)	GAL4 Acc# K10486 amino acids 1-881	very strong	(1)
	pPC86	no insert		

- three stably integrated single-copy GAL4-inducible reporter genes: *SPAL*10*::URA*3 integrated at *URA*3; *HIS*3$_{\text{UAS }GAL1}$*::HIS3* integrated at *LYS2*; and *GAL1::lacZ* integrated at an unknown locus, and
- the recessive drug resistance marker *cyh2*^{R} for curing the pDBLeu vector for plasmid shuffling (for review, see reference 17).

Yeast Controls

The reporter gene expression levels from a two-hybrid screen can vary from strong to quite weak (although these levels may not reflect the affinity of the protein:protein interaction observed in the native environment). To help determine which candidate clones likely represent true interactors, a collection of control strains (derivatives of MaV103) have been developed that contain plasmid pairs expressing fusion proteins with a spectrum of interaction strengths. The plasmids present in each control strain and the extent of cDNA contained are described in Table 1. The plasmid vectors used are pPC97 (*GAL4*-DB, *LEU*2), pPC97-*CYH2*^{S} and pPC86 (*GAL4*-AD, *TRP*1) (7,8,13). pDBLeu is derived from pPC97.

2.4 Verification of Candidate Clones

Fusion proteins (AD-Y) identified from a two-hybrid screen must be shown to retain the ability to induce the reporter genes when retested with the original test protein (DB-X). This re-evaluation eliminates false positives resulting primarily from self-activation by AD-Y or mutants of DB-X. For this analysis, plasmid shuffling or a retransformation assay can be used.

1. **Plasmid Shuffling**:

   Yeast cells isolated from a two-hybrid screen contain DB-X and AD-Y plasmids. In this procedure, cells that have spontaneously lost the DB-X plasmid, but retain AD-Y, are selected. The resulting AD-Y-containing cells are made competent and transformed with the DB-X. Transformants are then selected and retested for the interaction of X and Y. Those clones that induce the reporter genes are likely to contain interacting fusion proteins.

   Yeast cells that have spontaneously lost the DB-X plasmid are selected using the dominant *CYH2*^{S} gene present on the DB plasmid DB-X. The yeast strain MaV203 is resistant to cycloheximide (cyh^r) due to the recessive *cyh2*^{r} allele. Hence, MaV203 cells containing DB-X plasmid (*CYH2*^{S}) are sensitive to cycloheximide while those lacking this plasmid are cycloheximide resistant (17) (section 5.10 and Figure 15).

2. **Retransformation Assay:**

Yeast cells containing potentially interacting proteins harbor both DB-X and AD-Y. It is often desirable to isolate DB-X and AD-Y separately in *E. coli* to confirm the interaction and further characterize the candidate clones. To facilitate the isolation in *E. coli*, the pDEST™32 vector encodes gentamicin resistance while the pDEST™22 vector encodes ampicillin resistance. Plasmid DNA isolated from yeast cells containing DB-X and AD-Y is introduced into *E. coli* by electroporation and transformants containing AD-Y are selected with ampicillin (or DB-X with gentamicin). The plasmid DNA from these *E. coli* cells is transformed into MaV203 together with pDBLeu or DB-X and tested for induction of the reporter genes. True positives will induce the reporter genes with pDB-X but not with the pDBLeu vector alone.

3 Methods

3.1 Components

The components of the ProQuest™ Two-Hybrid System and their recommended storage conditions are listed below.

Component	Amount	Storage Temperature
Linearized pDEST™22 DNA (0.15 µg/µl)	30 µl	-20°C
Linearized pDEST™32 DNA (0.15 µg/µl)	30 µl	-20°C
pDBLeu DNA (0.1 µg/µl)	50 µl	-20°C
pEXP-AD502 DNA (50 ng/µl)	20 µl	-20°C
MaV203 Host Yeast Strain	0.5 ml	-70°C
Yeast Control Strain A	0.5 ml	-70°C
Yeast Control Strain B	0.5 ml	-70°C
Yeast Control Strain C	0.5 ml	-70°C
Yeast Control Strain D	0.5 ml	-70°C
Yeast Control Strain E	0.5 ml	-70°C

Prepare fresh glycerol stocks of the yeast strains by scraping the frozen glycerol stock with an autoclaved loop or toothpick (~20 µl) and suspend in 50 µl autoclaved, distilled water. Spread each mixture onto the center of a YPAD (for MaV203) or SC-Leu-Trp plate (for the 5 yeast control strains). Refer to section 5.3 for media preparation. Incubate for 24 to 48 h at 30°C. Scrape the confluent patch of cells and suspend completely in 500 µl autoclaved, distilled water. Add 500 µl autoclaved 50% glycerol. Mix well and store at -70°C.

3.2 Additional Materials

To incorporate a PCR product into pDEST™32 or pDEST™22, the PCR Cloning System with Gateway® Technology and LR Clonase™ Enzyme Mix are required. Please see the inside front cover for a Gateway® product selection guide. For additional information, please see the web site at www.invitrogen.com.

The following items are required for use with the ProQuest™ System, but are not included:

Equipment:

- water baths equilibrated at 30°C and 42°C
- stationary incubator (capable of maintaining a temperature of 30°C)
- incubator (capable of maintaining a temperature of 30°C and able to shake 500-ml and 1-L flasks at a setting of 250 rpm)
- 10 and 15-cm Petri plates
- 0.5-mm glass beads, acid washed
- autoclaved loop or toothpicks
- spreadbar
- nitrocellulose or nylon membranes
- replica plating device and velvets

Reagents:

- YPAD medium (rich medium for routine growth of yeast)
- Synthetic Complete dropout medium (SC)
- 3-Amino-1,2,4-Triazole (3AT)
- Luria broth (LB)

NOTE: Nitrocellulose membranes are fragile and can crack during freezing; therefore neutrally charged nylon membranes are recommended.

NOTE: The 10-cm and 15-cm replica plating blocks can be purchased from Qbiogene The velvets used should contain 100% cotton velveteen without rayon and should be cut in 220 × 220-mm squares. Twenty-five to fifty large and twenty-five small velvets will be required.

NOTE: See section 5.3 for media preparation.

- autoclaved, distilled water
- 10X TE [100 mM Tris-HCl (pH 7.5), 10 mM EDTA, autoclaved]
- sonicated Herring or Salmon Sperm DNA, boiled (10 mg/ml)
- 10X LiAc [1 M lithium acetate], filter-sterilized
- 30% PEG 8000/30 mM $MgCl_2$
- 5-fluoroorotic acid (5FOA)
- MAX Efficiency® *E. coli* DH5α™ or DH10B™ competent cells
- ElectroMAX™ DH10B™ cells
- MaV203 Competent yeast cells
- gentamicin
- kanamycin
- ampicillin
- dimethyl sulfoxide (DMSO)
- 0.9% sodium chloride, autoclaved

3.3 Preparation for Two-Hybrid Screening

Due to the complexity of this procedure, read this manual in its entirety prior to beginning a two-hybrid screen. An outline of the procedures are in Figures 6 and 7.

Prior to beginning a two-hybrid screen, determine as much information as possible regarding the test protein of interest (X) and those that might be expected to interact with the test protein (Y). Several issues that are of particular interest include:

- Does the test protein function as a transcription activator or contain other domains, (*e.g.,* repressor) of known function?
- Do the test or predicted target proteins belong to a protein family?

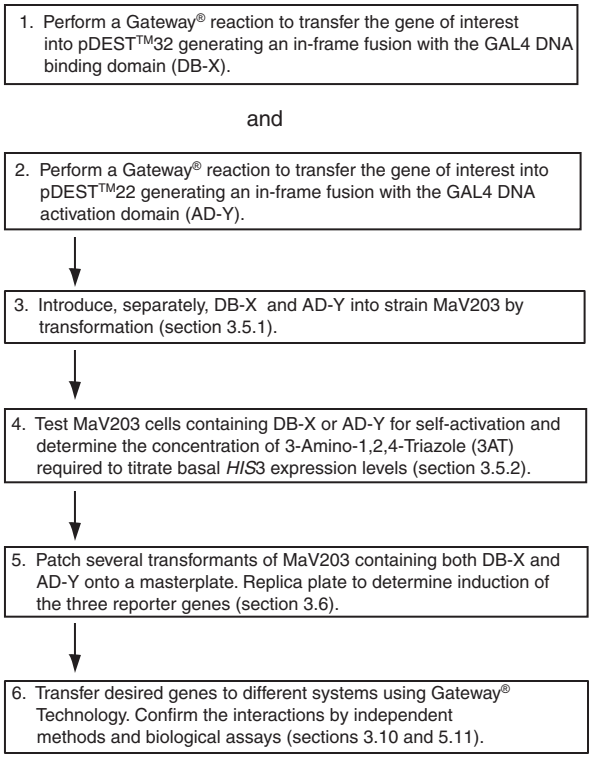


Figure 6. Summary of Procedure for Testing the Interaction of Two Known Proteins.

Methods

- Where and when is the test protein expressed?
- How will the interaction between proteins identified in the two-hybrid screen be confirmed, both biochemically and biologically?

These issues are considered in more detail in section 5.1.

These questions will aid in determining:

1. whether to use the entire protein or to use certain domains or motifs,
2. what libraries to screen,
3. a rough estimate of the number of candidate interacting proteins that might be obtained, and
4. how to further examine the clones obtained.

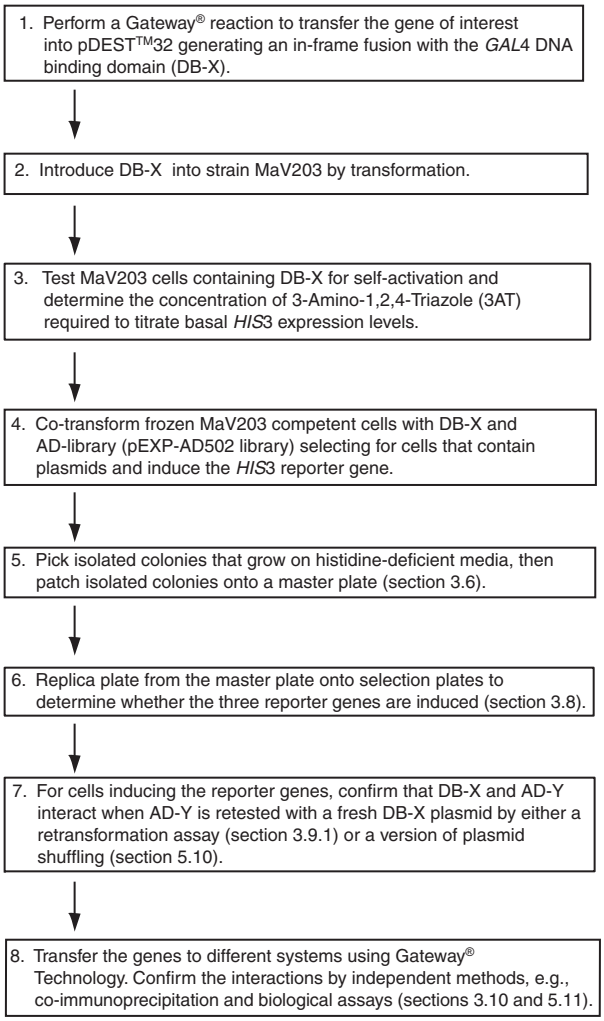


Figure 7. Summary of Procedure for Screening a Library with a Known Protein.

3.4 Construction of DB and AD Fusion Plasmids

3.4.1 Generating X and Y using PCR

This protocol uses PCR to create X and Y and the reagents in the PCR Cloning System with Gateway® Technology to create the DB-X and AD-Y plasmids.

1. Design primers containing the *att*B1 and *att*B2 sequences (Figure 8).

Figure 8. *att*B Primer Sequences for PCR Cloning into a pDONR™ Vector.

***att*B1 forward primer (amino-terminal):**

Lys-Lys

5′-GGGG -ACA-AGT-TTG -TAC-**AAA-AAA**-GCA-GGC-TNN--(template-specific sequence)-3′

*att*B1

***att*B2 reverse primer (carboxy terminal):**

Lys-Tyr

5′-GGGG -AC -CAC- **TTT- GTA**- CAA-GAA-AGC-TGG- GTN--(template-specific sequence)-3′

*att*B2

NOTE: For *att*B1, NN cannot be AA, AG, or GA because these would create stop codons.

2. Perform standard PCR to prepare the *att*B-PCR product.
3. Analyze 1 to 2 µl of the PCR on an agarose gel to assess yield.
   NOTE: Purification of the PCR product is recommended to remove *att*B primers and any *att*B primer-dimers which can clone efficiently into the Entry Vector.
4. Add 150 µl of TE to a 50-µl amplification reaction.
5. Add 100 µl of 30% PEG 8000/30 mM $MgCl_2$. Mix well and centrifuge immediately at 10,000×*g* for 15 min at room temperature. Remove the supernatant (pellet is clear and nearly invisible).
6. Dissolve the pellet in 50 µl TE. Check quality and recovery on a gel.

If the PCR template is a plasmid that contains the Km^r or Ap^r gene, treat the PCR products with *Dpn* I to degrade the plasmid. To a 50-µl reaction, add 5 µl of 10X REact® 4 Buffer and 5 units of *Dpn* I, and incubate for 15 min at 37°C. Heat-inactivate the *Dpn* I at 65°C for 15 min.

3.4.2 Cloning *att*B-PCR Products Directly into Destination Vectors

This one-tube protocol moves *att*B-PCR products into a pDEST™32 or pDEST™22 in two steps: a BP Reaction followed by an LR Reaction. This two-step protocol can also be used to transfer the gene from a linearized Expression Clone into pDEST™32 or pDEST™22.

1. Add the following to a 1.5-ml tube at 25°C.

Component	Volume (µl)
PCR Product (*att*B DNA) (100-200 ng)	5
pDONR™201 (*att*P DNA) (150 ng/µl)	2.5
BP Reaction Buffer 5X	5
TE	sufficient to bring volume to 20 µl

Note: *E. coli* strains such as MAX Efficiency® DH5α™ or DH10B™ cells are appropriate for this procedure.

2. Remove BP Clonase™ Enzyme Mix from -70°C and thaw on ice (~2 min)
3. Vortex BP Clonase™ Enzyme Mix briefly (2 s) twice.
4. Add 5 µl of BP Clonase™ Enzyme Mix. Return BP Clonase™ Enzyme Mix to -70°C.
5. Mix and incubate for 4 h at 25°C.

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate 6 to 20 h.

6a. Remove 5 µl of the reaction to a separate tube. Add 0.5 µl of proteinase K solution to this tube. Incubate for 10 min at 37°C.

Do not use an *E. coli* strain containing an F episome. F episomes contain the *ccd*A gene which prevents negative selection with *ccd*B.

When the template for PCR has the same selectable marker as the final Destination Vector (most commonly Ap^r), plate on LB plates containing 50 µg/ml ampicillin to determine the amount of false-positive colonies carried over to the LR Reaction.

6b. Transform 100 µl of competent cells with 1 µl of the remaining mixture. Plate on LB plates containing 50 µg/ml kanamycin. **Note:** The number of colonies will indicate the efficiency of the BP reaction. In addition, isolated Entry Clones can be used in later protein expression and analysis.

7. To the remaining 20-µl reaction, add:

Component	Volume (µl)
NaCl (0.75M)	1
pDEST™32 or pDEST™22 (150 ng/µl)	3

8. Remove LR Clonase™ Enzyme Mix from -70°C and thaw on ice (~2 min).
9. Vortex LR Clonase™ Enzyme Mix briefly (2 s) twice.
10. Add 6 µl of LR Clonase™ Enzyme Mix. Return LR Clonase™ Enzyme Mix to -70°C.

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate 3 h to overnight.

11. Mix and incubate for 2 h at 25°C.
12. Add 3 µl of Proteinase K solution. Incubate for 10 min at 37°C.

Do not use an *E. coli* strain containing an F episome. F episomes contain the *ccd*A gene which prevents negative selection with *ccd*B.

