

Matchmaker™ Library Construction & Screening Kits User Manual

PT3529-1 (PR6Z2169)
Published 22 December 2006

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I. Introduction

Matchmaker™ Library Construction & Screening Kits provide a simple method for constructing cDNA libraries for yeast two-hybrid and one-hybrid screening. These kits combine Matchmaker Systems with SMART cDNA Synthesis technology, which allows you to construct cDNA libraries from any tissue source starting with as little as 1 µg of poly A⁺ RNA or total RNA. Following a routine *in vivo* cloning step, you can then screen these libraries for one-hybrid and two-hybrid interactions using a sensitive transcriptional assay provided by our Matchmaker Systems.

Principle of the one-hybrid assay—a protein-DNA interaction assay

One-hybrid assays enable you to identify and characterize proteins that bind to a target, *cis*-acting DNA sequence—an upstream element that enhances transcription from a downstream minimal promoter. The assay may also be used to map the DNA-binding domain of previously known, or newly identified, DNA-binding proteins. With the Matchmaker One-Hybrid System, you can readily obtain the genes encoding the corresponding DNA-binding protein.

In a Matchmaker one-hybrid assay, potential DNA-binding proteins are expressed as fusions to the GAL4 activation domain (AD) by cloning the corresponding cDNA into pGADT7-Rec2, a low-copy vector designed for one-hybrid screening. One or more tandem copies of the target DNA sequence are cloned into pHIS2, a reporter vector that contains the nutritional reporter gene *HIS3*. Interaction between a DNA-binding protein and the target sequence stimulates transcription of *HIS3* (Figure 1), enabling the yeast host strain, Y187, a His auxotroph, to grow on minimal media lacking histidine.

Principle of the two-hybrid assay—a protein-protein interaction assay

Two-hybrid assays can be used to identify novel protein-protein interactions, confirm suspected interactions, and define interacting domains. In a Matchmaker Two-Hybrid assay, a bait gene is expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while another gene or cDNA is expressed as a fusion to the GAL4 activation domain (AD; Fields & Song, 1989; Chien *et al.*, 1991). When bait and library fusion proteins interact in a yeast reporter strain such as AH109, the DNA-BD and AD are brought into proximity and activate transcription of the reporter genes: *ADE2*, *HIS3*, *lacZ*, and *MEL1* (Figure 2).

DNA-BD and AD fusions are created by cloning cDNAs into the yeast expression vectors pGBKT7 and pGADT7-Rec. pGBKT7 expresses proteins as fusions to the GAL4 DNA-BD, while pGADT7-Rec expresses proteins as fusions to the GAL4 AD. In yeast, both fusions are expressed at high levels from the constitutive *ADH1* promoter (P_{ADH1}). Other GAL4-based DNA-BD cloning vectors such as pGBT9, pAS2-1, and pBridge are also compatible with this kit. pBridge Vector can be used to perform *three*-hybrid assays to identify ternary protein complexes.

Biological basis for one-hybrid and two-hybrid assays

One-hybrid (and two-hybrid) methods are based on the finding that many eukaryotic transcription factors are modular; their transcription activating and DNA-binding domains are structurally and functionally distinct. This allows researchers to construct various gene fusions that, when expressed as fusion proteins in yeast, can simultaneously bind to a target DNA sequence and activate transcription of a downstream reporter (Figures 1 and 2). Matchmaker systems use the transcription activating and DNA-binding domains of GAL4, a well-characterized yeast transcription factor. To learn more about GAL4-based yeast hybrid technology, see Zhu, L. & Hannon, G. J., 2000.

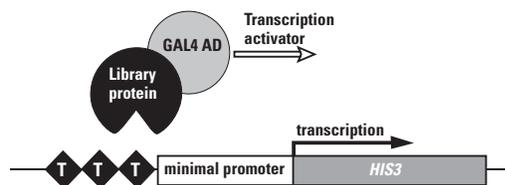


Figure 1. Screening for protein-DNA interactions with the Matchmaker One-Hybrid System. In this construct, three copies of the DNA target (T) have been inserted into the pHIS2 reporter vector.

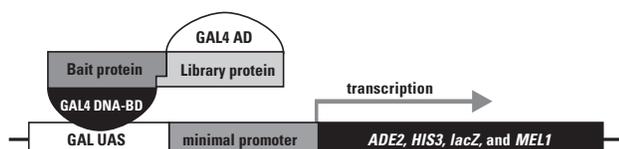


Figure 2. Screening for protein-protein interactions with the Matchmaker Two-Hybrid System.

I. Introduction *continued*

Constructing and screening Matchmaker one-hybrid and two-hybrid libraries

Constructing and screening Matchmaker one-hybrid and two-hybrid libraries consists of four main steps (Figure 3). Notice that both procedures follow the same general path.

If you intend to screen for two-hybrid interactions, the first step (Step 1) is to construct a DNA-BD fusion vector. If, on the other hand, you intend to screen for one-hybrid interactions, your first step is to construct a DNA target-*HIS3* reporter vector. Next (Step 2), use the SMART reagents we provide to generate a cDNA library from the poly A⁺ or total RNA that you provide.

In the case of yeast two-hybrid screening, you may skip RNA isolation, cDNA synthesis, and AD fusion library construction (Step 3) if, instead of preparing your own library, you intend to screen one of our many premade Matchmaker cDNA libraries. Representing a broad range of tissues, these libraries are available as glycerol stocks or pretransformed in yeast strain Y187. We also offer a Matchmaker Custom Library Service. To use this service, send us the tissue or cells you wish to screen, and we will make the AD fusion library for you. Please note, however, that many of our premade and pretransformed Matchmaker cDNA Libraries are constructed in high-copy yeast expression vectors, ideal for two-hybrid work, but less suitable for one-hybrid analysis. We recommend using low copy vectors such as pGADT7-Rec2 and pHIS2 for one-hybrid screening because they generate fewer false positives.

Following cDNA synthesis, construct a GAL4 AD fusion library by cloning the cDNA into one of our Matchmaker AD Cloning Vectors: pGADT7-Rec2 if you are constructing a one-hybrid library; pGADT7-Rec if you are constructing a two-hybrid library. The cloning takes place *in vivo* via homologous recombination (Figure 4). This step takes advantage of the highly efficient recombination system in yeast to fuse ds cDNA with the appropriate GAL4 AD plasmid. With recombination-mediated cloning, library construction and screening (Steps 3 and 4) can be completed in quick succession without the need for any bacterial transformation or amplification steps. Simply transform yeast with the cDNA library and the appropriate Matchmaker vectors; then spread the cells on dropout medium to select for one-hybrid or two-hybrid interactions.

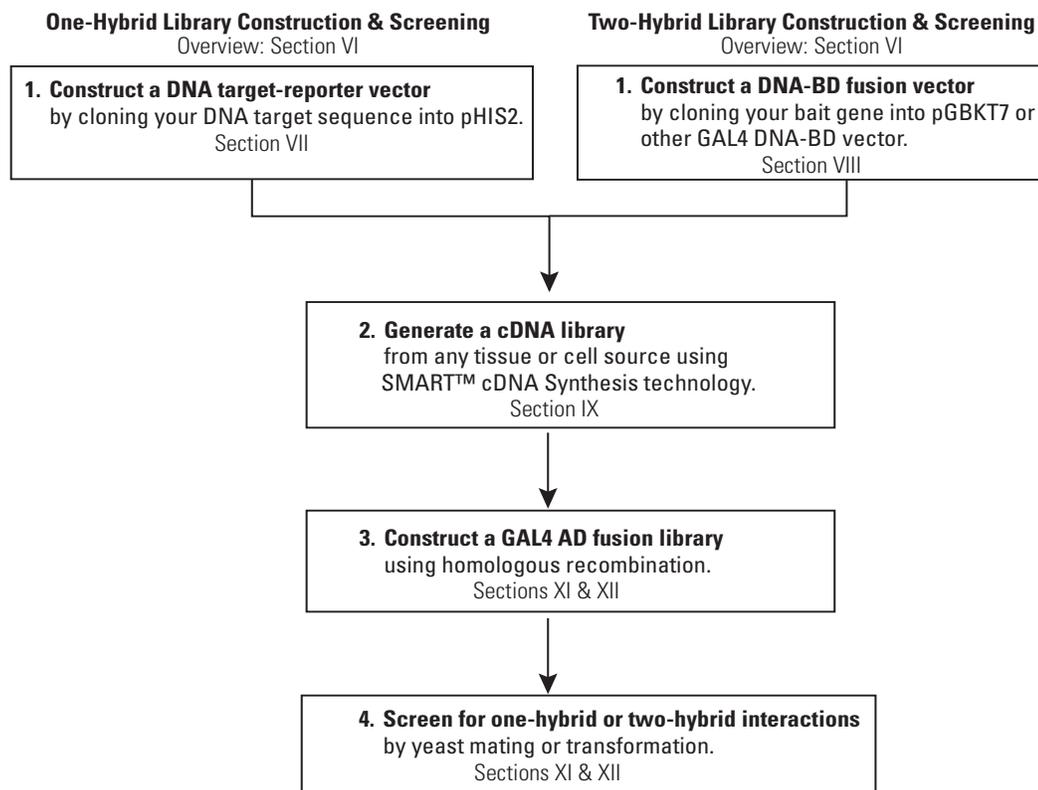


Figure 3. General steps of yeast one-hybrid and two-hybrid screening. For more detailed flow charts of the one-hybrid and two-hybrid procedures, please refer to Figures 6 and 7.

I. Introduction *continued*

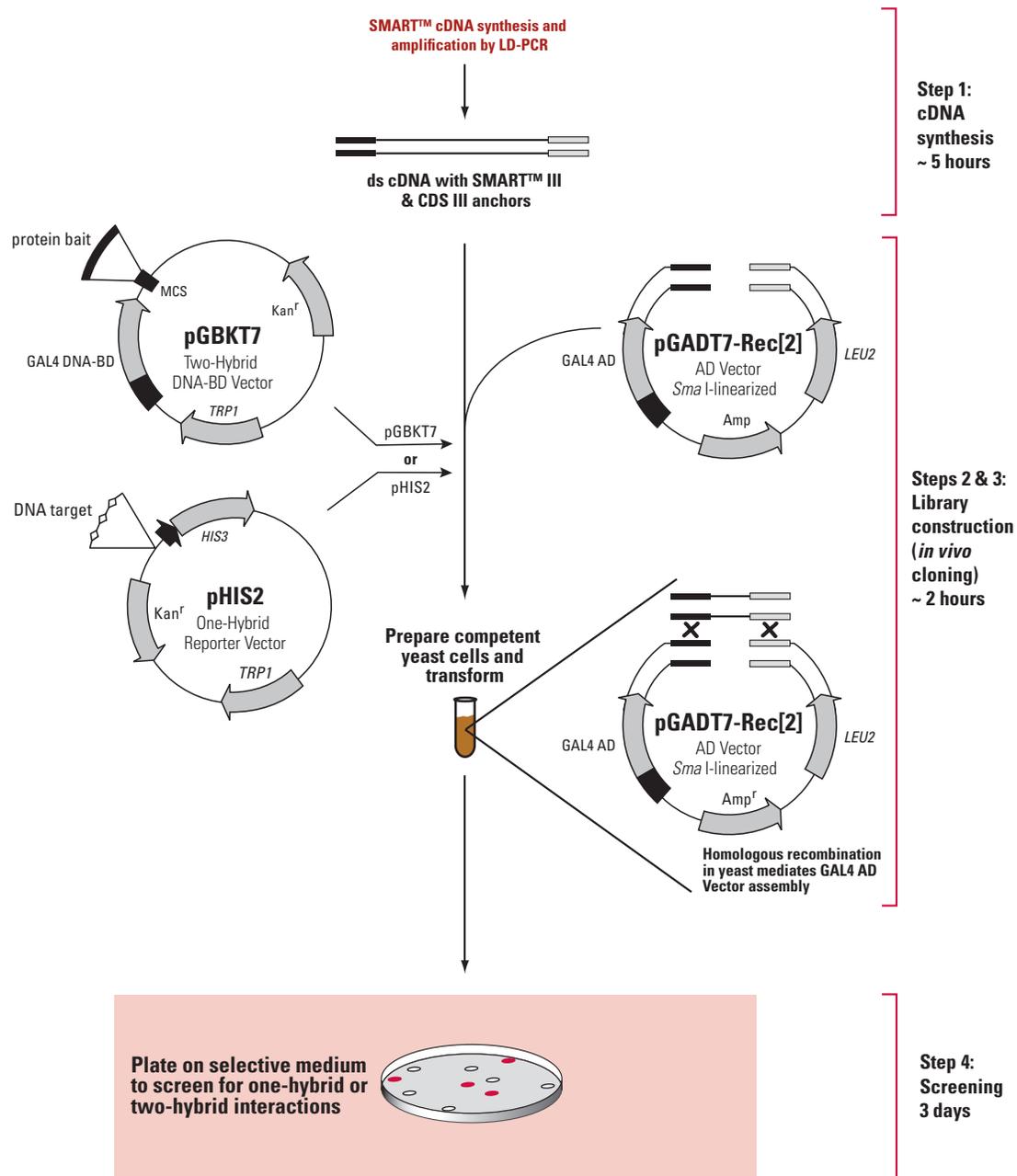


Figure 4. Constructing and screening Matchmaker One-Hybrid and Two-Hybrid libraries. As this diagram shows, recombination-mediated cloning makes library construction and screening quick and efficient. Though not shown here, two-hybrid libraries can also be screened by yeast mating (See Section XII for details). For details about SMART cDNA synthesis and amplification, please refer to Section IX. pGADT7-Rec is used for two-hybrid library construction and screening, while pGADT7-Rec2 is used for one-hybrid library construction and screening. Though related, the two vectors, denoted as pGADT7-Rec[2] in the figure, have different replication elements. See Section X and the corresponding Vector Information Packets for more information.