13. Transform 100 µl of DH5α™ or DH10B™ competent cells (>10^8 CFU/µg) with 1 µl of the reaction. Plate on LB plates containing 10 µg/ml gentamicin for pDEST™32 or 100 µg/ml ampicillin for pDEST™22. The resulting colonies are the DB-X Expression Clone and AD-Y Expression Clone, respectively.
Note: The total number of Expression Clone colonies (from step 13) is usually 10% to 20% of the total number of Entry Clone colonies (from step 6b).

3.4.3 Transferring Genes from Gateway® Entry Clones into pDEST™32 or pDEST™22 Using the LR Reaction

Use this protocol to transfer your gene of interest from an Entry Clone into pDEST™22 and pDEST™32 if the gene of interest is already in a Gateway® Entry Clone. To get your gene into an Entry Clone, refer to the specific entry vector manual. The reaction of an Entry Clone (*att*L) with a Destination Vector (*att*R) creates a new Expression Clone (*att*B).

Purify plasmid DNA with the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) for best results. Alternatively, DNA can be purified using an alkaline lysis protocol, with or without RNase treatment. During alkaline lysis treatment, keep the NaOH ≤0.125 M to minimize irreversible denaturation of the supercoiled plasmid DNA.

The efficiency of the LR Reaction depends upon the topology of the plasmids. The efficiency is greater when either or both plasmids are linear. All Destination Vectors are provided linearized.

1. Add the following to 1.5-ml tubes at room temperature and mix.

	Negative Control	Positive Control	Sample
Component	**Tube 1**	**Tube 2**	**Tube 3**
LR Reaction Buffer (5X)	4 µl	4 µl	4 µl
Entry Clone (*att*L DNA) (100 - 300 ng/reaction)	---	---	1-11 µl
pDEST™32 or pDEST™22 (*att*R DNA)(~300 ng/reaction)	1-11 µl	1-11 µl	1-11 µl
TE	to 16 µl	to 16 µl	to 16 µl

2. Remove LR Clonase™ Enzyme Mix from -70°C and thaw on ice (~2 min).
3. Vortex LR Clonase™ Enzyme Mix briefly (2 s) twice.
4. Add 4 µl of LR Clonase™ Enzyme Mix to each reaction. Return LR Clonase™ Enzyme Mix to -70°C.
5. Incubate reactions at 25°C for 1 h.

Do not use an *E. coli* strain containing an F episome. F episomes contain the *ccd*A gene which prevents negative selection with *ccd*B.

6. Add 2 µl of Proteinase K Solution to all reactions. Incubate for 10 min at 37°C.
7. Transform 1 µl of each reaction into 100 µl DH5α™ or DH10B™ competent cells (>10^8 CFU/µg). Incubate on ice for 30 min. Heat-shock the cells at 42°C for 30 s. Add 450 µl S.O.C. Medium and incubate at 37°C for 1 h.
8. Plate 20 µl and 100 µl of the transformation separately on LB plates containing 10 µg/ml gentamicin for pDEST™32 or 100 µg/ml ampicillin for pDEST™22.

   Note: *Bsr*G I cleaves within all *att* sites, and can be used to help characterize clones.

   - **To confirm that the design and construction of the fusion proteins are correct, it is strongly recommended that all vector/insert DNA junctions be sequenced to confirm the reading frame.**
   - **If available, a cDNA protein known to interact with the test DNA should be cloned into the AD vector pDEST™22 (in frame with the GAL4 sequence encoding the Activation Domain) to serve as a positive control for interaction.**

Note: Custom Gateway®-compatible two-hybrid cDNA libraries are available.

3.4.4 Construction of cDNA Libraries in AD-vectors

Premade cDNA libraries in pPC86 are available for use in the ProQuest™ Two-Hybrid System. pPC86 is a cloning vector similar to pEXP-AD502 with the GAL4 Activation Domain (AD) but without the recombination sites, *att*B1 and *att*B2. Gateway®-compatible ProQuest™ Two-Hybrid libraries will be available soon. Please check the web site at www.invitrogen.com for the most up-to-date list. cDNA libraries can be constructed using the SuperScript™ Plasmid System with Gateway® Technology for cDNA Synthesis and Plasmid Cloning in vector pEXP-AD502.

3.5 Assessing Self-Activation of DB-X and AD-Y

The following procedures require replica plating and replica cleaning. It is recommended that these techniques are practiced using Yeast Control Strains A-E following the method described in section 5.4 and the procedure outlined in Figure 10. The expected results are shown in Figure 11.

Before performing a two-hybrid screen, test DB-X (and AD-Y, if testing two known proteins) for self-activation and to determine the basal expression levels of the *HIS*3 reporter gene. Titrate *HIS*3 activity on plates lacking histidine containing 3-Amino-1,2,4-Triazole (3AT) to a point at which growth in the absence of histidine is inhibited.

The initial selection for interacting proteins involves induction of the *HIS*3 reporter gene. It is therefore critical to determine the DB-X induced basal levels of *HIS*3 reporter gene expression since some transcription factors, and many other proteins with no apparent function in transcription activation, may contain domains that cause some level of self-activation when fused to GAL4 DNA binding domain.

*HIS*3 encodes imidazole glycerol phosphate dehydratase, an enzyme involved in histidine biosynthesis. This enzyme can be specifically inhibited in a dose-dependent manner by 3-Amino-1,2,4-Triazole (3AT) (2,18). To maximize sensitivity of the *HIS3* reporter gene, the MaV203 strain expresses a basal level of *HIS3*. By determining the threshold of resistance to 3AT and including that concentration of 3AT in plates lacking histidine, even slight increases in *HIS*3 reporter gene expression can be detected. This enhances the likelihood of detecting even weak protein:protein interactions. It is best to test DB-X in the presence of the AD vector (pEXP-AD502) since there is a possibility that a DB-X/AD complex could form and activate transcription.

Important: The concentration of 3AT determined below must be included in all selection plates lacking histidine when performing two-hybrid screens.

Note: If preparing your own competent yeast, we recommend using the sequential transformation protocol found on the web site.

3.5.1 Small-Scale Transformation of MaV203 with DB-X (and AD-Y)

Use Table 2 as a guide for transformation. If screening a cDNA library, use transformations number 1 to 4. Store representative transformants in glycerol at -70°C for future use. If testing whether known protein X (DB-X) interacts with known protein Y (AD-Y), include transformations 5 and 6. This protocol uses Subcloning Scale MaV203 Competent Yeast cells (Cat. No. 11445-012).

Table 2. Small-Scale Transformation of MaV203.

Transformation	Plasmid 1	Plasmid 2	Selection	Purpose
1	control plasmid (pDBLeu)	none	SC-Leu	transformation control
2	none	none	SC-Leu and SC-Leu-Trp	transformation control
3	pDBLeu	pEXP-AD502	SC-Leu-Trp	self-activation control
4	DB-X	pEXP-AD502	SC-Leu-Trp	test self-activation of DB-X
5	pDBLeu	AD-Y	SC-Leu-Trp	self-activation of AD-Y if screening known X and Y
6	DB-X	AD-Y	SC-Leu-Trp	interaction test for known X and Y
7	DB-X	none	SC-Leu	for sequential transformation if screening cDNA libraries

1. Thaw the PEG/LiAc Solution (included with the competent cells) in a beaker containing room temperature water. Mix the solution well before dispensing.
2. Thaw the MaV203 competent cells in a 30°C water bath for no more than 90 s. Proceed immediately to step 3. Steps 3, 4, and 5 can be done at room temperature.
3. Once the cells are completely thawed, invert the cells several times and transfer 100 µl to a 1.5-ml autoclaved tube. (DO NOT VORTEX THE CELLS.)
4. To each 100-µl aliquot of cells, add 100 ng of each vector DNA. Mix well by tapping the tube. For a control transformation, add 5 µl of pMAB12 DNA to another tube containing 100-µl aliquot of cells.

Note: For a single plasmid transformation using 100 ng plasmid DNA, ~5×10^3 to 1×10^4 colonies are typically obtained. For co-transformation using 100 ng pDBLeu and 100 ng pEXP-AD502 vector DNAs, ~10^2 colonies are typically obtained.

5. Add 600 µl of the PEG/LiAC solution to each tube. Mix well by inversion.
6. Incubate for 30 min in a 30°C water bath. Invert the tubes every 10 min to resuspend the components.
7. Add 35.5 µl of DMSO to each tube. Mix well by inversion.
8. Heat shock the cells for 20 min at a 42°C water bath. Invert the tubes occasionally.
9. Centrifuge tubes for 5 s at 200 to 400 x *g* (1,800 rpm in most microcentrifuges). Carefully discard the supernatant.
10. Suspend each cell pellet in 1 ml of autoclaved saline (0.9% NaCl) by gentle pipetting.
11. Plate 100 µl of the undiluted transformation and 100 µl of a 1:10 dilution (in autoclaved saline) onto the appropriate selection plates (see Table 2). Incubate for 60 to 72 h at 30°C.

3.5.2 Determining the 3AT Concentration Necessary to Titrate Basal Levels of *HIS*3 Expression

1. After incubation, patch four different colonies from each transformation using autoclaved toothpicks on a single SC-Leu-Trp plate along with two colonies each from Yeast Control Strains A-E. Incubate for ~18 h at 30°C.

STOP!

Before proceeding: read section 5.4, Replica Plating/Replica Cleaning. The Key to success with the ProQuest™ system is successful replica plating and replica cleaning. The Replica Clean Step is Critical. Failure to properly replica clean plates can result in high background levels.

2. Replica plate from this SC-Leu-Trp master plate onto SC-Leu-Trp-His plates containing 3AT at concentrations of 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. Immediately replica clean the plates. Incubate for 24 h at 30°C.

   Note: Figure 9 illustrates the plating of two test colonies. It is important to plate four test colonies as described in the previous step, for more accurate determination of the optimum concentration of 3AT required.

Proper replica cleaning requires a fair amount of pressure. After replica cleaning the plate should not contain any visible cell material. If any cell material is present, repeat the replica clean procedure with a fresh velvet. It is sometimes necessary to replica clean a plate several times to remove all the cell material. See Figure 13 for replica plates with and without cleaning.

3. After incubation, replica clean again and incubate for 2 days (40-44 h) at 30°C. The lowest concentration of 3AT that inhibits the growth of the cells from transformation 4 (or 6) (Table 2) is the basal amount of 3AT added to all plates lacking histidine. A representative result from a titration is shown in Figure 9.

If cells from transformation 4 (or 6) grow even in the presence of 100 mM 3AT, the DB-X (or AD-Y) plasmid likely encodes a protein that directly or indirectly self-activates the reporter genes in this system. Such plasmids are not suitable for use in the two-hybrid screen.

An alternative approach may allow use of these test DNA segments in a two-hybrid screen. A portion of the test DNA segment that lacks the amino acids responsible for the high levels of self-activation (*e.g.,* deletion derivatives of the original clone) may be cloned into the DB vector.

3.6 Testing the Interaction of DB-X Against AD-Y

Note: If screening an AD-Library with DB-X, proceed to section 3.7.

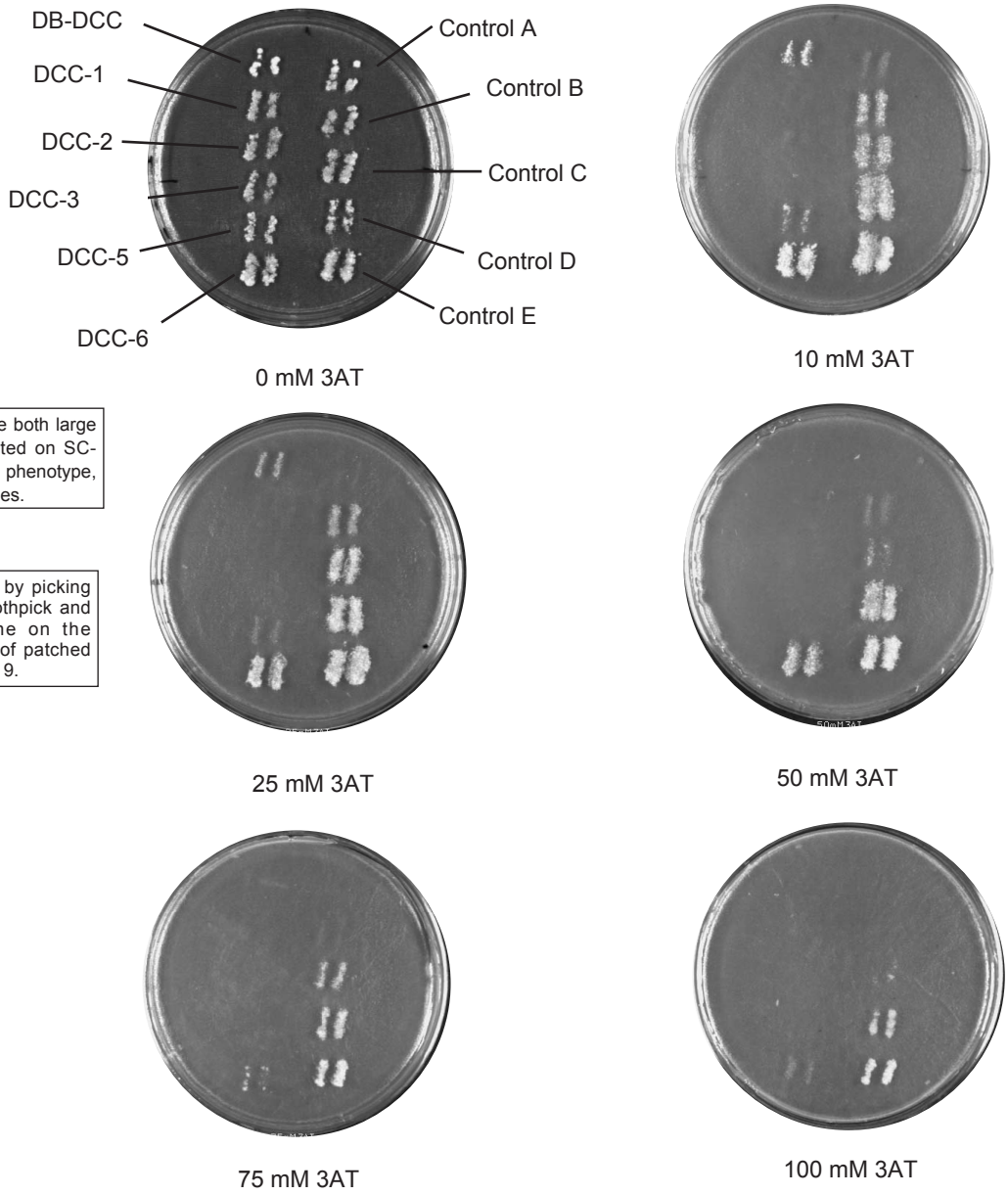
Because relatively few colonies are required for this assay, a small-scale co-transformation procedure can be used. If MaV203 containing DB-X and AD-Y was constructed and characterized as transformation 7 in the experiment described in section 3.5.1, proceed to step 3 below. If MaV203(DB-X)(AD-Y) was not constructed, proceed as follows:

1. Prepare small-scale DB-X and AD-Y plasmid DNA (18) and an appropriate number of 10-cm selection plates: YPAD (≥2 plates), SC-Leu (≥4 plates), SC-Leu-Trp (≥10 plates), SC-Leu-Trp-Ura (≥2 plates), SC-Leu-Trp-His+3AT (≥2 plates), SC-Leu-Trp+0.2% 5FOA (section 5.3).
2. Perform the transformations described in Table 3 using the procedure outlined in section 3.5.1. Incubate for 48 h at 30°C.

Table 3. Transformation to Test the Interaction of DBLeu-X Against pEXP-AD502.

Transformation	Plasmid 1	Plasmid 2	Selection	Purpose
A	control plasmid (pDBLeu)	none	SC-Leu	transformation control
B	none	none	SC-Leu and SC-Leu-Trp	transformation control
C	DB-X	pEXP-AD502	SC-Leu-Trp	test self-activation of DB-X
D	pDBLeu	AD-Y	SC-Leu-Trp	self-activation of AD-Y if screening known X and Y
E	DB-X	AD-Y	SC-Leu-Trp	interaction test for known X and Y

Methods



Note: Control D may generate both large and small colonies when plated on SC-Leu-Trp. To obtain the correct phenotype, always choose the small colonies.

Note: Colonies are patched by picking cell material with a sterile toothpick and lightly drawing a short line on the indicated plate(s). Examples of patched colonies are provided in Figure 9.

Figure 9. Titration of DB-X on 3AT Plates. The human DCC (deleted in colorectal cancer) gene cytoplasmic domain sequences were fused to the *GAL*4 sequence encoding the DNA Binding Domain (DB-DCC), as were derivatives deleted for increasing amounts of DNA at the 3′ end (DCC-3, DCC-2, and DCC-1, respectively) or deleted for increasing amounts of DNA at the 5′ end (DCC-5 and DCC-6). Each of these constructs was introduced, along with pPC86, into MaV203 (selecting SC-Leu-Trp), patched onto a SC-Leu-Trp master plate, incubated for 18 h at 30°C, replica plated to SC-Leu-Trp-His+3AT at 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM, immediately replica cleaned, and incubated for 24 h at 30°C. These plates were then replica cleaned again and incubated for 2 days at 30°C. SC-Leu-Trp-His plates using DCC-1, DCC-2 and DCC-3 contained 10 mM 3AT; DCC-5 was plated on 25 mM 3AT; and DB-DCC was plated on 50 mM 3AT. DCC-6 shows growth on 100 mM 3AT and should not be used. The DCC clones were the kind gift of Drs. Gang Hu, Marc Vidal and Eric Fearon (19).

STOP!

Please review section 5.4, Replica Plating/Replica Cleaning. The key to success with the ProQuest™ system is successful replica plating and replica cleaning.

3. Analyze the transformants as described in Figure 10.
   a. Patch 4 different colonies per transformation (from transformations C, D and E, Table 3) to SC-Leu-Trp plates. Also patch two isolated colonies of the 5 yeast control strains onto the same plate. Incubate for 18 h at 30°C.
   b. Replica plate to SC-Leu-Trp-His+3AT plates using the optimal concentration of 3AT determined in section 3.5.1, and to SC-Leu-Trp-Ura, SC-Trp-Leu+0.2% 5FOA, and onto a nitrocellulose or nylon membrane placed on the surface of a YPAD plate for use in the X-gal Assay (section 5.7). Immediately replica clean the 3AT and 0.2% 5FOA plates. Incubate all plates for 24 h at 30°C.

Note: When patching colonies to the master plate, remain a fair distance from the edge of the plate. Patches close to the edge replica plate poorly and can give inaccurate results.

Note: Control D may generate both large and small colonies when plated on SC-Leu-Trp. To obtain the correct phenotype, always choose the small colonies.

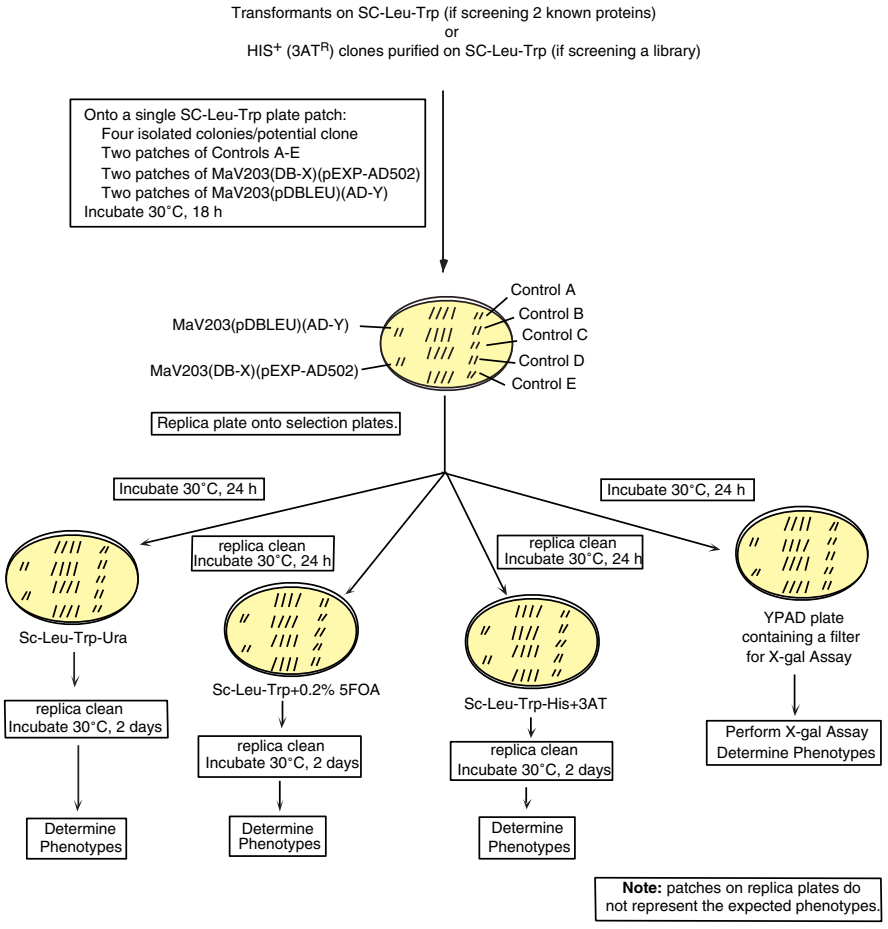


Figure 10. Screening Candidate Interactors for the Induction of Reporter Genes.

IMPORTANT: The Replica Clean Step is Critical. Failure to properly replica clean plates can result in high background levels.

Proper replica cleaning requires a fair amount of pressure. After replica cleaning, the plate should not contain any visible cell material. If any cell material is present, repeat the replica clean procedure with a fresh velvet. It is sometimes necessary to replica clean a plate several times to remove all the cell material. See Figure 13 for replica plates with and without cleaning.

c. After 18-24 h incubation of the YPAD plates containing a membrane, perform an X-gal Assay on the membrane as described in section 5.7.
d. After incubation of the selection plates for 24 h, replica clean the following plates:
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp+0.2% 5FOA
   - SC-Leu-Trp-His+3AT
e. Incubate for 2 additional days at 30°C.
f. Compare the phenotypes of the potential positives to Yeast Control Strains A-E (Figure 11). Only the colonies that induce the three reporter genes are further analyzed. However, weak phenotypic differences should be considered.

Note: As observed with Yeast Control Strain B (DB-Rb/AD-E2F1), intermediate levels of *URA*3 expression can result in both cell growth inhibition on 0.2% 5FOA and insufficient *URA*3 gene product to allow growth on plates lacking uracil (Figure 11). The $5FOA^S/Ura^-$ phenotype is often indicative of protein pairs that interact weakly in the ProQuest™ Two-Hybrid System.

Note: The strength of the three different reporter gene read-outs can vary dramatically between different pairs of interacting proteins. For example, DB-Rb/AD-E2F1 and DB-Jun/AD-Jun are both relatively weak interactions. However, in the ProQuest™ System, the DB-Rb/AD-E2F1 interaction confers a relatively strong growth inhibition ($5FOA^S$) phenotype but a low β-gal activity (Figure 11). On the other hand, DB-Jun/AD-Jun interaction confers a very weak growth inhibition ($5FOA^S$) and relatively high β-gal activity (7).

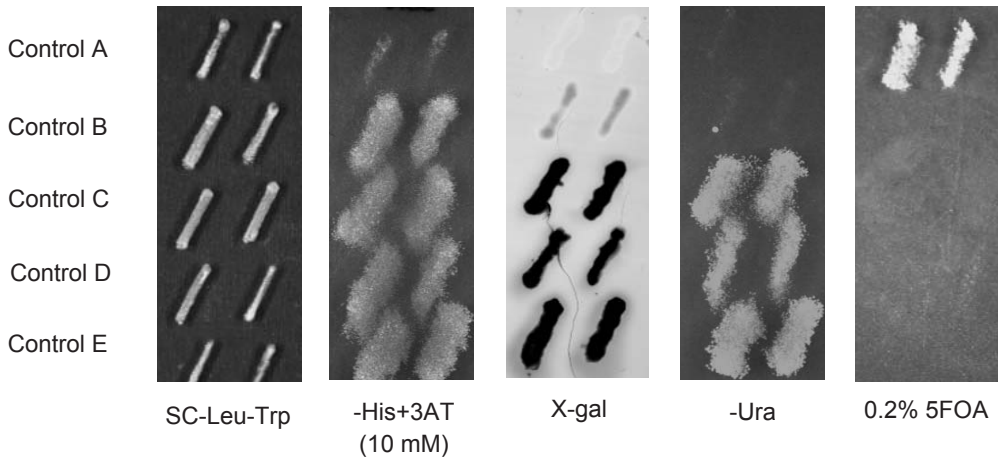


Figure 11. Reporter Gene Phenotypes of Yeast Control Strains A-E. Yeast Control Strains A-E were patched from isolated colonies onto an SC-Leu-Trp master plate and incubated for 18 h at 30°C. Cells from this master plate were replica plated onto SC-Leu-Trp-His+3AT (10 mM), a YPAD plate containing a nylon membrane (for X-gal Assay), SC-Leu-Trp-Ura, and SC-Leu-Trp+0.2% 5FOA, replica cleaned where indicated (Figure 10), and treated as described in Figure 10.

3.7 Screening AD-Libraries for cDNAs Encoding Proteins that Interact with DB-X

Introduce DB-X and AD-Y (library) plasmids into MaV203 using co-transformation (both vectors transformed simultaneously).

If you prepare your own competent yeast, sequential transformation is suggested because it is more efficient. .

3.7.1 Co-transformation with DB-X and the AD-Library

We recommend that you screen >10^6 yeast transformants for mammalian cDNA libraries. The protocol below achieves this number in a single co-transformation using Library-Scale Frozen Competent MaV203 Yeast cells.

500 µl of library-scale competent cells results in ≥2×10^6 colonies. Each tube contains 550 µl competent yeast cells. These can be divided into two 250-µl aliquots and used for two independent library screens (≥1×10^6 each), or both 250-µl aliquots can be used for a single large library screen. Plating each 250-µl aliquot of competent cells will require twenty 15-cm SC-Leu-Trp-His+3AT plates [the optimum concentration of 3AT for DNA binding domain vectors containing test gene X must be determined prior to doing a library transformation (Section 3.5.2)] and three 10-cm SC-Leu-Trp plates to determine the total number of transformants.

Include a control transformation using the “Control Transformation Procedure” provided with the Competent Yeast.

Note: The use of large amounts of DNA (10 µg DB-X and 10 µg AD-Y) can result in multiple AD-Y clones in a single transformant. Such transformants may show growth on 5FOA plates (see Table 4, section 4.2) and care must be taken to identify and retest all candidate AD-Y clones following isolation in *E. coli*. The use of 5 µg of each vector will reduce the number of transformants containing multiple copies of AD-Y, but will also reduce transformation efficiency slightly.

Note: Once thawed, do not refreeze competent yeast cells. Refreezing can result in a substantial loss in transformation efficiencies.

1. Thaw the PEG/LiAc Solution (included with the Competent MaV203) in a beaker containing room temperature water. Mix the solution well before dispensing.
2. Thaw competent yeast cells in a 30°C water bath for no longer than 90 s. Once thawed, keep the tubes at room temperature. Invert the tube of cells several times to mix (*Do not vortex the cells*). Immediately transfer 250 µl to two 15- or 50-ml polypropylene tubes.
3. Transfer 25 µl cells to two 1.5-ml tubes and simultaneously perform the control assay as directed, using the protocol provided with the competent yeast cells.

The following protocol is for 250 µl of cells.

4. Add 10 µg of DB-X and 10 µg of AD-library. Mix well by swirling the tubes.
5. Add 1.5 ml of the PEG/LiAc Solution. Mix well by swirling.
6. Incubate for 30 min in a 30°C water bath. Swirl occasionally (every 10 min) to resuspend.
7. Add 88 µl of DMSO. Mix well by swirling.
8. Heat shock the cells for 20 min in a 42°C water bath. Swirl the tubes occasionally.
9. Centrifuge for 5 min at 640 × *g*. Carefully remove the supernatant.
10. Suspend the pellet in 8 ml autoclaved saline (0.9% NaCl).
11. To estimate the total number of transformants, plate two dilutions of the transformation. Mix 10 µl of transformation with 90 µl autoclaved, distilled water. Plate 100 µl on a 10-cm SC-Leu-Trp plate (1:800 final dilution factor). Mix 10 µl of transformation with 990 µl autoclaved, distilled water. Plate 100 µl on a 10-cm SC-Leu Trp plate (1:8,000 final dilution factor).

12. Plate 400-µl aliquots of the transformation on 15-cm SC-Leu-Trp-His+3AT plates. Twenty plates will be required to plate all the cells.
13. Incubate for 60 to 72 h at 30°C.
14. Calculate the number of transformants on the 10-cm plates as follows:
    a. Count the number of colonies on the plates having 20 to 300 colonies.
    b. Multiply the number of colonies counted by the dilution factor.
       Example: 125 colonies are counted on the 1:8,000 plate.
       $125 \times 8{,}000 = 1 \times 10^6$ colonies per 250-µl transformation
15. Replica clean each 15-cm plate according to the procedure in section 5.4.
16. Incubate for 2 to 3 days at 30°C. Analyze the resulting colonies as described in section 3.8.

3.8 Characterization of Transformants that Induce the *HIS*3 Reporter Gene

Induction of the three reporter genes present in this system (*HIS*3, *URA*3, *lacZ*; Figure 5) is seen in MaV203 cells that contain DB-X and AD-Y interacting proteins. These colonies are identified by a series of patching and replica plating steps onto the selection/screen plates.

The growth properties of yeast cells on the selection/screen plates can be influenced by several parameters in addition to the induction levels of the reporter genes:

- Cells approaching stationary phase exhibit different expression levels of the hybrid proteins from cells growing in exponential phase.
- As the number of cells transferred by replica plating increases, the phenotypic differences between positive and negative controls decrease. Replica cleaning dilutes and normalizes the number of cells plated.
- The amount of growth of yeast patches on a particular selection plate will vary dramatically between 2 and 6 days of growth. Correct incubation times are critical.

Note: It is important to follow the incubation times indicated in Figure 10.

Figure 10 shows the scheme for testing putative His^+ ($3AT^r$) positive transformants for the other reporter genes.

STOP!

Please review section 5.4, Replica Plating/Replica Cleaning.

1. Streak transformants that grow on SC-Leu-Trp-His+3AT on SC-Leu-Trp plates to isolate single purified colonies. Incubate plates for 48 h at 30°C.
2. Prepare fresh colonies of the 5 yeast control strains by streaking from the glycerol stocks onto SC-Leu-Trp plates. Also prepare fresh colonies of MaV203 (DB-X)(pEXP-AD502) constructed in section 3.5.1. Incubate for 48 h at 30°C.

Note: When patching colonies to the master plate, remain a fair distance from the edge of the plate. Patches close to the edge replica plate poorly and can give inaccurate results.

3. Using an autoclaved toothpick or loop, patch onto a single SC-Leu-Trp plate the following: (Figure 10)
   - two isolated colonies of Yeast Control Strains A-E
   - two isolated colonies of MaV203 (DB-X)(pEXP-AD502)
   - four isolated colonies of each potential positive clone (up to four clones can be analyzed per plate)
   - If more than 4 His^+ clones were identified, use additional plates. On each plate include the 5 yeast controls and MaV203 (pDB-X)(pEXP-AD502).

Note: Control D may generate both large and small colonies when plated on SC-Leu-Trp. To obtain the correct phenotype, always choose the small colonies.

4. Incubate plates for 18 h at 30°C.

IMPORTANT: The Replica Clean Step is Critical. Failure to properly replica clean plates can result in high background levels.

Proper replica cleaning requires a fair amount of pressure. After replica cleaning the plate should not contain any visible cell material. If any cell

material is present, repeat the replica clean procedure with a fresh velvet. It is sometimes necessary to replica clean a plate several times to remove all the cell material. See Figure 13 for replica plates with and without cleaning.