II. List of Components

This kit contains sufficient reagents to make 5 one-hybrid (Cat. No. 630304) or 5 two-hybrid (Cat. No. 630445) libraries.

Store Deionized H₂O, CHROMA SPIN Columns, NaCl Solution, Dropout (DO) Supplements, NaOAc, LiAc, PEG, TE Buffer, and YPD Plus Medium at room temperature. Store yeast strains, Control Poly A⁺ RNA, and the SMART III Oligo at -70°C. Store all other reagents at -20°C.

First-strand cDNA synthesis

- 10 µl SMART III Oligo (10 µM; 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3')
- 10 µl CDS III Primer (10 µM; 5'-ATTCTAGAGCCGAGGCGGCCGACATG-d(T)₃₀VN-3')*
- 10 µl CDS III/6 Primer (10 µM; 5'-ATTCTAGAGCCGAGGCGGCCGACATG-NNNNNN-3')*
*N = A, G, C, or T; V = A, G, or C
- 20 µl MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase
- 7 µl RNase H
- 100 µl 5X First-Strand Buffer (250 mM Tris (pH 8.3); 30 mM MgCl₂; 375 mM KCl)
- 100 µl DTT (dithiothreitol; 20 mM)
- 5 µl Control Poly A⁺ RNA (Human Placenta; 1 µg/µl)
- 50 µl dNTP Mix (dATP, dCTP, dGTP, dTTP, 10 mM each)
- 500 µl Deionized H₂O (Cat. No. 630445 only)

cDNA amplification

- 50 µl 5' PCR Primer (10 µM; 5'-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3')
- 50 µl 3' PCR Primer (10 µM; 5'-GTATCGATGCCACCCTCTAGAGGCCGAGGCGGCCGACA-3')
- 500 µl 10X GC-Melt Solution

cDNA purification

- 10 CHROMA SPIN+TE-400 Columns
- 300 µl Sodium Acetate (3 M; pH 4.8)

One-Hybrid Library Construction (Cat. No. 630304)

- 20 µg pHIS2 Reporter Vector (500 ng/µl)
- 20 µg pGADT7-Rec2 AD Cloning Vector (*Sma* I-linearized; 500 ng/µl)
- 20 µg pGAD-Rec2-53 Control Vector (500 ng/µl)
- 20 µg p53HIS2 Control Vector (500 ng/µl)
- 0.5 ml *S. cerevisiae* strain Y187
- 50 ml NaCl Solution (0.9%)
- 10 g -Leu DO Supplement
- 10 g -Trp DO Supplement
- 10 g -Leu/-Trp DO Supplement
- 10 g -His/-Leu/-Trp DO Supplement

Two-Hybrid Library Construction (Cat. No. 630445)

- 20 µg pGBKT7 DNA-BD Cloning Vector (500 ng/µl)
- 25 µg pGADT7-Rec AD Cloning Vector (*Sma* I-linearized; 500 ng/µl)
- 20 µg pGBKT7-53 Control Vector (500 ng/µl)
- 20 µg pGBKT7-Lam Control Vector (500 ng/µl)
- 20 µl SV40 Large-T PCR Fragment (25 ng/µl)
- 0.5 ml *S. cerevisiae* strain AH109

II. List of Components *continued*

- 0.5 ml *S. cerevisiae* strain Y187
- 50 ml NaCl Solution (0.9%)
- 10 g –Leu DO Supplement
- 10 g –Leu/–Trp DO Supplement
- 10 g –Ade/–His/–Leu/–Trp DO Supplement

Yeastmaker Yeast Transformation System 2 (Cat. No. 630439) includes the following:

- 50 ml 1 M LiAc (10X)
- 50 ml 10XTE Buffer
- 50 ml YPD Plus Liquid Medium
- 20 µl pGBT9 (0.1 µg/µl; control plasmid)
- 2 x 1 ml Herring Testes Carrier DNA, denatured (10 mg/ml)
- 2 x 50 ml 50% PEG 3350

III. Additional Materials Required

The following reagents are required but not supplied. Store all reagents and solutions at room temperature (20–22°C) unless specified otherwise.

First-strand cDNA synthesis and SMART™ PCR cDNA amplification

- Advantage® 2 PCR Kit (Cat. Nos. 639206 & 639207)
- Sterile, 0.5-ml microcentrifuge tubes
- Poly A⁺ or total RNA
- Mineral oil
- Thermal Cycler
Note: The cycling parameters in this protocol were set using a hot-lid thermal cycler and may not be optimal for non hot-lid cyclers.
- DNA size markers (1-kb DNA ladder)
- 1.2% Agarose/EtBr gel

cDNA size fractionation

- 1.5-ml sterile microcentrifuge tubes
- Ring-stand with small clamp for holding CHROMA SPIN columns
- 95% ethanol (–20°C)
- 1% xylene cyanol

Constructing bait plasmids

- Competent *E. coli* cells. Use a general purpose strain such as DH5 α or Fusion-Blue Competent Cells (Cat. No. 636700)
Fusion-Blue Competent Cells are an *E. coli* K-12 strain that provides high transformation efficiency paired with blue-white screening capability when used with the appropriate plasmids. The strain carries *recA* and *endA* mutations that make it a good host for obtaining high yields of plasmid DNA.
- T4 DNA ligase
- 10XT4 ligation buffer (Sambrook *et al.*, 1989; or the buffer provided with the enzyme)
- LB/amp plates
- 50 mM NaCl
- Materials for purifying plasmid from *E. coli* transformants

Yeast transformation (Prepare reagents in sterile containers)

- PEG/LiAc Solution (polyethylene glycol 3350/lithium acetate)
Prepare a fresh 10-ml solution just prior to transformation using the stock solutions provided: Mix 8 ml of 50% PEG 3350 with 1 ml of 10XTE Buffer and 1 ml of 1M LiAc (10X).
- 1.1XTE/LiAc Solution
Should be freshly prepared before each transformation using the stock solutions provided: Combine 1.1 ml of 10XTE with 1.1 ml of 1 M LiAc (10X). Bring the total volume to 10 ml using sterile, deionized H₂O.
- Dimethyl Sulfoxide (DMSO; Sigma Cat. No. D8418)

III. Additional Materials Required *continued*

Yeast culture & mating

- YPD Medium Cat. No. 630409; or prepare your own; see the Yeast Protocols Handbook (PT3024-1)
- YPDA medium (YPD medium supplemented with 30 mg/L adenine hemisulfate; see the Yeast Protocols Handbook)
- TE buffer or sterile, distilled H₂O
- Appropriate sterile tubes or flasks for transformations
- 100- and 150-mm culture plates
- Sterile glass rod, bent pasteur pipette, or 5-mm glass beads for spreading cells on plates
- X- α -Gal (Cat. No. 630407) for blue/white screening of yeast two-hybrids expressing *MEL1* (α -galactosidase)
- Minimal SD Base with and without agar (Cat. Nos. 630412 and 630411)
- 3-amino-1,2,4-triazole (3-AT; Sigma Cat. No. A8056; for suppressing background growth on SD minimal media lacking His)
- Kanamycin stock solution (50 mg/ml in H₂O; 1000X); Store at –20°C.
- Ampicillin stock solution (50 mg/ml in H₂O; 1000X); Store at –20°C.

Long-term library storage

- 100% Glycerol
- Freezing Medium: YPD medium with 25% (v/v) glycerol

Two-Hybrid Library Construction & Screening

The following dropout (DO) supplements are **not supplied** with the Matchmaker Library Construction & Screening Kit (Cat. No. 630445). You must obtain these supplements separately from a commercial supplier or prepare them yourself using the recipe given in Appendix C of the Yeast Protocols Handbook (PT3024-1).

- –Trp DO Supplement
Required for selection of Matchmaker DNA-BD cloning vectors in yeast
- –His/–Leu/–Trp DO Supplement
Optional triple-dropout supplement for low stringency screening of yeast two-hybrid libraries
- –His/–Trp DO Supplement
Required for testing yeast strains transformed with a DNA-BD plasmid for background growth on SD minimal media lacking His
- –Ade/–Trp DO Supplement
Required for testing yeast strains transformed with a DNA-BD plasmid for background growth on SD minimal media lacking Ade

One-Hybrid Library Construction & Screening

The following dropout (DO) supplement is **not supplied** with the Matchmaker One-Hybrid Library Construction & Screening Kit (Cat. No. 630304). You must obtain this supplement separately from a commercial supplier or prepare it yourself using the recipe given in Appendix C of the Yeast Protocols Handbook (PT3024-1).

- –His/–Trp DO Supplement
Required for testing yeast strains transformed with a pHIS2 reporter plasmid for background growth on SD minimal media lacking His

PCR Colony-Screening

- Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433)

IV. Yeast Strains

For additional information on the growth and maintenance of yeast, see the Yeast Protocols Handbook (PT3024-1). We also recommend Guthrie & Fink's *Guide to Yeast Genetics and Molecular Biology* (1991) and Heslot & Gaillardin's *Molecular Biology and Genetic Engineering of Yeasts* (1992).

A. Genotypes

TABLE I. MATCHMAKER YEAST STRAIN GENOTYPES

Strain	Genotype ^a	References
AH109^b	<i>MAT_a</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>gal4Δ</i> , <i>gal80Δ</i> , <i>LYS2 :: GAL1_{UAS}-GAL1_{TATA}-HIS3</i> , <i>GAL2_{UAS}-GAL2_{TATA}-ADE2</i> , <i>URA3 :: MEL1_{UAS}-MEL1_{TATA}-lacZ</i> , <i>MEL1</i>	James <i>et al.</i> , 1996; Our unpublished observations
Y187	<i>MAT_α</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>gal4Δ</i> , <i>met⁻</i> , <i>gal80Δ</i> , <i>URA3 :: GAL1_{UAS}-GAL1_{TATA}-lacZ</i> , <i>MEL1</i>	Harper <i>et al.</i> , 1993

^a The *GAL1*, *GAL2*, and *MEL1* upstream activating sequences (UASs) are responsive to the GAL4 transcriptional activator. The *trp1*, *his3*, *gal4*, and *gal80* mutations are all deletions; *leu2-3, 112* is a double mutation.

^b AH109 is a derivative of strain PJ69-2A and includes the *ADE2* and *HIS3* nutritional markers and an endogenous *MEL1* gene (James *et al.*, 1996). The *lacZ* reporter gene was introduced into PJ69-2A to create strain AH109.

B. Phenotypes

It is important to verify the phenotypes of the AH109 and Y187 strains (Table II).

1. To recover strains from frozen stock, scrape a small amount of cells from the surface with a sterile loop or wooden stick and streak them onto YPDA plates.
2. Incubate plates at 30°C for 3–5 days until colonies appear. Propagate additional cultures only from isolated colonies on this working stock plate.