5. Replica plate onto the following plates, in the order listed. Replica clean where indicated (Figure 10).
   - YPAD containing a nitrocellulose or nylon membrane for an X-gal Assay
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp-His+3AT; replica clean
   - SC-Leu-Trp+0.2% 5FOA; replica clean
6. Incubate all plates for ~24 h at 30°C.
7. After 18 to 24 h incubation of the YPAD plates containing a membrane, perform an X-gal Assay on the membrane as described in section 5.7.
8. After incubation of the selection plates for 24 h, replica clean the following plates:
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp+0.2% 5FOA
   - SC-Leu-Trp-His+3AT
9. Incubate for 2 additional days at 30°C.
10. Compare the phenotypes of the potential positives to Yeast Control Strains A-E (Figure 11). Only the colonies that induce the three reporter genes are further analyzed. However, weak phenotypic differences should be considered (see also section 4.2 and the notes below for comments on interpretation of the phenotypes).

Note: As observed with Yeast Control Strain B (DB-Rb/AD-E2F1), intermediate levels of *URA*3 expression can result in both cell growth inhibition on 0.2% 5FOA and insufficient *URA*3 gene product to allow growth on plates lacking uracil (Figure 11). The $5FOA^S/Ura^-$ phenotype is often indicative of protein pairs that interact weakly in the ProQuest™ Two-Hybrid System.

NOTE: The strength of the three different reporter gene read-outs can vary dramatically between different pairs of interacting proteins. For example, DB-Rb/AD-E2F1 and DB-Jun/AD-Jun are both relatively weak interactions. However, in the ProQuest™ Two-Hybrid System, the DB-Rb/AD-E2F1 interaction confers a relatively strong growth inhibition ($5FOA^S$) phenotype but a low β-gal activity (Figure 11). On the other hand, DB-Jun/AD-Jun interaction confers a very weak growth inhibition ($5FOA^S$) and relatively high β-gal activity (7).

3.9 Reassessment of Interactions

Several types of “false positives” can result in phenotypes resembling true interactions (*e.g.,* a mutation in DB-X that converts it to a self activator). If the DB-X/AD-Y interactions identified above are authentic, the reporter gene phenotype should be reproduced when AD-Y is reintroduced into MaV203 with the original DB-X plasmid, but not when pDBLeu containing no insert is introduced.

Two strategies are typically employed to confirm the reporter gene phenotypes: retransformation assay and plasmid shuffling. Either method can be used with the ProQuest™ System. The plasmid shuffling method is fast and easy, but does not exclude false positives resulting from host cell mutations. The retransformation assay results in the isolation of the AD-Y plasmid in *E. coli*, which may facilitate additional analysis (*e.g.,* sequence analysis). The retransformation assay is described below and summarized in Figure 12. The plasmid shuffling assay is provided in section 5.10.

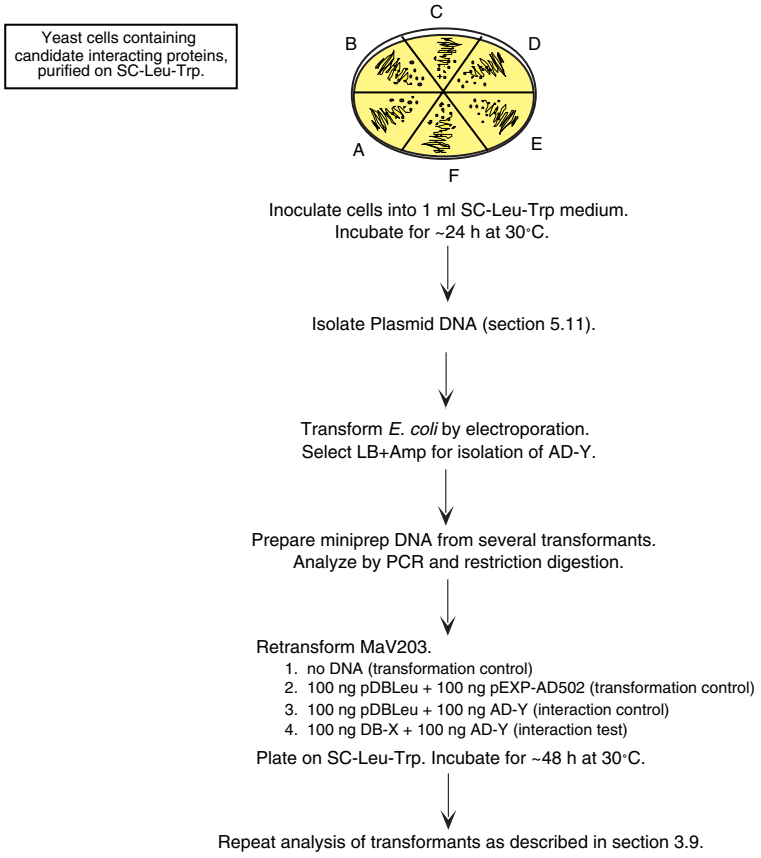


Figure 12. Retransformation Assay.

3.9.1 Retransformation Assay

1. Isolate plasmid DNA from candidate yeast strains (section 5.9).
2. Transform DNA into *E. coli* ElectroMAX™ DH10B™ cells. Plate on LB+100 µg/ml ampicillin to selectively isolate the AD-Y plasmid and not the DB-X plasmid. For isolation of DB-X plasmid, plate the transformation on LB+10 µg/ml gentamicin.
3. Cultivate several ampicillin resistant colonies per candidate clone in LB+100 µg/ml ampicillin broth. Prepare miniprep DNA (19) and examine by restriction analysis (typically *Sal* I + *Not* I to excise the cDNA insert) or by PCR. It is often useful to perform restriction digestion (using frequent cutters) on PCR-amplified cDNA inserts to identify similar clones based on common restriction fragments.
4. Reintroduce plasmid DNA from representative isolates by transformation into MaV203 using the procedure outlined in section 3.5.1. Perform two co-transformations: AD-Y + pDBLeu (vector alone), and AD-Y + DB-X. It is also useful to perform control transformations with no DNA (negative control) and with pDBLeu + pEXP-AD502 as a positive control for the transformation. Plate the transformation mixtures on SC-Leu-Trp plates and incubate for 48 h at 30°C.

5. Patch four different colonies from each transformation onto SC-Leu-Trp plates, along with the five yeast controls, and incubate for 18 h at 30°C (Figure 10). Replica plate this master plate onto the following plates. Replica clean where indicated.
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp+0.2% 5FOA; replica clean
   - SC-Leu-Trp-His+3AT; replica clean
   - YPAD containing a nitrocellulose or nylon membrane for an X-gal Assay
6. Treat as described in steps 6-10 of section 3.8 (Figure 10). Clones that reproduce the original phenotypes likely represent interacting DB-X:AD-Y fusion proteins.

3.10 Additional Characterization of Protein Interactions

Additional experiments must be performed to confirm that the interactions observed with a two-hybrid screen are direct interactions (*e.g.*, between DB-X and AD-Y) and if so, whether these interactions are biologically relevant. Several experiments and assays have been described that can be used as general methods to approach these issues (7,20):

- The cloned DNA can be sequenced and if the identity is known, the likelihood of biological relevance can often be surmised.
- The fusion proteins encoded by the cloned DNAs can be expressed and purified in *E. coli* or *in vitro*, and used in co-immunoprecipitation experiments with antibody to the test protein or using monoclonal antibodies to the GAL4-DB or GAL4-AD domains (4,7,20,21).
- For quantitation data on the *lacZ* reporter gene induction in liquid culture, refer to section 5.8.
- Other methods for the *in vitro* characterization of protein:protein interactions have been reviewed recently (22).

In addition to *in vitro* methods, assays demonstrating the biological relevance of the candidate interactions should be designed and tested. Mutations that affect the protein:protein interaction can be generated and these alleles can be tested in the ProQuest™ System (7).

4 Troubleshooting Guide

4.1 General Troubleshooting Guidelines

Note: For Troubleshooting Guidelines for the Gateway® Cloning System, please refer to the Gateway® Technology manual available from our web site (www.invitrogen.com) or by contacting Technical Service.

Problem	Possible Cause	Suggested Solution
Determining the basal levels of HIS3 expression:		
Growth of Yeast Control Strain A on SC-Leu-Trp-His+3AT	Plates not replica cleaned	Replica clean immediately after replica plating, and again after 24 h incubation (Figure 13).
	Inadequate replica cleaning	Review section 5.4. Immediately after replica cleaning, plate should contain no remaining visible cells.
	Too many cells transferred during replica plating	Review section 5.4. Transfer a minimal number of cells.
	Incorrectly prepared 3AT plates	Review section 5.3.4. Confirm that all stock solutions were fresh and prepared correctly. Confirm that the calculation for amount of 3AT addition was correct.
	Incorrect incubation times	Incubate plates no longer than 48 h (40-44 h is usually best).
Failure of Yeast Control Strains B-E to grow on SC-Leu-Trp-His +3AT	Incorrectly prepared 3AT plates	Review section 5.3.4. Confirm that all stock solutions were prepared correctly and fresh. Confirm that the calculation for amount of 3AT addition was correct.
	Control strains are too old or were mixed up	Return to the original glycerol stocks provided, streak on SC-Leu-Trp, and isolate fresh colonies.
	Uneven replica plating	When replica plating, maintain an even pressure across the entire surface of the master and selection plates. Uneven pressure can result in the failure of cells to transfer.
Failure of transformants to grow on any of the SC-Leu-Trp-His+3AT plates	Incorrectly prepared 3AT plates	Review section 5.3.4. Confirm that all stock solutions were prepared correctly and fresh. Confirm that the calculation for amount of 3AT addition was correct.
	Strains being tested do not contain DB-X and pEXP-AD502	Confirm growth on SC-Leu-Trp plates.
	Uneven replica plating	When replica plating, maintain an even pressure across the entire surface of the master and selection plates. Uneven pressure can result in the failure of cells to transfer.
Growth of MaV203(DB-X) (pEXP-AD502) transformants on all concentrations of 3AT	DB-X self activates	Subclone segments of X into pDEST™32 and retest.
	Incorrectly prepared 3AT plates	See above.
	Improper replica plating or replica cleaning	See above.
	Incorrect incubation times	See above.

Problem	Possible Cause	Suggested Solution
No transformants on SC-Leu-Trp-His+3AT (candidate interactors)	Gene of interest not in frame with *GAL*4 DNA Binding Domain-encoding sequence	Sequence the DB/test DNA junction.
	Poor quality cDNA library	Determine the percent of vectors containing inserts and their average size.
	Inadequate amount of cDNA library used in transformation	Confirm concentration of library.
	Test DNA (X) cloned into pDEST™32 lacks or masks a domain required for protein:protein interaction	Clone and test alternative segments of the test DNA (X).
	cDNA library used does not contain proteins that interact with test protein X	Screen a cDNA library from an alternative tissue, developmental time point, or organism.
		Determine whether the test gene is expressed in the library.
	AD-Y fusion that interacts with DB-X may be toxic, unstable or require post-translational modification	Some posttranslational modifications can not be accomplished in yeast (23).
		Make sure a cDNA library is constructed in pPC86 or pEXP-AD502 and not in other high-copy-number AD-vectors.
High background on SC-Leu-Trp-His+3AT	3AT concentration too low	Retest MaV203(DB-X)(pEXP-AD502) on various concentrations of 3AT (section 3.5.1, Figure 9).
	Plates made incorrectly	See “Growth of Yeast Control Strain A on SC-Leu-Trp-His+3AT” above.
	Improper replica cleaning	Replica clean where indicated. Review section 5.4. Immediately after replica cleaning, plate should contain no visible cell material (although a faint haze may be present on 3AT transformation plates).
	Improper incubation times	Do not incubate plates longer than 60 h. Colonies arising after 60 h are not likely to be of interest.
Reassessment of the Interaction/ Retransformation Assay:		
Failure to obtain *E. coli* transformants	*E. coli* not sufficiently competent	Use ElectroMAX™ DH10B™ cells.
	Too much DNA used	Use only 1 µl of DNA. Inhibitory compounds reduce electroporation efficiencies if more DNA is used.
	Incorrect selection or concentration	Select for DB-X plasmid on LB+gentamicin (10 µg/ml). Select for AD-Y plasmid on LB+ampicillin (100 µg/ml).
	Alternative yeast DNA preparation procedure used	Use the method described in 5.9. Other procedures designed for high-copy-number vectors may not work with the ARS/CEN-based vectors used here.
	DNA suspended in incorrect buffer	Electroporation is sensitive to ionic strength. Suspend DNA pellet in TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA].
Failure to obtain transformants of MaV203 with DNA isolated from *E. coli*	Failure to add both DB-X and AD-Y plasmids during transformation	Use DB-X and AD-Y plasmids simultaneously in co-transformation procedures.
	Incorrect selection plates	Plate co-transformations on SC-Leu-Trp plates.
Failure of candidate clones to reproduce the reporter gene readouts	Candidate clones were false positives	Candidate clones could have been mutants of DB-X that self-activate. See section 4.3 for additional information on false positives.
	Co-transformed pDEST™32 instead of DB-X	Confirm the reporter gene readouts by reconstructing the strain MaV203(DB-X)(AD-Y).
	Multiple AD-cDNA clones in the original 3AT^r transformants	Examine more Ap^r *E. coli* transformants (section 3.9) for additional AD-cDNA clones. Test each by reintroduction into MaV203.

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4.2 Interpretation of Results

Interpretation of the four reporter gene readouts is the most critical step in a two-hybrid screen. Several general comments are provided below along with unique features about each phenotypic test. A summary of likely interpretations of observed phenotypes is provided in Table 4.

Table 4: Summary of Potential Phenotypes.

-His	*lacZ*	-Ura	0.2% 5FOA	Easily-Interpreted Phenotypes
no growth				False positive/background
growth	blue	no growth	no growth	Interactor, probably weak
growth	blue	growth	no growth	Interactor
growth	white	no growth	growth	Non-interactor

-His	*lacZ*	-Ura	0.2% 5FOA	One Inconsistent Phenotype
growth	blue	growth	growth	Probable interactor Possible mixed population*
growth	white	growth	no growth	Probable interactor (check *lacZ* expression using CPRG assay)

-His	*lacZ*	-Ura	0.2% 5FOA	Two Inconsistent Phenotypes
growth	blue	no growth	growth	Possible non-interactor Possible weak interactor (look carefully for any inhibition on 5FOA) Possible mixed population*
growth	white	growth	growth	Possible interactor (check *lacZ* expression using CPRG assay) Possible mixed population*; look carefully for inhibition on 5FOA
growth	white	no growth	no growth	Probable weak interactor (check *lacZ* expression using CPRG assay; confirm 5FOA phenotype)

*Mixed populations can result from numerous causes. Examples include: More than one AD-Y in the cell resulting in one population that contains AD-Y interacting with DB-X (no growth on 5FOA), and another population that contains AD-Y that does not interact with DB-X, giving rise to growth on 5FOA; mutations or instability in DB-X, AD-Y or *URA*3 reporter or promoter; carry over of cells from the -His+3AT plate that do not contain interacting DB-X:AD-Y.

1. Difficulty in interpreting the reporter gene readouts is very often associated with replica plating and replica cleaning. These procedures take practice. The most important issues have been described in section 5.4. It is useful to practice (as outlined in Figure 10) with Yeast Control Strains A-E until the expected readouts (Figure 11) can be obtained consistently. Be sure plates and velvets are very dry. If Yeast Control Strains A-E do not give the expected results, confirm that the plates were prepared correctly and return to the glycerol stocks provided and purify isolated colonies on SC-Leu-Trp plates as described in section 3.1. Always include Yeast Control Strains A-E on each master plate (remember to keep the patches away from the edges of the plate) to confirm that the plates are correct, and the replica plating was successful. Alternatives to replica plating and replica cleaning are described in section 5.4.
2. The interpretation of growth/slight growth/no growth on the various selection plates is quite subjective. Yeast Control Strains A-E should show the expected growth profile on the selection plate being examined in order to make valid conclusions. If they do not, confirm that the plates were prepared correctly, that the cells of Yeast Control Strains A-E are fresh and correctly ordered (A to E). If necessary, return to the glycerol stocks provided and purify isolated colonies on SC-Leu-Trp plates as described in section 3.1.
3. Growth on the SC-Leu-Trp-Ura plate is the least sensitive selection method and

failure of candidate interactors to grow here should not exclude them as true interactors. This is because the *URA*3 promoter region contains the URS1 sequence that strongly represses transcription (Figure 5) (7). Only strong protein:protein interactions induce this gene sufficiently to allow growth on SC-Ura plates. Furthermore, certain DB-X fusions, while showing strong induction of the *HIS*3 and *lac*Z reporter genes, may show weak induction of *URA*3.