Notes:

- AH109 (and transformants derived from this strain) should be maintained on adenine-supplemented YPD (i.e., YPDA) for optimal viability of the strain and to prevent selection of spontaneous *ade1* or *ade5* mutations (Guthrie & Fink, 1991).
 - If you cannot recover the strain by scraping the frozen stock, the cells may have settled to the bottom of the tube before the stock was frozen. If this happens, thaw the frozen culture on ice and vortex it before restreaking.
 - Although nonlibrary stock cultures may be thawed and refrozen several times without significantly decreasing the viability, we recommend that you divide the once-thawed stock into aliquots before you refreeze it. This will keep the viability higher and will reduce the risk of bacterial contamination.
3. Test for the nutritional requirements shown in Table II.
 - a. Using a sterile loop or toothpick, streak 3–4 colonies from the working stock onto separate, appropriate SD selection plates.
 - b. Incubate plates at 30°C for 4–6 days. Yeast grows slower on SD selection medium than on YPDA.
 - c. Compare your results with those shown in Table II. Proceed only if AH109 and Y187 have the expected phenotypes.
 4. Use well-isolated colonies from the verified working stock plate to inoculate liquid cultures for mating or for preparing competent cells. Seal the verified working stock plate with Parafilm and store at 4°C.

TABLE II. MATCHMAKER YEAST STRAIN PHENOTYPES

Strain	SD/–Ade	SD/–Met	SD/–Trp	SD/–Leu	SD/–His	SD/–Ura	YPDA
AH109	–	+	–	–	–	+	+
Y187	–	–	–	–	–	+	+

Notes:

- AH109 and Y187 can grow on SD/–Leu/–Trp if functional *TRP1* and *LEU2* genes are introduced.
- AH109 and AH109/Y187 diploids can grow on SD/–Ade/–His if the *ADE2* and *HIS3* genes—carried by AH109—are activated (i.e., in the presence of GAL4).

IV. Yeast Strains *continued*

C. Mating type compatibilities

- Y187 (*MAT α*) can mate with AH109, HF7c, CG-1945, Y190, or SFY526 (all *MAT a*).

D. Colony Color and Size

- Y187 carries the *ade2-101* mutation and AH109 exhibits the Ade⁻ phenotype in the absence of GAL4. On medium with low amounts of adenine, the colonies will turn pink after a few days and may turn darker as the colony ages. When grown on Ade-supplemented medium, the color change may not be noticeable. These colonies grow to >2 mm in diameter. However, small (<1 mm) white colonies will form at a rate of 1–2% due to spontaneous mutations that eliminate mitochondrial function (Holm, 1993). Avoid these white colonies when inoculating cultures.
- Y187 grows more slowly and forms noticeably smaller colonies (on average) than AH109.

E. Reporter genes

AH109 contains four reporters—*ADE2*, *HIS3*, *MEL1*, and *lacZ*—under the control of three distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Figure 5). The *ADE2* reporter alone provides strong nutritional selection. For higher stringency, and to reduce the incidence of false positives, select for *ADE2* and *HIS3* (James *et al.*, 1996). You also have the option of assaying for *MEL1*, which encodes α -galactosidase. *MEL1* is endogenous to both Y187 and AH109. Because α -galactosidase is a secreted enzyme, its activity can be detected by adding X- α -Gal (Cat. No. 630407) to the selection plate: If *MEL1* is active and X- α -Gal is present, the colony will turn blue.

lacZ in Y187 exhibits a high level of induced β -galactosidase activity in a positive two-hybrid assay because it is under the control of the intact *GAL1* UAS.

AH109 Constructs

GAL1 UAS	GAL1 TATA	<i>HIS3</i>
GAL2 UAS	GAL2 TATA	<i>ADE2</i>
MEL1 UAS	MEL1 TATA	<i>lacZ</i>
MEL1 UAS	MEL1 TATA	<i>MEL1</i>

Y187 Constructs

GAL1 UAS	GAL1 TATA	<i>lacZ</i>
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Figure 5. Reporter gene constructs in yeast strains AH109 and Y187. Strain AH109 is a derivative of strain PJ69-2A and includes the *ADE2* and *HIS3* nutritional markers (James *et al.*, 1996). *MEL1* is an endogenous GAL4-responsive gene. The *lacZ* reporter gene was introduced into PJ69-2A to create strain AH109. The *HIS3*, *ADE2*, and *MEL1/lacZ* reporter genes are under the control of three completely heterologous GAL4-responsive UAS and promoter elements—GAL1, GAL2, and MEL1, respectively.

IV. Yeast Strains *continued*

F. Leaky *HIS3* expression

- 3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the yeast *HIS3* protein (His3p). 3-AT is used to inhibit low levels of His3p expressed in a leaky manner and thus to suppress background growth on SD medium lacking histidine (Fields, 1993; Durfee *et al.*, 1993).
- Transformants derived from AH109 may show slightly elevated *HIS3* expression because of intrinsic DNA-binding properties of the bait protein. A small amount of 3-AT is generally sufficient to suppress background growth on SD/-His. However, if you are selecting for both *HIS3* and *ADE2* expression, it is generally not necessary to suppress *HIS3* leakiness in the initial library screen.
- Some yeast strains have relatively high basal levels of His3p. If you use Y190 (*MATa*) as a host strain, 25–45 mM 3-AT will be required in the medium to suppress background growth.
- **To optimize the 3-AT concentration in your selection medium:**

Before starting this procedure, note that -His/-Trp Dropout Supplement is not supplied with this kit. You must purchase -His/-Trp dropout supplement separately or prepare your own using the recipe given in Appendix C of the Yeast Protocols Handbook (PT3024-1).

1. Plate yeast transformants on a series of SD/-His/-Trp plates containing different concentrations of 3-AT.
 - If you are working with AH109 transformants containing DNA-BD plasmids such as pGBKT7, we recommend you start by testing [3-AT] in the range 0 to 15 mM (e.g., 0, 2.5, 5, 7.5, 10, 12.5, and 15 mM).
 - If you are working with Y187 transformants containing pHIS2 reporter plasmids, we recommend you start by testing [3-AT] in the range 10 to 60 mM.
Note: These are recommendations only. The optimal concentration may be slightly higher or lower depending on the construct and strain used.
2. Use the lowest concentration of 3-AT that, after one week, allows only small (<1 mm) colonies to grow. Too much 3-AT in the medium can kill freshly transformed cells.

V. Yeast Vectors

A. One-Hybrid System

1. Cloning Vectors

- **pHIS2** is a one-hybrid reporter vector that contains the *HIS3* nutritional reporter gene. It has a multiple cloning site (MCS) upstream of the *HIS3* reporter gene so that a *cis*-acting DNA target sequence can be inserted, and therefore, linked to the minimal promoter of the *HIS3* locus ($P_{minHIS3}$). It also contains a *CEN6/ARS4* sequence for stable, low-copy propagation in yeast.
- **pGADT7-Rec2** is a cloning vector that can be used to express a protein of interest as a fusion with the GAL4 activation domain (AD). This vector is engineered for homologous recombination-mediated cloning in yeast (Figure 4). Thus, you can construct cDNA/AD fusion libraries by transforming yeast with *Sma*I-linearized pGADT7-Rec2 (provided) and ds cDNA generated with the SMART library construction protocol (Section IX).

2. Control Vectors (Appendix C)

- **p53HIS2** is a positive control reporter vector that contains three tandem copies of the *cis*-acting DNA consensus sequence recognized by p53. p53HIS2 was constructed by inserting the DNA targets into the multiple cloning site of pHIS2. As a result, the DNA targets are positioned just upstream of the minimal promoter of the *HIS3* locus ($P_{minHIS3}$) and the *HIS3* reporter gene.
- **pGAD-Rec2-53** is a positive control vector that encodes murine p53 as a fusion with the GAL4 AD. Yeast cells that contain both p53HIS2 and pGAD-Rec2-53 should grow on minimal SD media lacking histidine—i.e., on SD/-His/-Leu/-Trp.

TABLE III. ONE-HYBRID SYSTEM VECTORS

	Use	Epitope ^a	Yeast selection ^b	Bacterial selection ^c
Cloning vectors				
pHIS2	Link any DNA target to <i>HIS3</i>		<i>TRP1</i>	kanamycin
pGADT7-Rec2 (<i>Sma</i> I-linearized)	Express potential DNA-binding proteins as AD fusions	HA	<i>LEU2</i>	ampicillin
Control vectors				
p53HIS2 ^b	Detect DNA-binding activity of p53		<i>TRP1</i>	kanamycin
pGAD-Rec2-53	Express p53 as an AD fusion	HA	<i>LEU2</i>	ampicillin

^a HA = hemagglutinin; These epitope tags can be used to verify protein-protein interactions *in vitro* by coimmunoprecipitation (Co-IP) using the antibodies and protocol provided with the Matchmaker Co-IP Kit (Cat. No. 630449). They are not intended to be used for detection, affinity purification, or Co-IP of hybrid proteins expressed in yeast.

^b The reporter and AD vectors have different nutritional markers, so they can be independently selected when yeast transformants are plated on SD minimal medium lacking specific nutrients. The selection medium you choose depends on which plasmids you are using, whether you are selecting for one or two plasmids, and whether you are selecting for colonies in which one hybrid interactions are occurring.

^c The vectors carry different antibiotic markers so that they can be independently selected in *E. coli*.

V. Yeast Vectors *continued*

B Two-Hybrid System

1. Cloning Vectors

- **pGBKT7:** Used to express a protein of interest as a fusion with the GAL4 DNA binding domain (DNA-BD).
- **pGADT7-Rec:** Used to express a protein of interest as a fusion with the GAL4 activation domain (AD). This vector is engineered for homologous recombination-mediated cloning. pGADT7-Rec is provided as *Sma* I-digested linear DNA.

2. Control Vectors

a. Positive Control

- **pGBKT7-53** encodes a fusion between the GAL4 DNA-BD and murine p53.
- **SV40 LargeT PCR Fragment** encodes SV40 largeT-antigen. Use this DNA fragment together with pGADT7-Rec to check the transformation-recombination efficiency in yeast. *In vivo*, SV40 LargeT PCR Fragment and pGADT7-Rec recombine to form pGADT7-RecT, which encodes a fusion between the GAL4 AD and large T-antigen.
- p53 and SV40 largeT-antigen interact in a yeast two-hybrid assay (Li & Fields, 1993; Iwabuchi *et al.*, 1993).

b. Negative Control

- **pGBKT7-Lam** encodes a fusion of the GAL4 DNA-BD with human lamin C and provides a control for a fortuitous interaction between an unrelated protein and either the pGADT7-RecT control or your AD/library plasmid. Lamin C neither forms complexes nor interacts with most other proteins (Bartel *et al.*, 1993b; S. Fields, pers. comm.; Ye & Worman, 1995).

TABLE IV. TWO-HYBRID SYSTEM VECTORS

	Use	Epitope ^a	Yeast selection ^c	Bacterial selection ^d
Cloning vectors				
pGBKT7	Express any protein as a GAL4 DNA-BD fusion	c-Myc	<i>TRP1</i>	kanamycin
pGADT7-Rec (<i>Sma</i> I-linearized)	Express any protein as a GAL4 AD fusion	HA	<i>LEU2</i>	ampicillin
Control vectors				
pGADT7-RecT ^b	Express SV40 largeT-antigen as a GAL4 AD fusion	HA	<i>LEU2</i>	ampicillin
pGBKT7-53	Express p53 as a GAL4 DNA-BD fusion	c-Myc	<i>TRP1</i>	kanamycin
pGBKT7-Lam	Express lamin C as a GAL4 DNA-BD fusion	c-Myc	<i>TRP1</i>	kanamycin

^a HA = hemagglutinin; These epitope tags can be used to verify protein-protein interactions *in vitro* by coimmunoprecipitation (Co-IP) using the antibodies and protocol provided with the Matchmaker Co-IP Kit (Cat. No. 630449). They are not intended to be used for detection, affinity purification, or Co-IP of hybrid proteins expressed in yeast.

^b Created by homologous recombination *in vivo* by cotransforming yeast with SV40 LargeT PCR Fragment and pGADT7-Rec.