4. Inhibition of growth on 5FOA is more sensitive than growth on SC-Leu-Trp-Ura. Compare the amount of growth of the candidate clone with Yeast Control Strain A or with MaV203(DB-X)(pEXP-AD502). Yeast Control Strain A should show good growth (little growth inhibition) on SC-Leu-Trp+0.2% 5FOA; Yeast Control Strain B will show a slight amount of growth; and Yeast Control Strains C-E should be completely inhibited. The replica cleaning step is critical for good results on 5FOA (Figure 13). If the controls do not show the expected results (Figure 11), confirm the amount of 5FOA added and the media composition.
5. X-gal assays for examining induction of the *lac*Z reporter gene are quite sensitive if done correctly. Always use a fresh X-gal solution. The addition of excess X-gal/Z-buffer during incubation of the membrane will cause diffusion of the blue color into adjacent colonies. Use the minimal amount (7-8 ml) of buffer to saturate the paper filters. It is useful to tip the plate slightly during incubation to allow any excess buffer to accumulate below the filter papers. Yeast Control Strain E gives a very strong blue reaction. Keep this patch at the bottom of the tipped plate to avoid diffusion into adjacent patches. Efficient cell lysis is critical. Cells are lysed by immersion in liquid nitrogen. Typically 20-30 s is sufficient. Be careful with the membrane after lysing the cells. The patches become viscous and touching them will cause smearing. Be certain that the patches are incubated facing up. Nitrocellulose filters often crack in liquid nitrogen. Yeast Control Strains A-E must give the expected result (Figure 11) to accurately interpret the results. Yeast Control Strain B should give a very faint blue when lysis is good. For those candidate clones showing very weak or questionable *lac*Z activity, it is often useful to use the CPRG assay (section 5.8.2) to confirm the phenotype.
6. It is essential to add 3AT to all SC-Leu-Trp-His plates. Yeast Control Strains A-E require only 10 mM 3AT. These controls will show different growth patterns as the 3AT concentration is increased (Figure 9). This must be considered when determining whether cells containing candidate interactors induce the *HIS*3 reporter gene. It is important to properly replica clean 3AT plates and to interpret the results at the indicated times. As the incubation time is increased beyond the indicated time, growth of cells not inducing *HIS*3 can become indistinguishable from those inducing the *HIS*3 reporter gene.

4.3 False Positives

False positives have been defined as clones containing DB-X and AD-Y that induce the reporter genes in a two-hybrid screen, but where X and Y do not interact; or those protein:protein (or protein:DNA, etc.) interactions where X and Y do interact in the context of the two-hybrid system, but these interactions are biologically irrelevant. Several recent references have discussed false positives (7,24-27).

False positives in which DB-X or AD-Y self-activate (*e.g.*, mutations in X that result in self-activation or AD-Y clones that activate transcription by binding to the promoters or proteins bound there) are usually eliminated by retesting AD-Y with DB and DB-X (section 3.9). Strong evidence confirming the interaction of X and Y can be obtained by swapping the fusions: construct and test DB-**Y** and AD-**X**. However, several examples have been described where X, when fused to DB, is shown to interact with AD-Y, but not when the fusions are swapped, *i.e.* DB-Y:AD-X. This is presumably due to steric issues, hence failure to retest in this experiment should not necessarily exclude the interaction. On occasion, a false positive AD-Y will not induce the reporter genes when tested with DB alone, but will when an

Troubleshooting Guide

unrelated protein is fused to DB. Therefore, it is useful to test candidate AD-Y clones against an irrelevant DB-X (27). It is possible to isolate the DB-X plasmids [DB control plasmids are derivatives of vector pPC97 (13)] from Yeast Control Strains B (DB-Rb), C (DB-dDP), and D (DB-Fos) in *E. coli* on LB+Amp plates and test these against candidate AD-Y fusions (sections 3.9 or 5.10). This assumes that the DB-X fusions in the control plasmids do not interact with candidate AD-Y fusions.

Certain proteins, such as those with low affinities for many different proteins (*e.g.*, containing large hydrophobic domains) or those that may recognize structural features of proteins (*e.g.,* heat shock proteins) may be identified as false positives in a two-hybrid screen. More complex structures, where X and Y are bridged by a third protein or RNA, may also account for certain false positives.

It is critical to:

- confirm that X and Y interact by retesting the interaction and showing that it is specific to the test DB-X,
- perform an independent assay such as co-immunoprecipitation, and
- devise experiments to demonstrate that the interaction is biologically relevant.

Additional Information

5

5.1 Considerations in Designing a Two-Hybrid Screen

Prior to beginning a two-hybrid screen, determine as much information regarding the protein of interest and those interactions that might be expected to be detected. Several issues that are of particular interest are listed below.

1. Does the test protein function as a transcription activator or contain other domains (*e.g.*, repressor) of known function?

   The fusion of proteins containing domains capable of functioning as transcription activators to the GAL4 DB will induce the reporter genes in the absence of interacting proteins and can not be used in a typical two-hybrid screen. For example, roughly 0.1% of random *E. coli* sequences behave as transcriptional activation domains when fused to the GAL4 DB (28). Segments of such proteins that lack the activation domain can conceivably be constructed and tested. It is also possible to perform a swapped two-hybrid screen where the AD-fusion vector contains the test protein of interest and is used to screen a cDNA library constructed in the DB-vector (7,16). Other domain functions should also be considered; *e.g.,* several proteins have been examined that contain domains exhibiting repressor activity that function generally and reduce expression of the reporter genes when fused to GAL4 DB (Vidal, unpublished).
2. Do the test or predicted target proteins belong to a protein family?

   It is often useful to anticipate the number of interacting proteins one might expect to recover from a two-hybrid screen. Test proteins that are members of large protein families may interact with other members at varying degrees, generating a spectrum of reporter gene readout profiles. The prevalence of these proteins should be considered when determining the number of colonies required for a two-hybrid screen and the predicted strength of the reporter gene expression (*e.g.,* strong interactors or weak interactors).
3. Where and when is the test protein expressed?

   The choice of which cDNA library to screen is critical and depends primarily upon the expression pattern of the protein used in the screen. To help in the choice of an AD cDNA library, verify by PCR the presence of a cDNA corresponding to the DB-X.
4. How will the interaction between proteins identified in the two-hybrid screen be confirmed, both biochemically and biologically?

   The interaction of two fusion proteins in a two-hybrid screen is not necessarily an indication that these proteins interact *in vivo* under native conditions. Often only segments of the protein are analyzed, revealing (or masking) domains that might otherwise be unavailable. Even full-length proteins (when present as DB or AD fusion proteins) may bear little structural resemblance to the native protein. Similarly, many posttranslational modifications present in higher eukaryotic cells are absent (or incorrectly modified) in yeast, which may preclude or provide a basis for protein:protein interactions. As previously described, interactions can be mediated non-specifically (*e.g.,* by large hydrophobic regions) or occur between proteins that are biologically irrelevant (*e.g.,* the proteins exist in different cell types, compartments or at different times during development or cell cycle). Consequently, it is important to confirm the interactions between protein pairs detected in a two-hybrid screen

by biochemical methods (section 3.10). Ideally, the purified protein of interest and antibodies raised against the protein of interest, preferably monoclonal antibodies, for immunoprecipitation and Western blot experiments, will have been generated. In some cases, antibodies raised against the GAL4-DB or AD regions, or other epitopes included in the fusion protein, can be used. Finally, it is important to design in advance a functional test for the biological relevance of the protein:protein interactions.

5.2 Basic Manipulation and Storage of Yeast

Methods for the growth, manipulation and storage of yeast can be found in several general references (29-32).

Yeast strains can be stored for short periods (weeks) on YPAD or SC plates or for longer periods (6-12 months) on tightly sealed agar slants at 4°C.

Yeast strains can be stored indefinitely in 15-50% (v/v) glycerol at -70°C or lower. To store cells at -70°C, spread on the surface of a YPAD or appropriate SC agar plate. Following incubation, scrape with an autoclaved applicator stick and suspend cells in autoclaved glycerol solution. Alternatively, cells can be cultivated in liquid medium, centrifuged, and suspended in an autoclaved glycerol solution. The frozen yeast can be revived by transferring a small portion of the frozen sample onto an appropriate agar plate and incubating at 30°C for at least 48 h.

5.3 Media

Methods for the preparation of the following media and additional yeast media can be found in several references (29-31).

5.3.1 LB Medium (Luria Broth) (for Growth of *E. coli*)

Component	Amount
Bacto-yeast extract	5 g
Peptone	10 g
NaCl	10 g
Autoclaved, distilled water	to 1 liter

For agar plates, add 20 g bacteriological-grade agar per liter of non-autoclaved LB medium. Autoclave at 121°C for 25 min. Cool to 55°C, add antibiotics (or other selection reagent) as appropriate, and dispense into sterile petri dishes. Store plates when solidified upside down at 4°C.

5.3.2 YPAD Medium (Rich Medium for the Routine Growth of Yeast)

Component	Amount
Bacto-yeast extract	10 g
Bacto-peptone	20 g
Dextrose	20 g
Adenine sulfate	100 mg
Autoclaved, distilled water	to 1 liter

For agar plates, add 20 g bacteriological-grade agar per liter of non-autoclaved YPAD medium. Adjust the pH to 6.0 with HCl. Autoclave at 121°C for 25 min. Cool to 55°C and dispense into sterile Petri dishes. Store plates when solidified upside down at 4°C.

5.3.3 Synthetic Complete Medium (SC Medium)

Synthetic Complete medium consists of a nitrogen base, a carbon source, and a “dropout” solution containing essential amino acids, nucleic acids, trace elements and vitamins. For selection purposes, certain amino acids are omitted or “dropped out” (*e.g.,* leucine, tryptophan, histidine) from the dropout solution. For liquid medium, the agar is omitted. Alternative recipes that use yeast nitrogen base with ammonium sulfate are available (29-31).

1. Prepare the following solutions.
   40% glucose
   20 mM uracil
   100 mM histidine-HCl
   100 mM leucine
   40 mM tryptophan
   Autoclave the 40% glucose and filter sterilize the amino acids. Store the amino acids in the dark or wrapped in foil.
2. Prepare an amino acid powder mix of purine and amino acids by mixing equal weights (for example 2-3 g for each compound) of the following: adenine sulfate, alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine.
3. The liquid medium and the agar are autoclaved in two separate 2-L flasks. For 2 L of medium, to one flask add:

Component	Amount
Yeast nitrogen base without amino acids	13.4 g
Amino acid powder mix	2.7 g

4. Add a clean stir bar, suspend in 1 L distilled water and adjust the pH to 5.9 with NaOH. Keep the stir bar in the flask to stir the medium after autoclaving.
5. To the second flask add 40 g of agar in 900 ml distilled water. The agar will be solubilized during autoclaving.
6. Autoclave both flasks for 20 min on the liquid setting.
7. After autoclaving, pour the contents of the flask containing agar into the flask containing medium. Cool in a 50°C water bath for about 1 h. Add 100 ml autoclaved 40% glucose.
8. Depending on the auxotrophies to be tested with the dropout medium, also add the appropriate amino acids (*e.g.,* for SC-Leu, add all except Leucine):
   16 ml of 20 mM uracil
   16 ml of 100 mM histidine-HCl
   16 ml of 100 mM leucine
   16 ml of 40 mM tryptophan

Note: Approximately twenty 15-cm Petri plates (100 ml medium/plate) can be poured with 2 L of agar-containing medium.

5.3.4 Plates Containing 5-Fluoroorotic Acid (5FOA) or 3-Amino-1,2,4-Triazole (3AT)

Follow the previous recipe for YPAD or SC medium supplemented with the appropriate amino acids. Cool to approximately 65°C. Add 5FOA or 3AT as powders. Stir to dissolve (a few minutes for 3AT and ~30 min for 5FOA), then pour plates without further adjusting the pH.

5.3.5 Plates Containing Cycloheximide

Follow the recipe above for YPAD or SC medium supplemented with the appropriate amino acids. Cool to approximately 65°C. Add cycloheximide (filter-sterilized stock at 10 mg/ml, stored at -20°C) to a final concentration of 10 µg/ml.

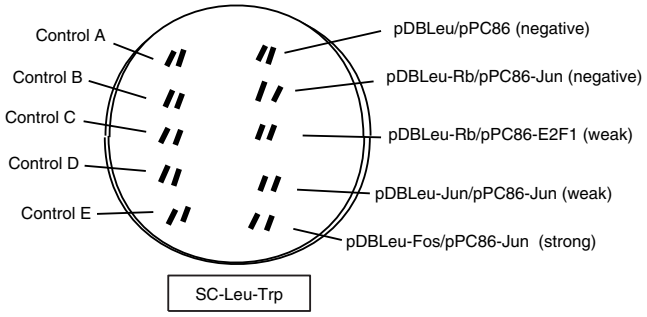
5.4 Replica Plating/Replica Cleaning

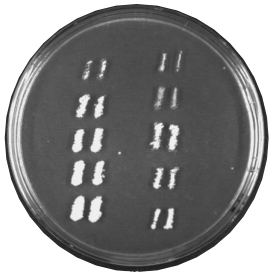
Replica plating is performed by gently pressing a master plate onto an autoclaved velvet to transfer the colonies or patches to a selection plate. Replica cleaning serves to remove excess cell material transferred to the selection plates. Figure 13 shows plates that have not been replica cleaned and plates that have been replica cleaned properly.

Essential tips for successful replica plating/replica cleaning:

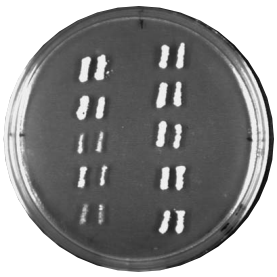
- As the number of cells transferred by replica plating onto the selection plates increases, the phenotypic differences between positive and negative controls decreases. Therefore, it is crucial to transfer a minimal number of cells to the selection plates.

Additional Information

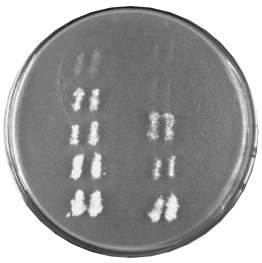




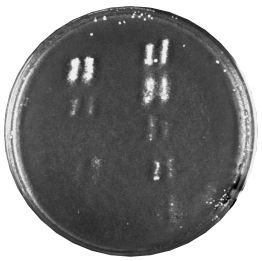
SC-Leu-Trp-His+3AT (10 mM)
Not replica cleaned



SC-Leu-Trp+0.2% 5FOA
Not replica cleaned



SC-Leu-Trp-His+3AT (10 mM)
Replica cleaned



SC-Leu-Trp+0.2% 5FOA
Replica cleaned

Figure 13. Selection Plates With and Without Replica Cleaning. It is critical to replica clean plates. Yeast Control Strains A-E and the indicated pDBLeu-X:pPC86-Y containing strains were patched onto an SC-Leu-Trp master plate and incubated for 18 h at 30°C. Cells from this master plate were replica plated onto SC-Leu-Trp-His+3AT (10 mM) and SC-Leu-Trp+0.2% 5FOA and either replica cleaned or not replica cleaned, and incubated as described (Figure 10). Strains lacking interacting proteins (*i.e.,* Yeast Control Strain A, DB/AD, and DB-Rb/AD-Jun) show growth similar to that of clones containing interacting fusion proteins on 3AT plates that were not replica cleaned, but are clearly distinguishable when correctly replica cleaned. Similarly, substantial growth of cells containing interacting proteins is observed on 5FOA plates if not replica cleaned, whereas the expected growth inhibition of cells containing interacting proteins is readily observed on correctly replica cleaned plates.