^c The DNA-BD and AD vectors have different nutritional markers, so they can be independently selected when yeast transformants are plated on SD minimal medium lacking specific nutrients. The selection medium you choose depends on which plasmids you are using, whether you are selecting for one or two plasmids, and whether you are selecting for colonies in which two hybrid proteins are interacting.

^d The vectors carry different antibiotic markers so that they can be independently selected in *E. coli*.

VI. Protocol Overview: One-Hybrid Library Construction & Screening

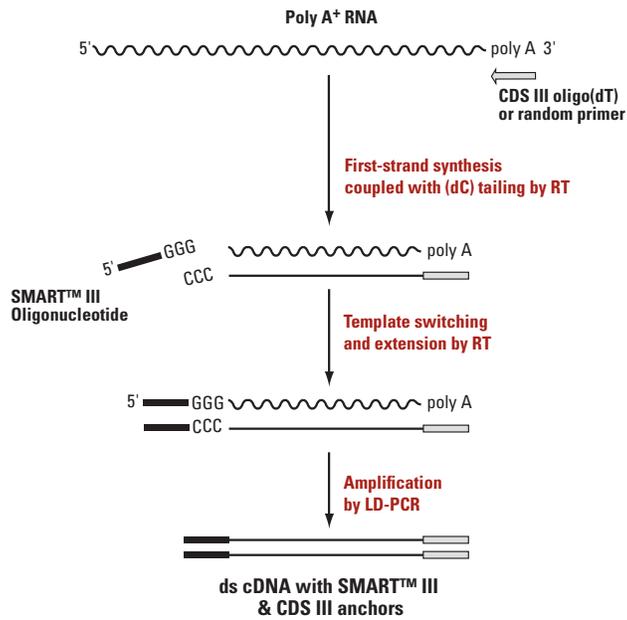


Figure 6. Matchmaker One-Hybrid Library Construction & Screening.

VI. Protocol Overview: Two-Hybrid Library Construction & Screening

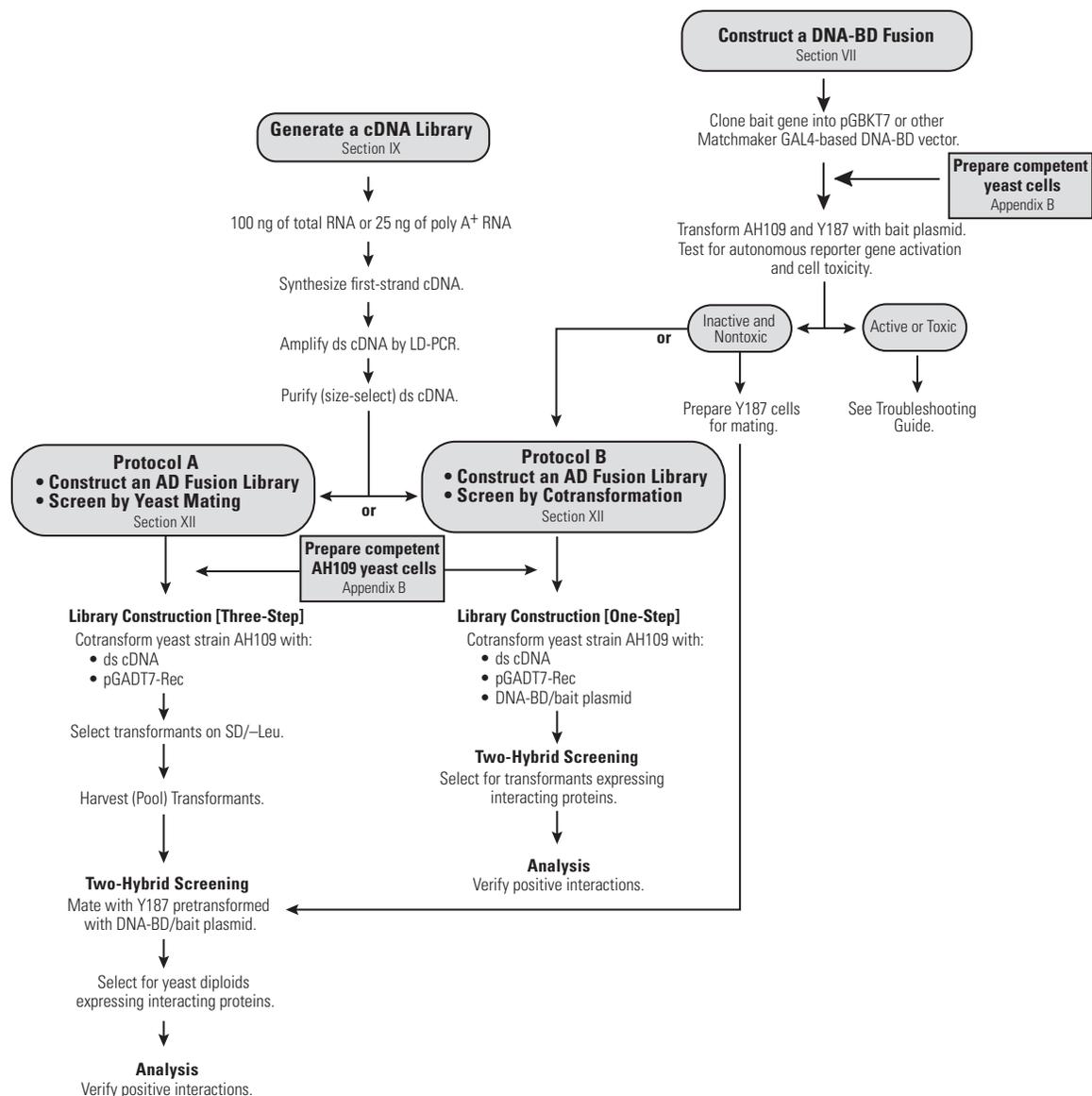


Figure 7. Matchmaker Library Construction & Screening Kit. Two-hybrid libraries may be screened by either yeast mating or cotransformation.

VII. Constructing a Reporter Vector for One-Hybrid Analysis

A. Background

To use the Matchmaker One-Hybrid System to screen a cDNA library for DNA-binding proteins, you must have identified a true or putative target element. It must be precisely defined using, for example, deletion and/or point mutation analysis. A construct composed of one or more tandem copies of your target regulatory element bordered by restriction sites is then prepared and inserted into the multiple cloning site (MCS) of pHIS2. This links the target element to the *HIS3* reporter gene. Inserting your target element may alter the level of background *HIS3* expression. Therefore, constructs should be tested for background (leaky) *HIS3* expression before you start a one-hybrid analysis. Background growth due to leaky *HIS3* expression is controlled by adding 3-AT to the selection medium, as described in Section IV.F.

B. Synthesize Your Target Element

Each target-reporter construct should contain at least one copy of the DNA target element inserted upstream of the reporter gene. Many early studies indicated that the reporter should contain at least three tandem copies of the DNA target. However, as Wei *et al.* (1999) have demonstrated, a single copy may be sufficient in many cases. For more information about target copy number, see Ghosh *et al.*, 1993. Tandem copies may be generated by various methods, but we have found the most convenient and reliable method for generating them to be oligonucleotide synthesis. It works nicely because well-defined regulatory elements are usually ≤ 20 bp.

1. Design two antiparallel oligonucleotides, one representing the sense strand and the other its antisense complement.

Note: The sense strand should consist of one or more copies of the target element with a different restriction site on each end. When the two strands are annealed, the resulting double-stranded DNA will have a different overhang at each end for directional cloning into pHIS2. See the pHIS2 Vector Information Packet (PT3705-5) for a diagram of the multiple cloning site.

2. Synthesize both strands without 5' phosphates (according to the protocol of the synthesizer manufacturer).

C. Insert Your DNA Target into the Multiple Cloning Site of pHIS2

1. For each construct planned, mix 0.1 μg of sense-strand and 0.1 μg of antisense-strand oligonucleotide in 10 μl of 50 mM NaCl.
2. Anneal the oligonucleotides by heating at 70°C for 5 min and then slowly cooling to room temperature (~30 min).
3. **Completely** digest 0.1 μg of pHIS2 in a 20- μl double digest using an appropriate pair of restriction enzymes. Incubate at 37°C for 2 hr, or as directed by the enzyme manufacturer.
4. Electrophorese a 2- μl sample of the digest on a 1% agarose gel to confirm that the plasmid has been efficiently linearized.
5. Combine 5 μl of digested plasmid with 1 μl of annealed oligo and 4 μl of H₂O.
6. Add 1.2 μl of 10X T4 ligation buffer and 0.8 μl (at least 0.8 units) of T4 DNA ligase, and incubate at room temperature for 4 hr.

Note: Since the molar ratio of oligonucleotide to vector is 100:1 or greater, gel purification to remove the stuffer fragment is unnecessary.

7. Separately transform competent *E. coli* cells with each construct using a standard method (Sambrook *et al.*, 1989). We recommend using a general purpose strain such as DH5 α or Fusion-Blue Competent Cells.
8. Plate transformants on LB/kan plates, and incubate at 37°C overnight.
9. Prepare plasmid using any standard method that yields highly pure DNA (Sambrook *et al.*, 1989). Check for inserts by electrophoresing on a 2% agarose gel and sequencing across the junctions.

D. Test your Target-Reporter Construct for Background *HIS3* Expression

1. Transform Y187 with the target-reporter construct using a small-scale protocol. (For example, see the small-scale protocol used in Section XI.C.)
2. Follow the procedure in Section IV.F to determine the optimum concentration of 3-AT to use in the selection medium. For example, we find that 10 mM 3-AT is sufficient to suppress background growth of Y187 cells transformed with p53HIS2 Control Vector.

VIII. Constructing a DNA-BD Fusion Vector for Two-Hybrid Analysis

A. Construct a DNA-BD Fusion

- You can generate a GAL4 DNA-BD fusion gene if compatible restriction sites are present in the test gene and the corresponding vector (Table V). If not, generate the gene fragment by PCR using primers that contain the desired restriction site (Scharf, 1990). A restriction site at the end of a gene can often be changed into a different site or put into a different reading frame by using a PCR primer that incorporates the desired mutation. Alternatively, if you have already cloned your gene into a Creator™ Donor Vector, use Cre recombinase to transfer your gene to pLP-GBKT7. Refer to the Creator DNA Cloning Kits User Manual (PT3460-1) for details.
- For more detailed information on cloning, see Sambrook *et al.* (1989).

TABLE V. COMPARISON OF MATCHMAKER DNA-BD VECTORS

DNA-BD vector	Description	Size	Protein Expression
pGBKT7	<i>GAL4</i> ₍₁₋₁₄₇₎ DNA-BD <i>TRP1</i> , Kan ^r	7.3 kb	High
pBridge ^a	<i>GAL4</i> ₍₁₋₁₄₇₎ DNA-BD <i>TRP1</i> , Amp ^r	6.5 kb	Low
pGBT9 ^b	<i>GAL4</i> ₍₁₋₁₄₇₎ DNA-BD, <i>TRP1</i> , Amp ^r	5.4 kb	Low
pAS2-1 ^b	<i>GAL4</i> ₍₁₋₁₄₇₎ DNA-BD, <i>TRP1</i> , Amp ^r , <i>CYH</i> ^{s2}	8.4 kb	High
pLP-GBKT7 ^c	<i>GAL4</i> ₍₁₋₁₄₇₎ DNA-BD, <i>TRP1</i> , Kan ^r , <i>loxP</i>	7.5 kb	High

^a Contains two distinct expression cassettes for investigating ternary protein complexes.

^b DNA-BD vectors used in previous Matchmaker Two-Hybrid Systems.

^c Creator Acceptor Vector (LP = *loxP*). Accepts a gene of interest from any Creator Donor Vector and expresses it as a GAL4 DNA-BD fusion.

1. Purify the gene fragment.

Note: We recommend the NucleoSpin Extraction Kit (Cat. No. 635961) for rapid isolation of DNA fragments.

- Digest the DNA-BD vector with the appropriate restriction enzyme(s), treat with phosphatase, and purify.
- Ligate the appropriate vector and insert. Transform ligation mixtures into *E. coli*.
- Identify insert-containing plasmids by restriction analysis or PCR.

B. Test the DNA-BD Fusion for Transcriptional Activation

- Transform AH109 and Y187 with the hybrid construct using a small-scale transformation protocol such as the one given in Section XII.A.7. Plate transformants on the following media*:

- SD/-Trp/X- α -Gal
- SD/-His/-Trp/X- α -Gal
- SD/-Ade/-Trp/X- α -Gal

Include a negative control. For example, transform cells with an "empty" DNA-BD vector.

- * **Note:** The dropout supplements required to make these media are not supplied with the Matchmaker Library Construction & Screening Kit (Cat. No. 630445). You must purchase these supplements separately from a commercial supplier, or prepare them yourself using the recipe given in Appendix C of the Yeast Protocols Handbook (PT3024-1).
- Analyze results.
 - Bait protein is inactive** if the transformant colonies are *white* and do not grow on SD/-His/-Trp or SD/-Ade/-Trp. Go to Steps 5–6.
 - Bait protein is active** if transformant colonies are *blue* and grow on SD/-His/-Trp or SD/-Ade/-Trp. Continue with Steps 3–4.
 - If a bait strain exhibits background growth on -His medium, you may be able to eliminate (or reduce) the background by adding 3-AT to the selection medium (Section IV.F). Alternatively, use -Ade/-His/-Leu/-Trp medium for the library screening.
 - If a bait strain exhibits background growth on -Ade and -His medium, it will be difficult to use this bait protein in a two-hybrid library screening. See Troubleshooting Guide.

VIII. Constructing a DNA-BD Fusion Vector for Two-Hybrid Analysis *continued*

5. [Optional] If a known protein partner for your bait protein is available, use it to check whether a two-hybrid interaction is detectable with this particular DNA-BD/bait fusion.
6. [Optional] Verify protein expression:

- Prepare Western blots from the lysate of transformants and probe the blots with antibodies to the GAL4 DNA-BD (Cat. No. 630403). Use untransformed yeast lysate as a control.

Notes:

- Using polyclonal antibodies may result in multiple cross-reacting bands.
- The level of expression from pGBT9 or pBridge is too low to permit detection using our GAL4 DNA-BD Monoclonal Antibody.

C. Test the DNA-BD Fusion for Toxicity

- Compare the growth rate in liquid culture of Y187 cells transformed with the "empty" DNA-BD vector and cells transformed with the DNA-BD/bait plasmid. If the bait strain grows noticeably slower, your DNA-BD/bait protein may be toxic.

Note: We also recommend checking for toxicity in strain AH109.

- Use a Small-Scale Yeast Transformation Protocol (such as the one given in Section XII.A.7) to prepare transformants. Select transformants on SD/-Trp, then prepare an overnight culture as follows:

1. Inoculate 50 ml of SD/-Trp/Kan (20 µg/ml) with one large (2–3 mm) colony.
2. Incubate at 30°C overnight (16–24 hr) with shaking at 250–270 rpm.
3. Check the OD₆₀₀ of the culture; it should be ≥0.8. If the OD₆₀₀ is much less than 0.8, the DNA-BD fusion may be **toxic** (see the Troubleshooting Guide). If the fusion does not appear to hamper yeast growth—i.e. is **nontoxic**—and you plan to screen your two-hybrid library by yeast mating, continue with Steps 4–7. If you are planning to screen by cotransformation, you may stop here and proceed to Section IX.
4. Centrifuge at 600 x g for 5 min.
5. Remove supernatant.
6. Resuspend in ~5 ml SD/-Trp liquid medium. Count cells using a hemacytometer. The cell density should be ≥1 x 10⁹ cells/ml.
7. Mate this bait strain with your AD fusion library host strain (Section XII.A.4).

Note: Y187 (*MAT α*) can mate with AH109, HF7c, CG-1945, Y190, or SFY526 (all *MAT α*).

IX. Generating a cDNA Library

A. Generating a cDNA library with SMART™ technology

Messenger RNA transcripts are efficiently copied into ds cDNA using SMART (Switching Mechanism at 5' end of RNA Transcript) technology (Zhu, Y. Y., *et al.*, 2001). This cDNA synthesis and amplification system is particularly well suited for one-hybrid and two-hybrid library construction because it consistently delivers high yields of cDNA while maintaining sequence representation. By maintaining the complexity of the original tissue, the SMART procedure provides you with the best opportunity of detecting rare and potentially novel interactions during yeast one-hybrid and two-hybrid screening.

B. How SMART cDNA Synthesis and Amplification Works

In the first-strand cDNA synthesis step, MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (RT) is used to transcribe RNA into DNA. To prime RNA for cDNA synthesis, you may use either a modified oligo(dT) primer (our CDS III Primer) or a random primer (our CDS III/6 Primer).

The composition of the resulting cDNA library may differ depending on which primer you choose. If you use the CDS III Primer, which hybridizes to the 3'-end of polyA⁺ RNA, sequences close to the 5'-end of the transcript may be slightly under-represented. If instead you use the CDS III/6 Primer, a random primer that can hybridize to many different sequences on the RNA template, your library should contain a variety of 5'- and 3'-end sequences, which are represented in near equal proportions.

When MMLV RT encounters a 5'-terminus on the template, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART III™ Oligonucleotide, which has an oligo(G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template (Figure 8). RT then switches templates and continues replicating to the end of the oligonucleotide. In the majority of syntheses, the resulting ss cDNA contains the complete 5' end of the mRNA as well as the sequence complementary to the SMART III Oligo, which then serves as a universal priming site (SMART III anchor) in the subsequent amplification by long-distance PCR (LD PCR; Chenchik *et al.*, 1998). Only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and be exponentially amplified by long-distance PCR (LD PCR).

In the second step, ss cDNA is amplified by LD PCR to produce a ds cDNA library. We recommend using the Advantage® 2 PCR Kit (Cat. Nos. 639206 & 639207) to generate and amplify ds cDNA. The Advantage 2 Polymerase Mix consists of TITANIUM *Taq* DNA Polymerase (a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase), *Taq*Start Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994), and a minor amount of a proofreading polymerase. This polymerase system lets you amplify cDNA (as large as 20 kb) with a fidelity rate significantly higher than that of conventional PCR (Barnes, 1994).

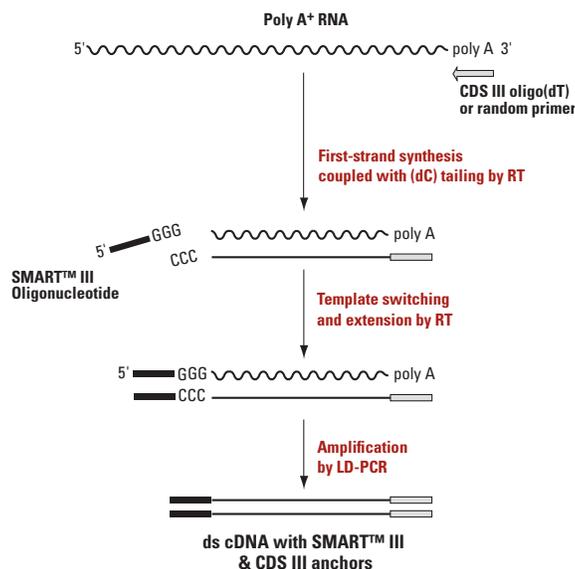


Figure 8. Synthesis of high-quality ds cDNA using SMART technology.

IX. Generating a cDNA Library *continued*

C. Good Laboratory Practices

- Wear gloves to protect your RNA and cDNA samples from degradation by nucleases.
- When resuspending pellets or mixing reactions, gently pipet the solution up and down or tap the bottom of the tube. Spin briefly to bring contents to the bottom of the tube. Do not vortex samples when resuspending pellets; vortexing may shear your cDNA.
- Perform all reactions on ice, unless otherwise indicated.
- Add enzymes to reaction mixtures last. Be sure the enzyme is thoroughly blended into the reaction mixture by gently pipetting the mixture up and down.
- **Do not increase the size (volume) of any of the reactions. All components have been optimized for the volumes specified.**
- Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing this reagent. For more information, see Sambrook *et al.* (1989). BondEx Ethidium Bromide Detoxification Cartridges are available from Clontech.
- Phenol is a corrosive. Always wear gloves and protective clothing when handling solutions containing this reagent. If possible, handle solutions containing phenol and/or chloroform under a chemical fume hood.
- In preparing your reactions, use the Deionized H₂O supplied. Otherwise, use freshly deionized (e.g., Milli-Q-grade) H₂O, without treatment with DEPC (diethyl pyrocarbonate). Avoid using autoclaved H₂O because recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- Rinse all glassware with 0.5 N NaOH, followed by deionized H₂O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips when handling RNA.

D. RNA Isolation

- Many procedures are available for the isolation of total RNA and polyA⁺ RNA (Chomczynski & Sacchi, 1987; Farrell, 1993; Sambrook *et al.*, 1989). Clontech offers several kits for the isolation of total RNA and subsequent isolation of poly A⁺ RNA (see Related Products).
- The minimum amount of starting material for cDNA synthesis is 100 ng of total RNA or 25 ng of poly A⁺ RNA. In general, the more RNA you start with, the fewer PCR cycles will be required for amplification (see Table VI). Fewer thermal cycles are less likely to generate nonspecific PCR products, and therefore are best for optimal cDNA and library quality. Thus, if your RNA sample is not limiting, use the higher starting amounts of RNA shown in the table.

E. RNA Analysis

- The sequence complexity of the ds cDNA synthesized, and ultimately of the cDNA library constructed, depends on the quality of the experimental RNA starting material. Therefore, before you use it in a first-strand synthesis, we recommend you estimate the integrity of the RNA by examining a sample on a denaturing formaldehyde/agarose gel. Total RNA from mammalian sources should appear as two bright bands (28S and 18S ribosomal RNA) at approximately 4.5 and 1.9 kb. The ratio of intensities of the 28S and 18S rRNA bands should be 1.5–2.5:1. Intact mammalian poly A⁺ RNA should appear as a smear (usually 0.5–10 kb [or more]) with faint 28S and 18S rRNA bands. The size distribution may be considerably smaller (0.5–3 kb) for nonmammalian species (e.g., plants, insects, yeast, amphibians, *etc.*).
- If the ratio of intensity of 28S RNA to 18S RNA (for total RNA) is less than 1:1 or if your experimental poly A⁺ RNA appears significantly smaller than expected (e.g., no larger than 1–5 kb), we suggest you prepare fresh RNA after checking your RNA purification reagents for RNase and other impurities. If problems persist, you may need to find another source of tissue or cells.

IX. Generating a cDNA Library *continued*

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

- We suggest you also perform a positive control cDNA synthesis with Human Placenta Poly A⁺ RNA. This control lets you verify that all components are working properly and lets you evaluate the yield and sizes of the ds cDNA synthesized from your RNA sample.
- In the protocols that follow, you have the option of priming first-strand cDNA synthesis with an oligo (dT) or random primer (Sections F and G, respectively). The reaction conditions vary slightly depending on the primer used.

F. Synthesize First-Strand cDNA using an Oligo (dT) Primer

- Combine the following reagents in a sterile 0.25-ml microcentrifuge tube:

1–2	μl	RNA sample (0.025–1.0 μg poly A ⁺ or 0.10–2.0 μg total RNA)
		(For the control reaction, use 1 μl [1 μg] of the control RNA.)
1.0	μl	CDS III Primer
1–2	μl	Deionized H ₂ O to bring volume up to 4.0 μl.
<hr/>		
4.0	μl	Total volume
- Mix contents and spin briefly.
- Incubate at 72°C for 2 min.
- Cool on ice for 2 min.
- Spin briefly.
- Add the following to the reaction tube:

2.0	μl	5X First-Strand Buffer
1.0	μl	DTT (20 mM)
1.0	μl	dNTP Mix (10 mM)
1.0	μl	MMLV Reverse Transcriptase
<hr/>		
9.0	μl	Total volume
- Mix gently by tapping. Spin briefly.
- Incubate at 42°C for 10 min.
- Add 1.0 μl SMART III Oligonucleotide.
- Incubate at 42°C for 1 hr in an air incubator or hot-lid thermal cycler.