- The time of incubation of the master plate can affect the results. This is probably due to yeast cells approaching stationary phase which can exhibit different expression levels of the hybrid proteins from cells growing in exponential phase.
- During replica plating, do not press the plate onto the replica velvet too hard. This smears the cells and increases surface moisture.
- **Replica cleaning requires a fair amount of pressure in order to remove all excess cell material. After replica cleaning, the recipient plate should not contain visible cell material. At most, a shadow of cells should be observed when held to the light. If substantial cell material is present, repeat the replica clean procedure with a fresh velvet. It is often necessary to replica clean a plate 2-3 times to remove all of the cell material. A successfully replica cleaned plate will typically contain a faint imprint of the velvet on the surface of the agar.**
- Replica cleaned 15-cm SC-Leu-Trp-His+3AT transformation plates (section 3.7.1) may retain a slight haze of cell material due to the large number of non-transformed cells plated.
- The moisture content of the plates will affect the phenotypes. Too much moisture causes smearing of the patches. Be sure plates are dry. Allow the freshly poured agar plates to dry for 3 or 4 days before use.
- Do not replica plate onto cold plates. A prewarming step at 30°C helps keep surface moisture to a minimum.
- Be sure the replica velvets are clean and very dry. Replica cleaning works poorly with moist velvets.

5.4.1 Procedure for Replica Plating and Replica Cleaning

Note: During replica cleaning, each velvet is used only once. Because replica cleaning often requires 2 or 3 velvets per 10-cm plate to remove all cell material, a large supply of sterile velvets will be required.

1. Replica Plating:
   After incubation of the master plate (18 h, 30°C, Figure 10), gently press the master plate with a light, consistent pressure onto an autoclaved velvet. Be sure to transfer only a slight haze of cells and avoid cell clumps on the velvet. This will make subsequent replica cleaning easier. The selection plate(s) of interest are then gently pressed onto this “inoculated” velvet to transfer the colonies or patches. A single “inoculated” velvet can be used to inoculate up to five selection plates. Place asymmetric marks on the master plate and selection plates to allow realignment.
2. Replica Cleaning:
   Immediately following replica plating or following incubation (Figure 10), the cells on the selection plate are replica cleaned or “diluted” by pressing a new autoclaved velvet onto the surface. A greater amount of pressure is required in this procedure than replica plating. The cells adhering to the velvet are discarded by washing the velvet.
3. Where indicated, the plates should be replica cleaned again after an initial 24-h incubation.
4. In order to control the efficiency of these different steps, Yeast Control Strains A-E should be patched on every single master plate, and their phenotypes verified on the selection plates (Figure 11).
5. After replica plating, remove the cell material from the velvets by light brushing with a bottle brush and immerse them in water. Autoclave them in the water with a 25-min cycle. Air dry the velvets by hanging them. Pack the velvets flat in aluminum foil, approximately 30/pack, and autoclave with a 20-min cycle and a 99-min drying cycle (if available). After several uses, the velvets can be machine washed using cold water. It is very important not to add soap. Machine dry using low heat. Pack the velvets as before and autoclave.

Additional Information

5.4.2 Alternatives to Replica Plating and Replica Cleaning

Alternatives to replica plating and replica cleaning exist. For example, candidate yeast colonies can be suspended in sterile saline, and 1:10 dilutions prepared. Samples from each of these dilutions (*i.e.,* 10 µl) can be spotted onto each of the selection plates (include SC-Leu-Trp), and following incubation, the amount of growth for each dilution determined. By comparing growth on SC-Leu-Trp to the same dilution on each selection plate, the extent to which the reporter gene is induced can be determined. With care, several candidate clones can be examined on each selection plate. Similarly, candidate yeast colonies can be streaked (using a sterile loop or toothpick) for isolated colonies on each of the selection plates and, following incubation, the extent of growth determined.

While these alternatives can be used, replica plating/replica cleaning are the fastest, easiest and most reproducible methods for identifying candidate clones.

5.5 Alternative Procedure for Screening AD-Libraries with DB-X

This procedure is available from Technical Service.

In this procedure, the transformation is first plated onto SC-Leu-Trp plates to recover cells containing both DB-X and AD-Y, then replica plated onto SC-Leu-Trp-His+3AT to identify candidates containing interacting protein pairs. This approach may be effective in identifying very weakly interacting proteins by allowing accumulation of the HIS3 protein prior to selection. The method will require twenty-five 15-cm SC-Leu-Trp plates and fifty 15-cm SC-Leu-Trp-His+3AT plates.

5.6 Preparation of Competent Yeast for Large-Scale Co-transformation with DB-X and AD-Y-Library

This procedure is available from Technical Service.

This protocol typically gives 2 x 10^4 to 5 x 10^4 colonies after transformation with 5 µg of DB-fusion plasmid and 5 µg of AD-library. Screen greater than 10^6 yeast transformants when using mammalian cDNA libraries. Hence, 10^6 yeast transformants will require 125 to 250 µg of each DB-X and AD-Y (cDNA library) plasmid DNAs, 25 to 50 transformations, and between twenty-five to fifty 15-cm plates (SC-Leu-Trp-His+3AT).

5.7 X-gal Assay

1. From the master plate containing Yeast Control Strains A-E (Figure 11), replica plate the patches directly onto a membrane (nylon or nitrocellulose) that has been placed on the surface of a YPAD agar plate. **Note: Be sure to make asymmetric marks on the membrane to allow for realignment on the master plate.** Incubate for 18 to 24 h at 30°C.
2. For each membrane, dissolve 10 mg X-gal (5-bromo-5-chloro-3-indolyl-β-D-galactoside) in 100 µl N,N-dimethyl formamide (DMF). Prepare Z buffer [16.1 g $Na_2HPO_4 \bullet 7H_2O$ (or 8.52 g anhydrous), 5.5 g $NaH_2PO_4 \bullet H_2O$ (or 4.8 g anhydrous), 0.75 g KCl, 0.246 g $MgSO_4 \bullet 7H_2O$ (or 0.12 g anhydrous), dissolved in 1 L distilled water and adjusted to pH 7.0 and filter sterilized]. Combine 100 µl X-gal in DMF, 60 µl 2-mercaptoethanol and 10 ml Z buffer.
3. Stack two round 125-mm Whatman 541 filter papers in a 15-cm petri plate. Saturate with ~8 ml of the X-gal solution. Remove any air bubbles.
4. Using forceps, carefully remove the membrane from the surface of the YPAD plate. Completely immerse the membrane in liquid nitrogen for 20-30 s. Place the frozen membrane on top of the soaked Whatman filters colony side up. Remove any air bubbles. Tip the plates slightly and remove excess X-gal solution.

Note: Control D may generate both large and small colonies when plated on SC-Leu-Trp. To obtain the correct phenotype, always choose the small colonies.

Note: Nitrocellulose membranes are fragile and can crack during freezing; therefore neutrally charged nylon membranes are recommended.

Note: Handle liquid nitrogen with care. Always wear thick gloves and goggles.

5. Cover the plates and incubate at 37°C. Tip the plates at a slight angle so excess X-gal solution does not accumulate on the membrane. Monitor the appearance of blue color over a 24-h period. Score final results at 24 h.

   Note: When scoring the results, the membrane will be a mirror image of the master plate. Strong interactors show blue color within 1 h (*e.g.,* Yeast Control Strain E). Weak interactors show blue color within 24 h (*e.g.,* Yeast Control Strain B). The β-gal phenotype of the weakly interacting proteins in Yeast Control Strain B, when tested with X-gal, can be very faint blue to white. The controls must show the expected phenotypes in order to draw conclusions from this experiment (Figure 11).

5.8 Quantitative Assays for β-Galactosidase

Quantitative assays for β-galactosidase (β-gal) activity in liquid cultures can be performed using either o-nitrophenyl-β-D-galactopyranoside (ONPG) or Chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. As a substrate, CPRG is more sensitive and faster than ONPG and is therefore particularly useful for clones exhibiting weak or moderate expression levels of β-galactosidase.

NOTE: Both ONPG and CPRG will stain. Wear gloves during these procedures.

5.8.1 Assay for β-Gal in Liquid Culture Using ONPG

For each strain, test 3 to 5 independent isolated colonies. Assay each sample extract in triplicate to reduce variability. The ONPG assay typically requires 45 min of incubation for Yeast Control Strains D and E, while Yeast Control Strains A, B, and C require 21 to 24 h. Interactions as weak as Yeast Control Strain B show activities barely over background. Consequently, ONPG may not be appropriate for analysis of weak interactions.

1. Inoculate an isolated single colony into 2.5 ml SC-Leu-Trp using an entire yeast colony/tube. Incubate overnight with shaking (230-250 rpm) at 30°C.
2. Inoculate 5 ml YPAD medium with 1 ml overnight culture giving a starting OD_{600} of ~0.5. Incubate at 30°C with shaking (230-250 rpm) until the OD_{600} = 1.0-1.5.

Note: It may be necessary to dilute or concentrate the cells to remain within the linear range of the assay.

3. Dissolve ONPG at 4 mg/ml in Z buffer [16.1 g $Na_2HPO_4 \bullet 7H_2O$ (or 8.52 g anhydrous), 5.5 g $NaH_2PO_4 \bullet H_2O$ (or 4.8 g anhydrous), 0.75 g KCl, and 0.246 g $MgSO_4 \bullet 7H_2O$ (or 0.12 g anhydrous) dissolved in 1 L autoclaved, distilled water and adjusted to pH 7.0 and filter sterilized] with shaking for 1 to 2 h.
4. Determine and record the final OD_{600}. Be sure the cells are well suspended (no clumps) by vortexing or by gently pipetting up and down. Accurate readings require OD_{600} < 1.0.
5. Place 1.5 ml culture in each of three 1.5-ml microcentrifuge tubes. Centrifuge at 14,000 × *g* for 30 s. Carefully remove and discard supernatant (avoid cell loss in all steps as this will affect the final activity calculations).

Note: Cells can also be lysed by three cycles of immersion in liquid nitrogen until the cells are frozen (10 s), followed by a brief (90 s) incubation in a 37°C water bath (33).

6. Resuspend each cell pellet in 1.5 ml Z buffer. Centrifuge at 14,000 × *g* for 30 s. Carefully remove and discard supernatant.
7. Resuspend each cell pellet in 300 µl Z buffer. Transfer 100 µl of the cell suspension to a fresh graduated microcentrifuge tube. Add autoclaved, acid-washed 0.5-mm glass beads to a final volume of 200 µl. (Place the remaining 200 µl on ice for repeat assays if necessary.)
8. Vortex 1-2 min.
9. Prepare a stock of 700 µl Z buffer + 1.9 µl 2-mercaptoethanol per sample to be assayed.
10. Set up a blank tube with 100 µl Z buffer.
11. Add 700 µl Z buffer + 2-mercaptoethanol to each extract and the blank.
12. Start timer. Immediately add 160 µl of ONPG in Z buffer to the reaction and blank tubes and place tubes in a 30°C water bath.
13. Monitor color development. After a medium-yellow color develops, stop the reaction by addition of 400 µl 1 M Na_2CO_3 to each reaction and the blank. Record elapsed time. Reaction times vary from 5 min to 24 h.
14. Centrifuge reaction tubes for 5 min at 14,000 × *g*.

Additional Information

15. Carefully transfer supernatants to clean cuvettes, avoiding all cellular debris.
16. Calibrate the spectrophotometer against the blank at OD_{420}.
17. Measure the OD_{420} of each sample. The linear range of this assay at OD_{420} is 0.02-1.0. For accuracy, the A_{420} is best read between 0.3-0.7.
18. Calculate β-gal units, where 1 unit of β-gal is defined as the amount that hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per minute (34):
    β–gal units = $1{,}000 \times OD_{420}/(t \times V \times OD_{600})$; where
    t = elapsed time (in min) of incubation
    V = volume of culture used in the assay (ml)
    OD_{420} = absorbance by o-nitrophenol (and light scattering by cell debris)
    OD_{600} = cell density at the start of the assay

5.8.2 Assay for β-Gal in Liquid Culture Using CPRG

For each strain, test 3 to 5 independent isolated colonies. Assay each sample extract in triplicate to reduce variability. The CPRG assay typically requires 3-15 min incubation for Yeast Control Strain E, 10-20 min for Yeast Control Strain D, 1-2 h incubation for Yeast Control Strain C, and 24 h for Yeast Control Strains B and A. Interactions as weak as Yeast Control Strain B show activities approximately 10 times background (Yeast Control Strain A) making CPRG the preferred substrate for weak interactors.

1. Prepare Buffer 1, Buffer 2, and 6 mM $ZnCl_2$.
   Buffer 1: For 100 ml, dissolve 2.38 g HEPES, 0.9 g NaCl, 0.065 g L-aspartate (hemi-Mg salt), 1.0 g BSA, and 50 µl Tween 20® in 75 ml distilled water and adjust the pH to 7.25-7.3. Adjust volume to 100 ml. Filter sterilize and store at 4°C for up to 3 months.
   Buffer 2: Dissolve 27.1 mg CPRG in 20 ml Buffer 1 (to give 2.23 mM CPRG). Filter sterilize and store in the dark at 4°C for up to 3 months.
2. Inoculate an isolated single-colony into 2.5 ml SC-Leu-Trp using an entire yeast colony per tube. Incubate overnight with shaking (230-250 rpm) at 30°C.
3. Inoculate 5 ml YPAD medium with 1.0 ml overnight culture giving a starting OD_{600} of ~0.5. Incubate at 30°C with shaking (230-250 rpm) until the OD_{600}= 1.0-1.5.
4. Determine and record the final OD_{600}. Be sure the cells are well suspended (no clumps) by vortexing or by gently pipetting up and down. Accurate readings require OD_{600} <1.0.
5. Place 1.5 ml culture in each of three graduated 1.5-ml microcentrifuge tubes. Centrifuge at 14,000 × *g* for 30 s. Carefully remove and discard supernatant (avoid cell loss in all steps as this will effect the final activity calculations).
6. Suspend each cell pellet in 1.0 ml Buffer 1. Centrifuge at 14,000 × *g* for 30 s. Carefully remove and discard supernatant.
7. Suspend each cell pellet in a final volume of 100 µl Buffer 1. Add autoclaved, acid-washed 0.5-mm glass beads to a final volume of 200 µl.
8. Vortex 1-2 min.
9. Prepare a buffer blank by combining 100 µl Buffer 1 and 900 µl Buffer 2.
10. Add 900 µl Buffer 2 to each sample. Vortex to mix thoroughly. Start timer/record time.
11. Monitor color development. After a rusty yellow to a red-brown color develops, stop the reaction by addition of 250 µl of 6 mM $ZnCl_2$ to the sample and the buffer blank. Record elapsed time. Reaction times can vary from seconds to 24 h.
12. Centrifuge samples at 14,000 × *g* for 1 min to pellet cell debris.
13. Carefully transfer supernatants to clean cuvettes, avoiding all cellular debris.
14. Calibrate the spectrophotometer against the blank at OD_{574}.

15. Measure the OD_{574} of each sample. The linear range of this assay at OD_{574} is 0.25-1.8.
16. Calculate β-gal units, where 1 unit of β-gal is defined as the amount that hydrolyzes 1 μmol of CPRG to chloramphenicol red and D-galactose per minute (34):

    β-gal units = $1{,}000 \times OD_{574}/(t \times V \times OD_{600})$; where

    t = elapsed time (in min) of incubation

    V = volume of culture used in the assay (ml)

    OD_{574} = absorbance by chloramphenicol red (and light scattering by cell debris)

    OD_{600} = cell density at the start of the assay

5.9 Plasmid DNA Extraction from Yeast for Use in the Transformation of *E. coli*

Plasmid DNA isolated from yeast is typically not suitable for restriction analysis. Consequently, plasmids isolated from yeast are first introduced into *E. coli*, then miniprep DNA from the resulting transformants is characterized. Several quick yeast plasmid preparations have been described (35-37). The following modification of the method described by Polaina and Adam (37) has routinely provided the most reproducible results. ARS/CEN-based plasmids isolated from MaV203 and introduced into ElectroMAX™ DH10B™ cells typically yield 10 to 100 colonies when transformed with 1 μl DNA (plating 20% of the expression mixture). The procedure can be scaled up to at least 5 ml of starting yeast culture. However, due to apparent inhibitory compounds in the final preparation, scale the final resuspension volume proportionally and use only 1 μl of purified DNA for electroporation.