Note: If you use a water bath or non hot-lid thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.
- Place the tube at 75°C for 10 min to terminate first-strand synthesis.
- Cool the tube to room temperature, then add 1.0 μl RNase H.
- Incubate at 37°C for 20 min.
- If you plan to proceed directly to the LD-PCR step, take a 2-μl aliquot from the first-strand synthesis and place it in a clean, prechilled, 0.5-ml tube. Place the tube on ice, and proceed to Section H. If you used mineral oil in your first-strand reaction tube, be sure to take the 2-μl sample from the **bottom** of the tube to avoid the oil.
- Any first-strand reaction mixture that is not used right away should be placed at –20°C. First-strand cDNA can be stored at –20°C for up to three months.

G. Synthesize First-Strand cDNA using a Random Primer

- Combine the following reagents in a sterile 0.25-ml microcentrifuge tube:

1–2	μl	RNA sample (0.025–1.0 μg poly A ⁺ or 0.10–2.0 μg total RNA)
		(For the control reaction, use 1 μl [1 μg] of the control RNA.)
1.0	μl	CDS III/6 Primer
1–2	μl	Deionized H ₂ O to bring volume up to 4.0 μl.
<hr/>		
4.0	μl	Total volume
- Mix contents and spin briefly.
- Incubate at 72°C for 2 min.
- Cool on ice for 2 min.
- Spin briefly.

IX. Generating a cDNA Library *continued*

6. Keep the tube at room temperature and add the following:

2.0 µl	5X First-Strand Buffer
1.0 µl	DTT (20 mM)
1.0 µl	dNTP Mix (10 mM)
1.0 µl	MMLV Reverse Transcriptase
<hr/>	
9.0 µl	Total volume

7. Mix gently by tapping. Spin briefly.

8. Incubate at 25–30°C for 10 min at room temperature.

9. Incubate at 42°C for 10 min.

10. Add 1.0 µl SMART III Oligonucleotide.

11. Incubate at 42°C for 1 hr in an air incubator or hot-lid thermal cycler.

Note: If you use a water bath or non hot-lid thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.

12. Place the tube at 75°C for 10 min to terminate first-strand synthesis.

13. Cool the tube to room temperature, then add 1.0 µl (2 units) RNase H.

14. Incubate at 37°C for 20 min.

15. If you plan to proceed directly to the LD-PCR step, take a 2-µl aliquot from the first-strand synthesis and place it in a clean, prechilled, 0.5-ml tube. Place the tube on ice, and proceed to Section H. If you used mineral oil in your first-strand reaction tube, be sure to take the 2-µl sample from the **bottom** of the tube to avoid the oil.

16. Any first-strand reaction mixture that is not used right away should be placed at –20°C. First-strand cDNA can be stored at –20°C for up to three months.

H. Amplify ds cDNA by Long Distance PCR (LD-PCR)

Table VI shows the optimal number of thermal cycles to use based on the amount of RNA used in the first-strand synthesis. Fewer cycles generally mean fewer nonspecific PCR products. The optimal cycling parameters in Table VI were determined using the Control Poly A⁺ Human Placenta RNA; these parameters may vary with different templates and thermal cyclers.

TABLE VI. RELATIONSHIP BETWEEN AMOUNT OF RNA AND OPTIMAL NUMBER OF THERMAL CYCLES

Total RNA (µg)	Poly A ⁺ RNA (µg)	Number of Cycles
1.0–2.0	0.5–1.0	15–20
0.5–1.0	0.25–0.5	20–22
0.25–0.5	0.125–0.25	22–24
0.05–0.25	0.025–0.125	24–26

IX. Generating a cDNA Library *continued*

1. Preheat the PCR thermal cycler to 95°C.
2. To prepare sufficient ds cDNA for transformation, set up two 100- μ l PCR reactions for each experimental sample. Set up one reaction for the Control sample. In each reaction tube, combine the following components:

2	μ l	First-Strand cDNA
70	μ l	Deionized H ₂ O
10	μ l	10X Advantage 2 PCR Buffer
2	μ l	50X dNTP Mix
2	μ l	5' PCR Primer
2	μ l	3' PCR Primer
10	μ l	10X GC-Melt Solution
2	μ l	50X Advantage 2 Polymerase Mix
<hr/>		
100	μ l	Total volume

3. Mix gently by flicking the tube. Centrifuge briefly.
4. Overlay the reaction mixture with 2 drops of mineral oil if necessary. Cap the tube and place it in a preheated (95°C) thermal cycler.
5. Begin thermal cycling. If you have a hot-lid thermal cycler, use the following program:

- 95°C 30 sec
- x cycles^a:

95°C	10 sec
68°C	6 min ^b
- 68°C 5 min

^a Refer to Table VI to determine the optimal number of cycles to use.

^b Program the cycler to increase the extension time by 5 sec with each successive cycle. For example, in the second cycle, the extension should last 6 min and 5 sec; in the third, 6 min and 10 sec. And so on.

Note: These cycling parameters may not be optimal for non hot-lid thermal cyclers.

6. When the cycling is complete, analyze a 7- μ l aliquot of the PCR product from each sample alongside 0.25 μ g of a 1-kb DNA size marker on a 1.2% agarose/EtBr gel. Typical results obtained with Human Placenta Poly A⁺ RNA are shown in Appendix A. If your PCR product does not appear as expected, refer to the Troubleshooting Guide.
7. Proceed with Section I or store ds cDNA at -20°C until use.

I. Purify ds cDNA with a CHROMA SPIN™ TE-400 Column

CHROMA SPIN Columns are packed with resins that fractionate molecules based on size. Molecules larger than the pore size are excluded from the resin. These molecules quickly move through the gel bed when the column is centrifuged, while molecules smaller than the pore size are held back. In the following protocol, a CHROMA SPIN TE-400 Column is used to select for DNA molecules >200 bp. For more information about CHROMA SPIN Columns, please refer to the CHROMA SPIN Columns User Manual (PT1300-1), available at our website at www.clontech.com.

Note: We recommend centrifuging CHROMA SPIN Columns in a swinging bucket or horizontal rotor. Fixed-angle rotors can also be used, but there is a risk that a portion of the sample will slide down the inner side of the column instead of passing through the gel matrix, resulting in reduced or inconsistent purification. Perform the following steps for each experimental and control sample.

1. Remove the CHROMA SPIN Column from the protective plastic bag and invert it several times to resuspend the gel matrix completely. Use one column for each ~95- μ l cDNA sample.
2. Holding the CHROMA SPIN Column upright, grasp the break-away end between your thumb and index finger and snap off (Figure 9). Place the end of the spin column into one of the 2-ml microcentrifuge (collection) tubes, and lift off the top cap. Save the top cap and the white-end cap.

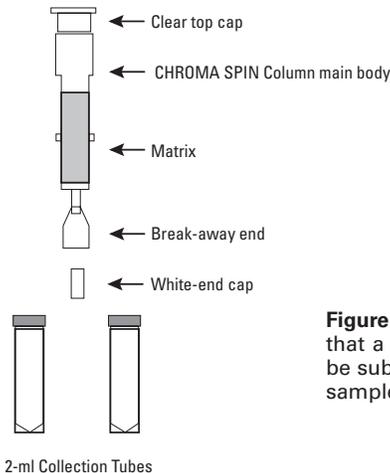
IX. Generating a cDNA Library *continued*

Figure 9. CHROMA SPIN Column and Collection Tubes. Note that a conventional, tapered 1.5-ml microcentrifuge tube can be substituted for the 2-ml collection tube. This will allow the sample to be confined to a narrower area for easier handling.

3. Centrifuge at 700 x g for 5 min. After centrifugation, the column matrix will appear semi-dry. This step purges the equilibration buffer from the column and re-establishes the matrix bed.

Note: The column fitted in the 2-ml microcentrifuge tube can be placed inside a 17 x 100-mm polypropylene tube during centrifugation in a swinging bucket rotor.
4. Remove the spin column and collection tube from the centrifuge rotor, and discard the collection tube and column equilibration buffer.

Note: Always hold both the CHROMA SPIN Column and the 2-ml microcentrifuge tube when removing them from the rotor.
5. Place the spin column into the second 2-ml microcentrifuge tube. Carefully and slowly apply your cDNA sample (~95 µl from Step IX.H.7) to the **center** of the gel bed's flat surface. Do not allow any sample to flow along the inner wall of the column.

Note: A conventional, tapered 1.5-ml microcentrifuge tube can be substituted for the 2-ml collection tube. This will allow the sample to be confined to a narrower area for easier handling.
6. Centrifuge at 700 x g for 5 min.
7. Remove the spin column and collection tube from the rotor and detach them from each other. The purified sample is at the bottom of the collection tube.

Note: Hold the sample collection tube to prevent it from detaching from the spin column.
8. Combine duplicate experimental samples in a single tube.
9. Add the following reagents:
 - 1/10 vol. Sodium Acetate (3 M; pH 4.8)
 - 2.5 vol. 95% ethanol (-20°C)
10. Mix gently by rocking the tube back and forth.
11. Place the tube in a -20°C freezer or a dry-ice/ethanol bath for 1 hr. (**Optional:** You may incubate at -20°C overnight, which may result in better recovery.)
12. Centrifuge the tube at 14,000 rpm for 20 min at room temperature.
13. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
14. Briefly centrifuge the tube to bring all remaining liquid to the bottom.
15. Carefully remove all liquid and allow the pellet to air dry for ~10 min.
16. Resuspend the pellet in 20 µl of Deionized H₂O and mix gently. The cDNA is now ready for *in vivo* recombination (Library Construction) with pGADT7-Rec or pGADT7-Rec2. Proceed with One-Hybrid or Two-Hybrid Library Construction, or store the cDNA at -20°C until you are ready.

X. Constructing & Screening One-Hybrid and Two-Hybrid Libraries

A. Constructing GAL4 AD Fusion Libraries for One-Hybrid and Two-Hybrid Screening

1. Whether you plan to screen for one-hybrid or two-hybrid interactions, the methods used to construct the library are the same. In both cases, recombination-mediated cloning is used to fuse SMART ds cDNA with the GAL4 AD (Figure 10). While the cloning methods are the same, the GAL4 AD cloning vectors are not—

- To construct a one-hybrid library, use pGADT7-Rec2.
- To construct a two-hybrid library, use pGADT7-Rec.

pGADT7-Rec2 and pGADT7-Rec differ in their mode of replication. pGADT7-Rec is a high-copy plasmid; it contains a 2 μ origin of replication, which enables it to replicate multiple times during the cell cycle. pGADT7-Rec2, on the other hand, is a low-copy plasmid; it contains an autonomous replication sequence, or *ARS* element, which allows the vector to replicate only once during the cell cycle. pGADT7-Rec2 also contains a centromeric sequence, or *CEN* element, to ensure stable segregation of the plasmid during mitosis and meiosis. Low-copy plasmids such as pGADT7-Rec2 are preferred for one-hybrid analysis because they generate fewer false positives.

2. A GAL4 AD fusion library is produced by cotransforming yeast with SMART ds cDNA and *Sma* I-linearized pGADT7-Rec or pGADT7-Rec2, depending on whether you are constructing a two-hybrid or one-hybrid library. SMART ds cDNA recombines with the AD cloning vector *in vivo* to yield a complete GAL4 AD expression vector (Figure 10). The resulting construct will express the cDNA insert as a GAL4 AD fusion protein. This one-step cloning procedure is possible because the SMART III and CDS III sequences—incorporated into the cDNA by RT and LD-PCR—have been engineered into the pGADT7-Rec and pGADT7-Rec2 plasmids. In yeast, the linear plasmid is restored to its circular form by recombination with overlapping sequences at the ends of the SMART cDNA. Successful plasmid assembly results in a positive (*Leu2*⁺) transformant.

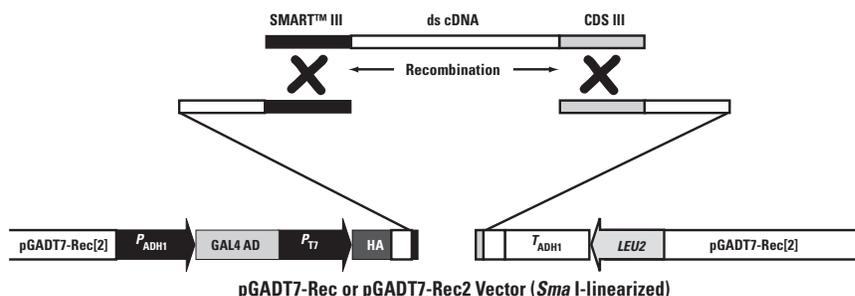


Figure 10. Constructing AD fusion libraries by recombination-mediated cloning in yeast. To clone cDNA into pGADT7-Rec or pGADT7-Rec2, cotransform yeast with ds cDNA (generated with our modified SMART procedure) and either pGADT7-Rec (two-hybrid screen) or pGADT7-Rec2 (one-hybrid screen). Yeast repair enzymes will restore the *Sma* I-linearized plasmid to its circular form by recombining sequences at the ends of the cDNA with homologous sequences at the ends of pGADT7-Rec(2). The outcome is a fully functional GAL4 AD/cDNA expression vector.