1. Suspend an isolated colony in 1 ml SC-Trp-Leu and incubate at 30°C with shaking (285 rpm) for ~24 h.
2. Centrifuge the 1 ml culture at 14,000 × *g* for 30 s at room temperature. Remove all residual liquid.
3. Suspend the cells in 100 μl freshly prepared 3% sodium dodecyl sulfate (SDS), 0.2 N NaOH.
4. Incubate 15 min at room temperature with occasional mixing by several rapid inversions.
5. Add 500 μl TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. Mix completely by several rapid inversions.
6. Add 60 μl 3 M sodium acetate (not pH adjusted). Mix completely by several rapid inversions.
7. Add 600 μl phenol [saturated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]: chloroform: isoamyl alcohol, 25:24:1.
8. Vortex for 2 min at full speed.
9. Centrifuge in a microcentrifuge at 14,000 × *g* for 2 min. Transfer the upper aqueous phase to a new microcentrifuge tube.
10. Repeat steps 7-9.
11. Add 650 μl ice cold isopropanol. Mix completely by several rapid inversions.
12. Incubate at -20°C for at least 20 min.
13. Centrifuge in a microcentrifuge at 14,000 × *g* for 5 min.
14. Carefully discard the supernatant. Centrifuge for 10 s and remove all residual supernatant.
15. Add 100 μl 70% ethanol.
16. Centrifuge in a microcentrifuge at 14,000 × *g* for 5 min.
17. Discard the supernatant. Centrifuge for 10 s and remove all residual supernatant.
18. Suspend the pellet in 10 μl TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA].

19. Use 1 µl of DNA to electroporate ElectroMAX™ DH10B™ cells according to the procedure provided with the cells. For isolation of *E. coli* transformants containing the AD-Y plasmid, plate ~20% of the transformation mixture on LB+100 µg/ml ampicillin. For isolation of the *E. coli* transformants containing the DB-X plasmid, plate ~20% of the transformation mixture on LB+10 µg/ml gentamicin.

5.10 Plasmid Shuffling: Curing of the DB-X Plasmid

The pDEST™32 plasmid contains the *CYH2*^{S} gene (section 2.4). Cells harboring this plasmid do not grow when plated on medium containing cycloheximide. When MaV203(DB-X)(AD-Y) cells are plated on SC-Trp+cycloheximide, surviving cells will have spontaneously lost the single-copy DB-X plasmid and will contain only the AD-Y plasmid; thus the cured cells will not grow when plated on SC-Leu. Also, the DB vector in Yeast Control Strain C contains the *CYH2*^{S} gene and can be used as a control in this protocol.

A transformation assay is used to reintroduce DB-X into AD-Y-containing cells. The reporter gene phenotype of the resulting transformants is determined. The procedure is summarized in Figure 14.

1. From an SC-Leu-Trp master plate (section 3.8, Figure 10) containing the patched cells with candidate interacting fusion proteins, streak colonies onto SC-Trp+cyh (10 µg/ml). Incubate 3-5 days at 30°C.
2. Suspend a good-sized isolated colony from the SC-Trp+cyh plate in ~50 µl autoclaved, distilled water and spread onto the center (~5 cm^2) of a 10-cm YPAD plate using an autoclaved loop or toothpick. Incubate overnight at 30°C. Repeat for each candidate clone.
3. For each clone, scrape the cells from the YPAD plate and completely suspend (by brief vortexing and pipetting up and down) in 5 ml autoclaved, distilled water. Add a sufficient amount of this cell suspension to 100 ml YPAD broth in a 500-ml flask to produce a final OD_{600} of 0.1. Reserve approximately 10 ml YPAD medium to use as a blank in the spectrophotometer.

   Note: Perform serial 1:10 dilutions in water of the 5-ml cell suspension then determine the OD_{600} of each dilution to allow an estimate of the amount of cell suspension required to produce the desired OD of 0.1. Accurate cell densities require that the measured OD < 1.0. Use plastic cuvettes.
4. Follow the sequential transformation procedure described on the web site. The method can be scaled down by half, if desired.
5. Perform transformations 1-3 as follows:

Tube	Plasmid	Purpose
1	None	Control for curing DB-X
2	pDBLeu	Test for self-activation of AD-Y
3	DB-X	Reconfirm interaction

6. For each transformation, combine 50 µl cells, 5 µl freshly boiled herring or salmon sperm carrier DNA and 100 ng of each plasmid DNA in an autoclaved 1.5-ml microcentrifuge tube. Mix gently by pipetting up and down. Add 300 µl PEG/LiAc solution and mix gently.
7. Incubate for 30 min in a 30°C water bath.
8. Heat shock for 15 min in a 42°C water bath.
9. Centrifuge in a microcentrifuge (6,000-8,000 × *g*) for 20-30 s at room temperature. Carefully remove the supernatant.

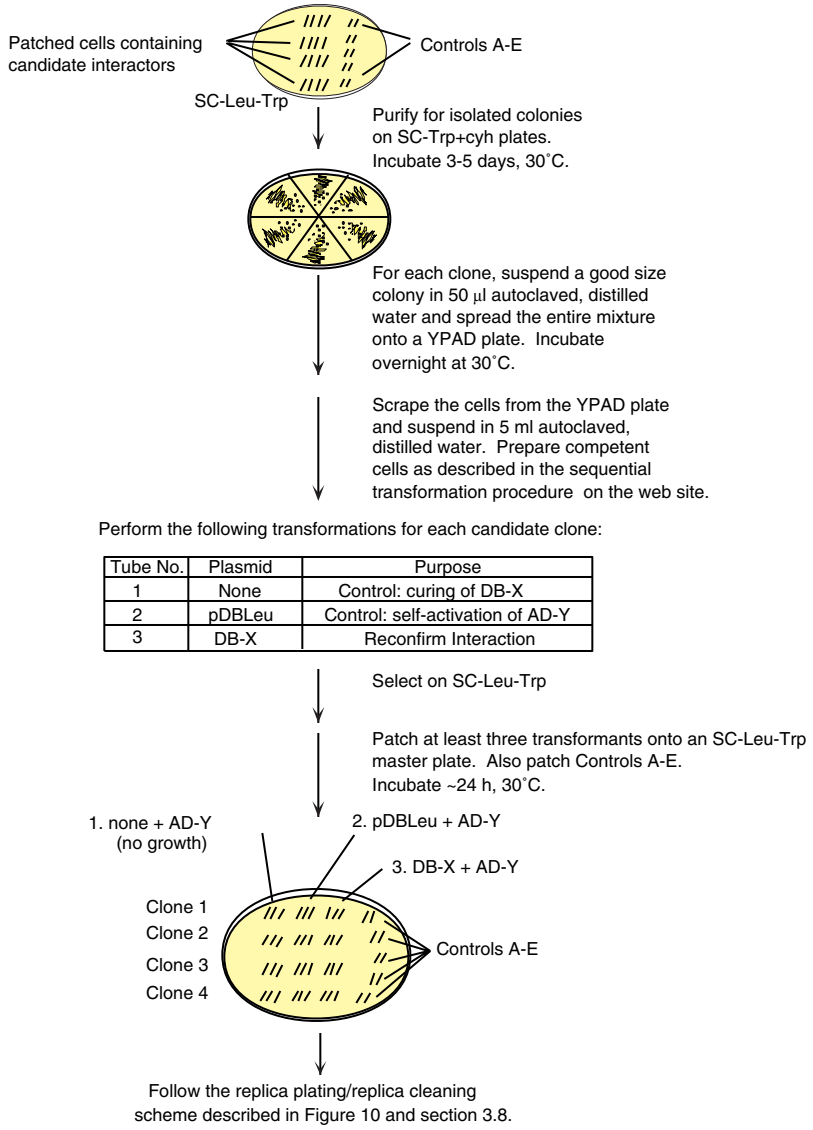


Figure 14. Plasmid Shuffling

10. Suspend the cell pellet in 500 µl autoclaved, distilled water. Plate 100 µl onto a SC-Leu-Trp selection plate. Incubate for 48-72 h at 30°C.
11. Patch at least three transformants from each transformation onto an SC-Leu-Trp plate. Also patch Yeast Control Strains A-E onto the same plate. Incubate for 24 h at 30°C.
12. Replica plate this master plate onto SC-Leu-Trp-His+3AT (replica clean), SC-Leu-Trp+0.2% 5FOA (replica clean), SC-Leu-Trp-Ura, and YPAD+nylon or nitrocellulose membrane (for X-gal Assay) and treat as described in section 3.8, step 5 (Figure 10, bottom).
13. Expected results:
    - Only transformations 2 and 3 should give growth on SC-Leu-Trp. Growth of transformation 1 on SC-Leu-Trp indicates incomplete curing of DB-X. In this case, repurify for isolated colonies from the SC-Trp+cyh plate to a new SC-Trp+cyh plate, incubate for 3 days at 30°C and repeat the transformation using an isolated colony.

Note: Control D may generate both large and small colonies when plated on SC-Leu-Trp. To obtain the correct phenotype, always choose the small colonies.

Additional Information

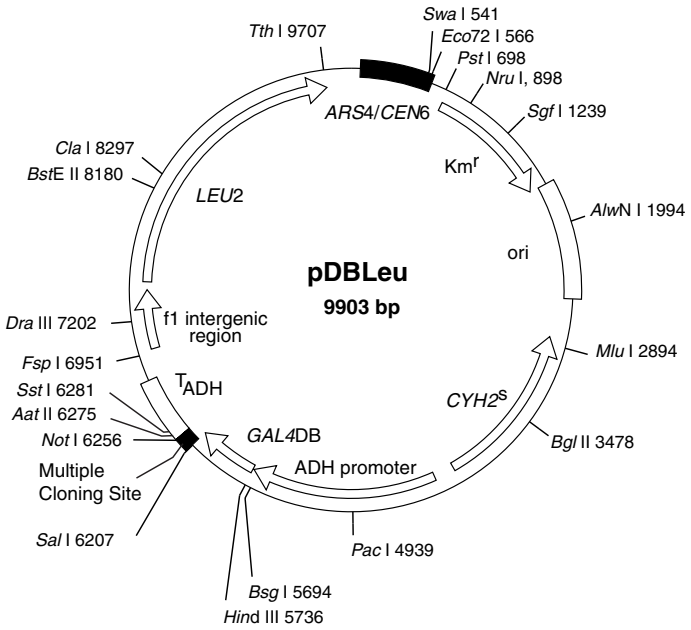
- Reporter gene induction should be observed only from transformation 3. Clones that reproduce the original phenotypes likely represent interacting DB-X:AD-Y fusion proteins. These should be further characterized (section 3.10).

5.11 Advanced Techniques Using the ProQuest™ Two-Hybrid System

The ProQuest™ Two-Hybrid System is an extremely flexible system that allows optimization of conditions for screening protein:protein interactions (by adjusting 3AT and 5FOA concentrations). In addition, the *URA*3/5FOA reporter gene provides a direct selection for mutations, peptides or compounds that effect the interaction of two proteins, allowing rapid characterization of protein:protein interactions or isolation of potentially valuable research reagents or pharmaceutical compounds (7).

Detailed descriptions of the use of this system for characterization of protein:protein interactions by mutagenesis, large-scale experiments, and screening DB-cDNA libraries have been published (4,7,8,16).

5.12 Vector Maps and Restriction Endonuclease Sites



pDBLeu Expression Vector. Restriction endonucleases that cleave pDBLeu once are shown on the outer circle. The nucleotide position refers to the 5′ base of the recognition sequence. The stop codons are in bold in all 3 reading frames following the *Not* I site.

Note: The multiple cloning site for pDBLeu and pDBTrp is different from the multiple cloning site for pPC86.

```
                                        6150                                                                    6200
                                         •                                                                       •
                                  suggested sequencing/PCR primer
                                  5′-GA ATA AGT GCG ACA TCA TCA TC-3′→                                                    Sal I   Sma I
5′-ACA TTG AGA CAG CAT AGA ATA AGT GCG ACA TCA TCA TCG GAA GAG AGT AGT AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG TCG AGG TCG ACC CCG GGT
                                                                                                  leu thr val ser ser arg ser thr pro gly
                                                                                                  -------GAL4 DB---------
                                        6250                                                                    6300
                                         •                                                                       •
  Nhe I     Stu I      Msc I     Nco I     Spe I     Not I                          Aat II   Sst I
GCT AGC AAG GCC TTG TGG CCA GCC ATG GCA ACT AGT GCG GCC GCT AAG TAA GTA AGA CGT CGA GCT CTA AGT AAG TAA CGG CCG CCA CCG CGG
ala ser lys ala leu trp pro ala met ala thr ser ala ala ala lys STOP

                                                    6350                                                                    6400
                                                     •                                                                       •
TGG AGC TTT GGA CTT CTT CGC CAG AGG TTT GGT CAA GTC TCC AAT CAA GGT TGT CGG CTT GTC TAC CTT GCC  AGA AAT TTA CGA AAA GAT GGA AAA GGG-3′
    ----ADH Terminator----                                                                    ←3′-CAG ATG GAA CGG TCT TTA AAT G-5′
                                                                                                suggested sequencing/PCR primer
```

Restriction endonucleases that do not cleave pDBLeu:

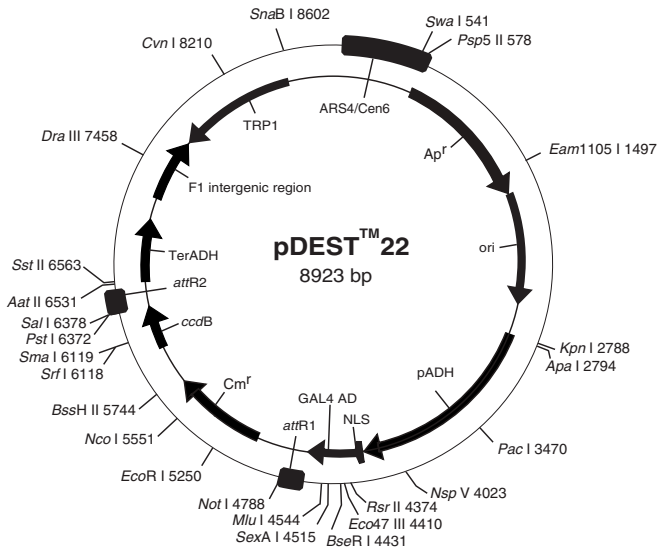
Apa I	*Bpu*1102 I	*Eco*47 III	*Pme* I	*Sfi* I	*Sse*8387 I
Asc I	*Bss*H II	*Fse* I	*Psh*A I	*Sgr*A I	*Sun* I
Avr II	*Cvn* I	*Kpn*2 I	*Rsr* II	*Sna*B I	*Xba* I
Bcl I	*Eam*1105 I	*Nde* I	*Sex*A I	*Srf* I	

Restriction endonucleases that cleave pDBLeu twice:

*Bam*H I	4263	6778	*Kpn* I	2805	8393	*Pvu* II	2586	6901
Bgl I	4468	6957	*Nar* I	4402	7696	*Sst* II	1114	6307
*Bse*R I	7492	9485	*Ngo*A IV	4468	7099	*Sap* I	408	2530
*Bsp*M I	5627	9149	*Nsi* I	1089	1355	*Sca* I	4102	4771
*Bss*S I	12	2235	*Pin*A I	7485	8396	*Xho* I	4272	5978
*Eco*R I	3459	8783	*Pvu* I	1240	6931	*Xma* III	6257	6298
Hpa I	6036	7727						

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com.