B. Screening GAL4 AD Fusion Libraries

1. One-Hybrid Library Screening (cotransformation)

With recombination-mediated cloning (Figure 10), library construction and screening can be carried out in the same host strain on the same day. If you prepare a DNA target/reporter plasmid—e.g., *pHIS2*/DNA target—in advance, you can include it in the cotransformation reaction together with your cDNA library and pGADT7-Rec2. With a single transformation step, all three DNA components can be introduced into the yeast reporter strain (Figure 4). Screening begins as soon as the pGADT7-Rec2 AD vector is assembled by the host's recombination processes. Positive one-hybrids can be identified immediately after cotransformation by plating the transformation mixture on medium that selects for the *HIS3* nutritional reporter. For protocol details, see Section XI.

2. Two-Hybrid Library Screening (yeast mating or cotransformation)

Two-Hybrid libraries can be screened by either yeast mating or cotransformation. As described above, cotransformation allows you to construct and screen your library in a single host strain. The procedure is quick and efficient. For details please review Protocols A and B in Section XII and see the Two-Hybrid Library Construction & Screening flow chart in Figure 7.

XI. Constructing & Screening a One-Hybrid Library

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Before starting library construction:

- Pour an appropriate number of SD agar plates:
 - **SD/–His/–Leu/–Trp + optimal [3-AT]** to select for one-hybrid interactions (150-mm)
 - **SD/–Leu** to measure the transformation efficiency of the library plasmid (100-mm)
 - **SD/–Trp** to measure the transformation efficiency of the reporter plasmid (100-mm)
 - **SD/–Leu/–Trp** to measure the number of clones screened (100-mm plates)
- Allow SD agar plates to dry at room temperature for 2–3 days or at 30°C for 3 hr.
- Prepare PEG/LiAc Solution (Section III).
 - Be sure to run the necessary controls (Part C) in parallel with your experimental sample.

Important: Note that you must prepare competent yeast cells before starting library construction. Please take some time to review the procedure in Appendix B, and plan your work accordingly.

A. Cotransform Yeast Strain Y187 with ds cDNA, pGADT7-Rec2, and pHIS2/target DNA.

1. Prepare competent yeast cells (Appendix B).
 2. In a sterile, 15-ml tube combine the following:
 - 20 µl ds cDNA (from Section IX.I, Step 16)
 - 6 µl pGADT7-Rec2 (0.5 µg/µl)
 - 5 µg pHIS2/target DNA (prepared in Section VII)
 - 20 µl Herring Testes Carrier DNA, denatured*
- Note:** The combined volume of these DNA components should not exceed 60 µl, or 1/10 the volume of the competent cells added at Step 3, below.
- * Transfer ~50 µl of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 15-ml reaction tube.
3. Add 600 µl of competent cells to the DNA.
 4. Gently mix by vortexing.
 5. Add 2.5 ml PEG/LiAc Solution.
 6. Mix gently by vortexing for 3–5 sec.
 7. Incubate at 30°C for 45 min. Mix cells every 15 min.
 8. Add 160 µl DMSO, mix, and then place the tube in a 42°C water bath for 20 min. Mix cells every 10 min.
 9. Centrifuge at 700 x g for 5 min.
 10. Discard the supernatant and resuspend in 3 ml of **YPD Plus Liquid Medium**.

Note: YPD Plus is specially formulated to promote transformation. **Do not use** standard YPD medium for this step.
 11. Incubate at 30°C with shaking at ~265 rpm for 90 min.
 12. Centrifuge at 700 x g for 5 min.
 13. Discard the supernatant and resuspend in 6 ml of NaCl Solution (0.9%).

B. Select for One-Hybrid Interactions

1. To determine the transformation efficiency and to calculate the number of clones screened, spread 100 µl of a 1:10, 1:100, and 1:1,000 dilution onto 100-mm SD/–Leu, SD/–Trp, and SD/–Leu/–Trp agar plates.
2. Spread the remaining mixture on SD/–His/–Leu/–Trp + optimal [3-AT] plates (150 µl cells/150-mm plate) to select for one-hybrid interactions.
3. Incubate at 30°C for 3–7 days until colonies appear.
4. Calculate the transformation efficiency and number of clones screened:
 - a. Colonies on SD/–Leu x dilution factor ÷ volume (ml) plated x 6 ml = #transformants per 3 µg pGADT7-Rec2. Expected: $\geq 1 \times 10^6$ transformants / 3 µg pGADT7-Rec2
 - b. Colonies on SD/–Leu/–Trp x dilution factor ÷ volume (ml) plated x 6 ml = #clones screened
Expected: $\geq 3 \times 10^5$ clones / library

XI. Constructing & Screening a One-Hybrid Library *continued*

- Restreak the His⁺ colonies on fresh SD/–His/–Leu/–Trp + optimal [3-AT]. Incubate at 30°C for 2–4 days. Seal the plates with Parafilm and store at 4°C. For long-term storage, prepare glycerol stocks and store at –70°C.
- Analyze positive interactions as described in Section XIII.

C. One-Hybrid Controls

One-Hybrid Controls		
	Cotransform Y187 with:	Control Strain(plasmids)
1. Positive Control	<ul style="list-style-type: none"> pGAD-Rec2-53 p53HIS2 	Transform Y187 → Y187[pGAD-Rec2-53 + p53HIS2]
2. Negative Control	<ul style="list-style-type: none"> pGAD-Rec2-53 pHIS2 	Transform Y187 → Y187[pGAD-Rec2-53 + pHIS2]

TABLE VII. SET-UP FOR ONE-HYBRID CONTROL COTRANSFORMATIONS

Component	#1 Positive Control (μl)	#2 Negative Control (μl)
pGAD-Rec2-53 (500 ng/μl)	1.0	1.0
p53HIS2 (500 ng/μl)	1.0	—
pHIS2 (500 ng/μl)	—	1.0
Herring Testes Carrier DNA (10 mg/ml), denatured*	5	5
Y187 Competent Yeast Cells	50	50
PEG/LiAc Solution	500	500

* Transfer ~50 μl of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 1.5-ml reaction tube.

- Prepare competent yeast cells (Appendix B).
- Set up two 1.5-ml microcentrifuge tubes. Add DNA, competent yeast cells, and PEG/LiAc Solution using the volumes and in the order indicated (Table VII).
- Mix thoroughly by gently vortexing.
- Incubate in a water bath at 30°C for 30 min. Vortex gently every 10 min.
- Add 20 μl of DMSO to each tube, mix, and then place the tube in a 42°C waterbath for 15 min. Vortex gently every 7.5 min.
- Centrifuge at high speed for 15 sec.
- Remove supernatant and resuspend in 1 ml of **YPD Plus Liquid Medium**.
- Shake at 30°C for 90 min.
- Centrifuge at high speed for 15 sec.
- Discard the supernatant and resuspend in 1 ml of NaCl Solution (0.9%) by gently pipetting up and down.
- Spread 100 μl of a 1:10, 1:100, and 1:1,000 dilution on SD/–Leu, SD/–Trp, and SD/–Leu/–Trp to check the transformation efficiency, and on SD/–His/–Leu/–Trp + 10 mM 3-AT to select for positive one-hybrids.
- Incubate the plates at 30°C (face down) for 2–7 days, until colonies appear.
- Compare your results with those shown in Table VIII.

TABLE VIII. CONTROL ONE-HYBRID COTRANSFORMATIONS: EXPECTED RESULTS

Control Strain	Plated on SD Minimal Media	Phenotype (Growth)
Positive Control		
Y187[pGAD-Rec2-53 + p53HIS2]	–His/–Leu/–Trp + 10 mM 3-AT	+
Negative Control		
Y187[pGAD-Rec2-53 + pHIS2]	–His/–Leu/–Trp + 10 mM 3-AT	–

XII. Constructing & Screening a Two-Hybrid Library: Protocol A

There are two ways to screen a Matchmaker two-hybrid library:

- Yeast mating
- Cotransformation

The method you choose determines the protocol you will now use to construct (and screen) your library. To screen by yeast mating, construct your library using Protocol A. To screen by cotransformation, construct your library using Protocol B. For a quick comparison of these two protocols, refer back to Figure 7.

Important: Note that you must prepare competent yeast cells before starting library construction. Please take some time to review the procedure in Appendix B, and plan your work accordingly.

Protocol A: Screen by Yeast Mating

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Before starting:

- Pour SD agar plates. You will need:

SD/-Leu	~200	150-mm plates
SD/-Leu	~5-10	100-mm plates
SD/-Trp (see note below)*	~5-10	100-mm plates
SD/-Leu/-Trp	~5-10	100-mm plates
SD/-His/-Leu/-Trp (see note below)*	~50	150-mm plates
SD/-Ade/-His/-Leu/-Trp/X- α -Gal	~50	150-mm plates

Allow SD agar plates to dry at room temperature for 2-3 days or at 30°C for 3 hr before plating any transformation mixtures.

- * **Note:** As explained in the List of Additional Materials Required (Section III), the dropout supplements required to make these media are not supplied. You must obtain these supplements separately from a commercial supplier or prepare them yourself using the recipe in Appendix C of the Yeast Protocols Handbook (PT3024-1).
- Plan controls (Steps 7-8) Controls should be done in parallel with experimental work.
- Prepare Freezing Medium: YPD medium with 25% (v/v) glycerol.
- Prepare PEG/LiAc Solution (Section III).

1. Transform yeast strain AH109 with ds cDNA and pGADT7-Rec.

- a. Prepare competent yeast cells (Appendix B).
- b. In a sterile, prechilled, 15-ml tube combine the following:
 - 20 μ l ds cDNA (from Section IX.I, Step 16)
 - 6 μ l pGADT7-Rec (0.5 μ g/ μ l)
 - 20 μ l Herring Testes Carrier DNA, denatured*

*Transfer ~50 μ l of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 15-ml reaction tube.
- c. Add 600 μ l of competent cells to the DNA.
- d. Gently mix by vortexing.
- e. Add 2.5 ml PEG/LiAc Solution.
- f. Gently mix by vortexing.
- g. Incubate at 30°C for 45 min. Mix cells every 15 min.
- h. Add 160 μ l DMSO, mix, and then place the tube in a 42°C water bath for 20 min. Mix cells every 10 min.
- i. Centrifuge at 700 x g for 5 min.
- j. Discard the supernatant and resuspend in 3 ml of **YPD Plus Liquid Medium**.

Note: YPD Plus is specially formulated to promote transformation. **Do not use** standard YPD medium for this step.

XII. Constructing & Screening a Two-Hybrid Library: Protocol A *continued*

- k. Incubate at 30°C with shaking for 90 min.
- l. Centrifuge at 700 x g for 5 min.
- m. Discard the supernatant and resuspend in 30 ml of NaCl Solution (0.9%).

2. Select transformants on SD/-Leu plates.

- a. Spread 150 µl on each 150-mm plate (~200 plates total).
Note: To check the transformation efficiency, spread 100 µl of a 1:10, 1:100, 1:1,000, and 1:10,000 dilution on 100-mm SD/-Leu plates.
- b. Incubate plates upside down at 30°C until colonies appear (~3–6 days).
- c. Calculate the transformation efficiency.
 Expected results: $\geq 1 \times 10^6$ transformants / 3 µg pGADT7-Rec

3. Harvest (pool) transformants.

- a. Chill plates at 4°C for 3–4 hr.
- b. Add 5 ml Freezing Medium to each plate.
- c. Use sterile glass beads and gentle swirling to dislodge the cells into the liquid.
- d. Combine all liquids in a sterile flask. Mix well.
- e. Check the cell density using a hemacytometer. If the cell density $\leq 2 \times 10^7$ cells/ml, reduce the volume of the suspension by centrifuging.
- f. Aliquot (1-ml) and store at -80°C (not longer than 1 year).
- g. To determine the library titer, spread 100 µl of a 1:100, 1:1,000, and 1:10,000 dilution on 100-mm SD/-Leu plates. Incubate at 30°C until colonies appear (~2–3 days). Count the colonies (cfu) and calculate the number of clones in your library.