Additional Information



Gateway® pDEST™22 Vector. DNA from the Entry Clone replaces the region between nucleotides 4671 and 6492.

```
4540
|
tyr asn ala phe gly ile thr thr gly met phe asn thr thr thr met asp asp val tyr asn tyr leu phe asp asp
TAT AAC GCG TTT GGA ATC ACT ACA GGG ATG TTT AAT ACC ACT ACA ATG GAT GAT GTA TAT AAC TAT CTA TTC GAT GAT
ATA TTG CGC AAA CCT TAG TGA TGT CCC TAC AAA TTA TGG TGA TGT TAC CTA CTA CAT ATA TTG ATA GAT AAG CTA CTA
                                                        *
glu asp thr pro pro asn pro lys lys glu gly gly ser asn gln thr ser leu tyr lys lys ala gly
GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG GGT GGG TCG AAT CAA ACA AGT TTG TAC AAA AAA GCA GGC TNN -------
CTT CTA TGG GGT GGT TTG GGT TTT TTT CTC CCA CCC AGC TTA GTT TGT TCA AAC ATG TTT TTT CGT CCG ANN -------
                                                        attB1
                          ♦
----------NACCCAGCTT TCTTGTACAA AGTGGTTTGA TGGCCGCTAA GTAAGTAAGA CGTCGAGCTC TAAGTAAGTA ACGGCCGCCA
   GENE
----------NTGGGTCGAA AGAACATGTT TCACCAAACT ACCGGCGATT CATTCATTCT GCAGCTCGAG ATTCATTCAT TGCCGGCGGT
          attB2                                                                          6632
                                                                                         |
CCGCGGTGGA GCTTTGGACT TCTTCGCCAG AGGTTTGGTC AAGTCTCCAA TCAAGGTTGT CGGCTTGTCT
GGCGCCACCT CGAAACCTGA AGAAGCGGTC TCCAAACCAG TTCAGAGGTT AGTTCCAACA GCCGAACAGA
                                           3'CAGAGGTT AGTTCCAACA GCCGA 5'
```

Features of the pDEST™22 Recombination Region Sequence:

- Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST™22 Vector by recombination. Non-shaded regions are derived from pDEST™22 Vector.
- The nucleotides marked with * and ♦ correspond to bases 4670 and 6493, respectively, of the pDEST™22 Vector sequence.
- For sequencing at the 5´ end the following primer located at 4374-4396 is recommended: 5´ CGG TCC GAA CCT CAT AAC AAC TC. The primer recommended for 3´ sequencing is shown on the figure.

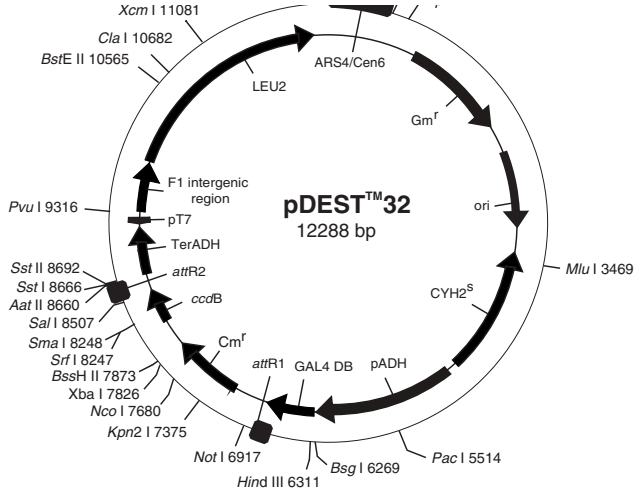
Restriction endonucleases that do not cleave pDEST™22

Afl II	*Bst*E II	*Nhe* I	*Sfi* I	*Sun* I
Asc I	*Cla* I	*Nru* I	*Sgf* I	*Tth*111 I
Avr II	*Eco*N I	*Nsi* I	*Sgr*A I	*Xcm* I
Bcl I	*Fse* I	*Pin*A I	*Spe* I	
Bgl I	*Hpa* I	*Pme* I	*Sse*8387 I	
*Bpu*1102 I	*Nde* I	*Psh*A I	*Stu* I	

Restriction endonucleases that cleave pDEST™22 DNA twice:

*Alw*N I	1976	6024	*Fsp* I	1277	7207	*Sap* I	408	2512
*Bam*H I	5703	7034	*Hin*d III	4267	7939	*Sph* I	3863	6895
Bbs I	357	3946	*Kpn*2 I	5246	8587	*Sty* I	3063	5551
Bsg I	4225	8060	*Mam* I	3794	6130	*Xba*	5697	8368
*Bsp*LU11 I	2390	3659	*Nar* I	2933	4303	*Xho* I	2803	4296
*Eco*57 I	815	1863	*Ngo*A IV	2999	7355	*Xma* III	4789	6554
*Eco*72 I	566	8494	*Pfl*M I	4909	5476			
*Eco*R V	3557	8169	*Pvu* I	1130	7187			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com.



Gateway® pDEST™32 Vector. DNA from the Entry Clone replaces the region between nucleotides 6800 and 8621.

```
6690
|
pro leu thr leu arg gln his arg ile ser ala thr ser ser ser glu glu ser ser asn lys gly
CCT CTA ACA TTG AGA CAG CAT AGA ATA AGT GCG ACA TCA TCA TCG GAA GAG AGT AGT AAC AAA GGT
GGA GAT TGT AAC TCT GTC GTA TCT TAT TCA CGC TGT AGT AGT AGC CTT CTC TCA TCA TTG TTT CCA

                                                        *
gln arg gln leu thr val ser ser arg ser asn gln thr ser leu tyr lys lys ala gly
CAA AGA CAG TTG ACT GTA TCG TCG AGG TCG AAT CAA ACA AGT TTG TAC AAA AAA GCA GGC TNN ---
GTT TCT GTC AAC TGA CAT AGC AGC TCC AGC TTA GTT TGT TCA ACA TG TTT TTT CGT CCG ANN ---
                                                  attB1

                                  ♦
----------NACCCAGCTT TCTTGTACAA AGTGGTTTGA TGGCCGCTAA GTAAGTAAGA CGTCGAGCTC TAAGTAAGTA
   GENE   NTGGGTCGAA AGAACATGTT TCACCAAACT ACCGGCGATT CATTCATTCT GCAGCTCGAG ATTCATTCAT
          attB2
                                                                                  8760
                                                                                   |
ACGGCCGCCA CCGCGGTGGA GCTTTGGACT TCTTCGCCA GAGGTTTGGT CAAGTCTCCA ATCAAGGTTG TCGGCTTGTC
TGCCGGCGGT GGCGCCACCT CGAAACCTGA AGAAGCGGT CTCCAAACCA GTTCAGAGGT TAGTTCCAAC AGCCGAACAG
                                                    3'CAGAGGT TAGTTCCAAC AGCCGA 5'
```

Features of the pDEST™32 Recombination Region Sequence:

- Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST™32 Vector by recombination. Non-shaded regions are derived from pDEST™32 Vector.
- The nucleotides marked with * and ♦ correspond to bases 6799 and 8623, respectively, of the pDEST™32 Vector sequence.
- For sequencing at the 5′ end the following primer located at 6409-6431 is recommended: 5′ AAC CGA AGT GCG CCA AGT GTC TG. The primer recommended for 3′ sequencing is shown on the figure.

Restriction endonucleases that do not cleave pDEST™32

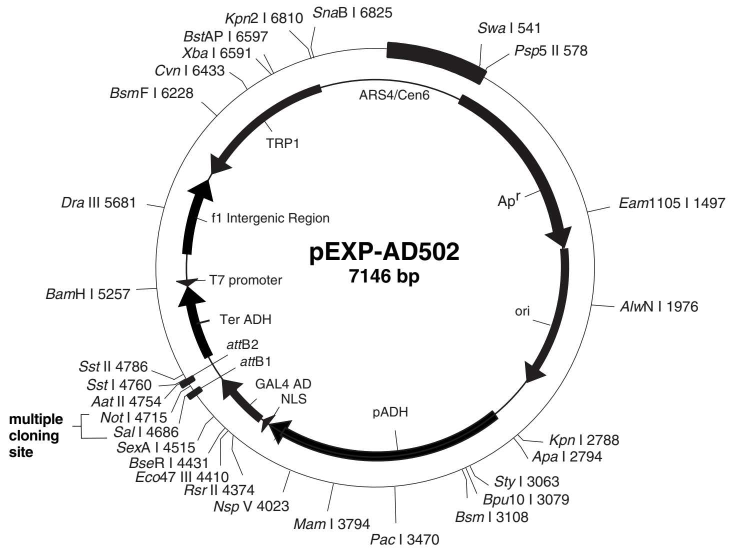
Asc I	*Eam*1105 I	*Nru* I	*Sex*A I	*Spe* I
Avr II	*Eco*47 III	*Nsi* I	*Sfi* I	*Sse*8387 I
Bcl I	*Fse* I	*Pme* I	*Sgf* I	*Stu* I
*Bpu*1102 I	*Nde* I	*Psh*A I	*Sgr*A I	*Sun* I
Cvn I	*Nhe* I	*Rsr* II	*Sna*B I	

Restriction endonucleases that cleave pDEST™32 twice:

*Alw*N I	2569	8153	*Fsp* I	704	9336	*Pin*A I	9870	10781
Bgl II	1763	4053	*Hpa* I	6611	10112	*Pst* I	698	8501
*Bss*S I	12	2810	*Kpn* I	3380	10778	*Sap* I	408	3105
Dra III	1199	9587	*Nar* I	4977	10081	*Tth*111 I	1552	12092
*Eco*N I	10796	11486	*Ngo*A IV	5043	9484	*Xho* I	4847	6553

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com.

Additional Information



Gateway® pEXP-AD502 Vector.

```
                                                                                    4700
4630                 4650                                                  Sal I  Sma I  EcoR I
CCA AAC CCA AAA AAA GAG GGT GGG TCG ATC ACA AGT TTG TAC AAA AAA GCA GGC TTG TCG ACC CCG GGA ATT CAG
GGT TTG GGT TTT TTT CTC CCA CCC AGC TAG TGT TCA AAC ATG TTT TTT CGT CCG AAC AGC TGG GGC CCT TAA GTC
pro asn pro lys lys glu gly gly ser ile thr ser leu tyr lys lys ala gly leu ser thr pro gly ile gln
----GAL4 AD------                                   attB1

Bgl II Spe I   Not I                       4750   Aat II Sst I                      Sst II
ATC TAC TAG TGCGGCCGCA CGCGTACCCA GCTTTCTTGT ACAAAGTGGT GACGTCGAGCT CTAAGTAAGT AACGGCCGCC ACCGCGGTGG
TAG ATG ATC ACGCCGGCGT GCGCATGGGT CGAAAGAACA TGTTTCACCA CTGCAGCTCGA GATTCATTCA TTGCCGGCGG TGGCGCCACC
ile tyr stop
                                attB2

  4800                                            4850                                  4884
AGCTTTGGAC TTCTTCGCCA GAGGTTTGGT CAAGTCTCCA ATCAAGGTTG TCGGCTTGTC TACCTTGCCA GAAATTTACG AAAAGATGGA
TCGAAACCTG AAGAAGCGGT CTCCAAACCA GTTCAGAGGT TAGTTCCAAC AGCCGAACAG ATGGAACGGT CTTTAAATGA TTTTCTACCT
------ADH terminator ------                                 3'-CAG ATGGAACGGT CTTTAAATG-5'
```

Features of the pEXP-AD502 Recombination Region Sequence:

- For sequencing at the 5´ end the following primer located at 4374-4396 is recommended: 5´ CGG TCC GAA CCT CAT AAC AAC TC. The primer recommended for 3´ sequencing is shown on the figure.

Restriction endonucleases that do not cleave pEXP-AD502 DNA:

Afl II	*Cla* I	*Nhe* I	*Pst* I	*Sun* I
Asc I	*Eco*N I	*Nru* I	*Sfi* I	*Tth*111 I
Avr II	*Fse* I	*Nsi* I	*Sgf* I	*Xcm* I
Bcl I	*Hpa* I	*Pfl*M I	*Sgr*A I	
*Bpu*1102 I	*Msc* I	*Pin*A I	*Srf* I	
*Bss*H II	*Nco* I	*Pme* I	*Sse*8387 I	
*Bst*E II	*Nde* I	*Psh*A I	*Stu* I	

Restriction endonucleases that cleave pEXP-AD502 DNA twice:

Bbs I	357	3946	*Fsp* I	1277	5430	*Pvu* II	2568	5380
Bsg I	4225	6283	*Gsu* I	1412	5177	*Sap* I	408	2512
*Bsp*LU11 I	2390	3659	*Hinc* II	4686	4913	*Sca* I	1019	3302
*Bsp*M I	4158	6233	*Hind* III	4267	6162	*Sph* I	3863	5118
Dsa I	3542	4786	*Mlu* I	4544	4723	*Xho* I	2803	4296
*Eco*57 I	815	1863	*Nar* I	2933	4303	*Xma* III	4716	4777
*Eco*72 I	566	6717	*Ngo*A IV	2999	5578			
*Eco*R V	3557	6392	*Pvu* I	1130	5410			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com.

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6

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Related Products

7

Product	Size	Cat. No.
ProQuest™ cDNA Libraries*		
Custom ProQuest™ cDNA Libraries**		
Systems:		
SuperScript™ Plasmid System with Gateway® Technology	3 reactions	18248-013
SuperScript™ Plasmid System with Gateway® Technology with Gateway® pEXP-AD502 *Not* I-*Sal* I Cut	3 reactions	18248-039
GeneTrapper® cDNA Positive Selection System	5 reactions	10356-020
Competent Cells:		
MaV203 Competent Yeast Cells, Library Scale	2 × 0.55 ml	11281-011
MaV203 Competent Yeast Cells, Subcloning Scale	4 × 0.1 ml	11445-012
MAX Efficiency® DH5α™ Competent Cells	5 × 0.2 ml	18258-012
MAX Efficiency® DH10B™ Competent Cells	5 × 0.2 ml	18297-010
ElectroMAX™ DH10B™ Competent Cells	5 × 0.1 ml	18290-015
MAX Efficiency® STBL2™ Competent Cells	5 × 0.2 ml	10268-019
UltraMAX™ DH5α-FT™ Competent Cells	5 × 0.1 ml	10643-013
Gateway® Technology Products:		
PCR Cloning System with Gateway® Technology with pDONR™221	20 reactions	12535-019
E. coli Expression System with Gateway® Technology	20 reactions	11824-026
Mammalian Expression System with Gateway® Technology	20 reactions	11826-013
Baculovirus Expression System with Gateway® Technology	20 reactions	11827-011
Gateway® Vector Conversion System	20 reactions	11828-019
Gateway® pDONR™201 Vector	6 µg	11798-014
Gateway® pDONR™207 Vector	6 µg	12213-013
Gateway® pDONR™221 Vector	6 µg	12536-017
SuperScript™ II RNase H- Reverse Transcriptase	10,000 units	18064-014
SuperScript™ Choice System for cDNA Synthesis	3 reactions	18090-019
SuperScript™ Lambda System for cDNA Synthesis and λ Cloning	3 reactions	19643-014
Gateway® pCMV•SPORT6, *Not* I -*Sal* I-Cut	5 µg	12209-011
Gateway® pEXP-AD502, *Not* I -*Sal* I-Cut	5 µg	12211-017
Reagents:		
Sal I	1,000 units	15217-011
Not I	200 units	15441-025
Herring Sperm DNA Solution	5 × 1 ml	15634-017
Salmon Sperm DNA Solution	5 × 1 ml	15632-011
Calf Intestinal Alkaline Phosphatase	1,000 units	18009-019
T4 DNA Ligase	100 units	15224-017
X-gal	100 mg	15520-034

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**Please see our web site for information about custom cDNA libraries.

Related Products

Product	Size	Cat. No.
Bovine Albumin Fraction V	25 g	11018-017
0.5 M EDTA (pH 8.0)	4 × 100 ml	15575-012
Sodium Dodecyl Sulfate	100 g	15525-017
Phenol: Chloroform:Isoamyl Alcohol (25:24:1, v/v)	100 ml	15593-031
Glycerol	500 ml	15514-011
S.O.C. Medium	10 × 10 ml	15544-034
Media Components:		
5-Fluoroorotic Acid	1 g	10836-013
Luria Broth Base, powder	500 g	12795-027
SELECT Yeast Extract, powder	500 g	30393-029
SELECT Peptone 140	500 g	30392-021
SELECT Agar, powder	500 g	30391-023
Yeast Nitrogen Base	500 g	25685-033
Sodium Chloride	1 kg	11830-023
Adenine Sulfate	25 g	16002-024
Ampicillin Sodium Salt, lyophilized	20 ml	11593-019
Kanamycin Sulfate	5 g	11815-024
Gentamicin Reagent Solution	10 ml	15710-064

Notes

Notes

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Italy 02 98 22 201
Japan 03 3663 7974
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