4. Mate the library host strain with your bait strain.

- a. Thaw a 1-ml aliquot ($\geq 2 \times 10^7$ cells) of your AH109[library] in a room temperature water bath.
- b. Combine the 5-ml Y187[bait] culture from Section VIII.C.7 ($\geq 1 \times 10^9$ cells/ml) and the 1-ml aliquot of AH109[library] cells ($\geq 2 \times 10^7$ cells/ml) in a sterile 2-L flask.
Note: The flask size must be at least 2 L to permit sufficient aeration of the mating culture at low-speed swirling.
- c. Add 45 ml 2XYFDA/Kan (50 µg/ml). Swirl gently.
- d. Rinse cells from library vial with two 1-ml aliquots of 2XYFDA/Kan (50 µg/ml).
- e. Incubate at 30°C for 20–24 hr with gentle swirling (30–50 rpm).
Note: Low-speed swirling is necessary to keep cells from settling to the bottom of the flask. However, shaking the culture at speeds >50 rpm will significantly reduce mating efficiency.
- f. After 20 hr of mating, check a drop of the mating culture under a phase-contrast microscope (400X). If zygotes are present, allow mating to continue for four more hours. Otherwise, continue to Step g.
Note: A zygote typically has a three-lobed shape, the lobes representing the two haploid (parental) cells and the budding diploid cell.
- g. Transfer the mating mixture to a sterile 100-ml centrifuge bottle. Centrifuge at 1,000 x g for 10 min. Meanwhile, rinse the mating flask twice (50 ml each rinse) with 0.5XYFDA/Kan (50 µg/ml). Combine the rinses and use them to resuspend the pellet.
- h. Centrifuge at 1,000 x g for 10 min. Resuspend the cell pellet in 10 ml of 0.5XYFDA/Kan (50 µg/ml). Measure the total volume of cells + medium.

5. Select for yeast diploids expressing interacting proteins

- a. To determine the mating efficiency, spread 100 µl of a 1:10,000, 1:1,000, 1:100, and 1:10 dilution of the mating mixture on three media (100-mm plates):
 - SD/-Leu
 - SD/-Trp
 - SD/-Leu/-Trp
- b. Spread remaining mating mixture on TDO or QDO plates (200 µl cells / 150-mm plate).
 - TDO stands for Triple Dropout Medium: SD/-His/-Leu/-Trp
 - QDO stands for Quadruple Dropout Medium: SD/-Ade/-His/-Leu/-Trp

See Figure 11.

XII. Constructing & Screening a Two-Hybrid Library: Protocol A *continued*

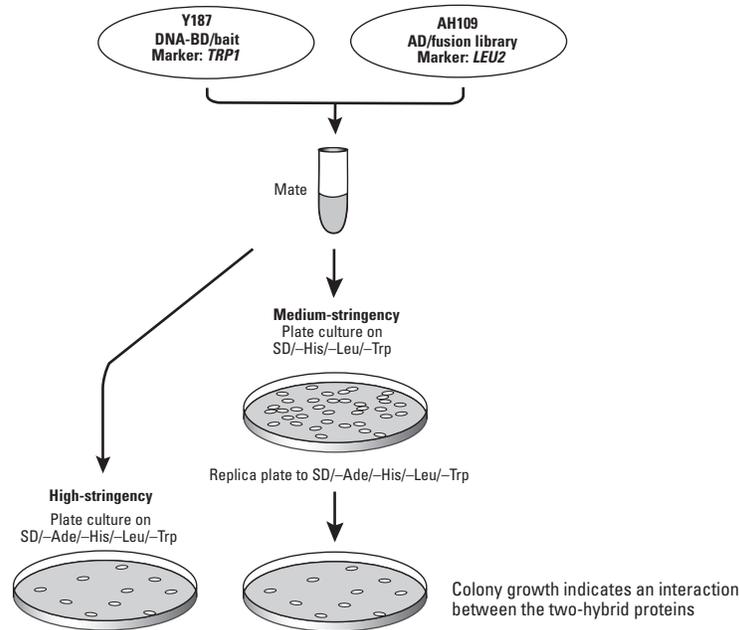


Figure 11. Screening an AD fusion library for two-hybrid interactions. Use the stringency of your choice to screen for interacting proteins. Note: high stringency selections result in fewer colonies, and reduce the number of false positives. However, weak interactions may be missed.

- c. Incubate at 30°C until colonies appear.

After 2–3 days, some colonies will be visible, but plates should be incubated for at least 5 days to allow slower growing colonies (i.e., weak positives) to appear. Ignore the small, pale colonies that may appear after 2 days but never grow to >1 mm in diameter. True Ade⁺, His⁺ colonies are robust and can grow to >2 mm. Also, Ade⁺ colonies are white or light pink, whereas Ade⁻ colonies will slowly turn red on adenine-limited medium.

- d. Score for growth on SD/-Leu, SD/-Trp, and SD/-Leu/-Trp. Calculate Mating Efficiency and Number of Colonies Screened (Part 6).
- e. For colonies growing on TDO medium: Replica plate colonies onto QDO medium. Incubate at 30°C for 3–8 days.
- f. Choose Ade⁺, His⁺ colonies for further analysis.

Not all of the colonies surviving this selection will be true two-hybrid positives. However, the most common class of false positives will be eliminated by screening for expression of *ADE2* and *HIS3* (James *et al.*, 1996). Other types of false positives can be eliminated as described in Section XIII.

- g. Streak out Ade⁺/His⁺ colonies on fresh SD/-Ade/-His/-Leu/-Trp/X- α -Gal master plates and grow for 2–4 days at 30°C. Having X- α -Gal present in the medium enables you to test Ade⁺/His⁺ colonies for the activation of a third reporter: *MEL1*, which encodes α -galactosidase, a secreted enzyme that hydrolyzes X- α -Gal to produce a blue end product.
- h. Seal the master plates with Parafilm and store at 4°C. If desired, prepare glycerol stock cultures of interesting clones and freeze at -70°C for long-term storage.

6. Calculate Mating Efficiency & Number of Clones Screened

- a. Count the colonies (cfu) growing on the SD/-Leu, SD/-Trp, and SD/-Leu/-Trp dilution plates that have 30–300 cfu.
- b. Calculate the viable cfu/ml on each type of SD medium:

$$\frac{\text{cfu}}{\text{Vol. plated (ml)} \times \text{dilution factor}} = \# \text{ viable cfu/ml}$$

cfu/ml on SD/-Leu = viability of Y187 partner
 # cfu/ml on SD/-Trp = viability of AH109 partner
 # cfu/ml on SD/-Leu/-Trp = viability of diploids

XII. Constructing & Screening a Two-Hybrid Library: Protocol A *continued*

- c. Compare the viable cfu/ml of the two mating partners. The strain with the lower viability is the “limiting” partner. In this library screening protocol, the AH109[library] strain should be the limiting partner to ensure that the maximum number of library cells find a mating partner. In a control cross, either strain could be limiting.

- d. Calculate the mating efficiency (i.e., % Diploid):

$$\frac{\text{\# cfu/ml of diploids}}{\text{\# cfu/ml of limiting partner}} \times 100 = \% \text{ Diploid}$$

Note: If the mating efficiency was <2%, and if you obtained few (if any) positive clones, you may wish to repeat the library screening with another 1-ml aliquot. But first, see the Troubleshooting Guide for tips on improving the mating efficiency.

- e. Estimate the number of clones screened:

$$\text{\# cfu/ml of diploids} \times \text{resuspension volume (ml)} = \text{\# of clones screened}$$

7. Controls for Protocol A: Small-Scale Yeast Transformation

Use this small-scale transformation protocol to produce the following three control strains.

Two-Hybrid Transformation Controls

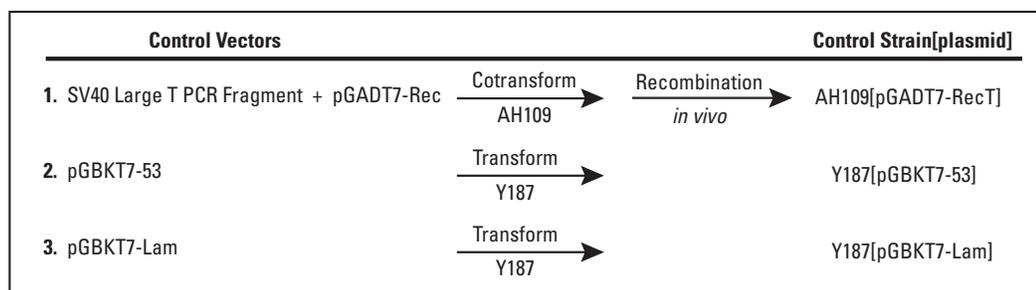


TABLE IX. SET-UP FOR CONTROL TWO-HYBRID TRANSFORMATIONS

Component	#1 (μl)	#2 (μl)	#3 (μl)
SV40 Large T PCR Fragment (25 ng/μl)	5.0	—	—
pGADT7-Rec (<i>Sma</i> I-linearized; 500 ng/μl)	0.5	—	—
pGBKT7-53 (500 ng/μl)	—	0.5	—
pGBKT7-Lam (500 ng/μl)	—	—	0.5
Herring Testes Carrier DNA (10 mg/ml), denatured*	5	5	5
AH109 competent yeast cells	50	—	—
Y187 competent yeast cells	—	50	50
PEG/LiAc Solution	500	500	500
Select for transformants by plating on:	SD/–Leu	SD/–Trp	SD/–Trp

* Transfer ~50 μl of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 1.5-ml reaction tube.

- Prepare competent yeast cells (Appendix B).
- Set up three 1.5-ml microcentrifuge tubes. Add DNA, competent yeast cells, and PEG/LiAc Solution using the volumes and in the order indicated (Table IX).
- Mix thoroughly by gently vortexing.
- Incubate at 30°C for 30 min. Vortex gently every 10 min.
- Add 20 μl of DMSO to each tube, mix, and then place the tube in a 42°C waterbath for 15 min. Vortex gently every 5 min.
- Centrifuge at high speed in a microcentrifuge for 15 sec.

XII. Constructing & Screening a Two-Hybrid Library: Protocol A *continued*

- g. Remove supernatant and resuspend in 1 ml of **YPD Plus Liquid Medium**.
- h. Incubate at 30°C for 90 min.
- i. Centrifuge at high speed for 15 sec.
- j. Discard the supernatant and resuspend in 1 ml of NaCl Solution by gently pipetting up and down.
- k. Spread 100 µl of a 1:10, 1:100, and 1:1,000 dilution onto 100-mm plates containing SD/-Leu or SD/-Trp, depending on the nutritional marker carried by the plasmid (Table IX).
- l. Incubate the plates at 30°C (face down) for 2–4 days, until colonies appear.
- m. Pick the largest colonies and restreak them on the same selection medium. Seal these master plates with Parafilm and store at 4°C (not longer than 1 month). Use these colonies for the control matings in Section 8.

8. Controls for Protocol A: Small-Scale Yeast Mating

- a. Pick one colony of each type—i.e., AH109[pGBKT7-RecT], Y187[pGBKT7-53], and Y187[pGBKT7-Lam]—to use in the mating. Use only large (2–3-mm), fresh (<2 weeks old) colonies from the master plates.
- b. Place both colonies in one 1.5-ml microcentrifuge tube containing 0.5 ml of 2XYFDA medium. Vortex the tube for 1 min to completely resuspend the cells.
- c. Incubate at 30°C overnight (20–24 hr) with shaking.
Note: Use the lowest shaking speed possible to prevent cells from settling. Vigorous shaking can reduce the mating efficiency.
- d. Plate cells on SD minimal media (Table X; 100 µl cells/100-mm plate). Use sterile 5-mm glass beads to promote even spreading of the cells.
- e. Incubate plates (colony side down) at 30°C for 3–5 days to allow diploid cells to form visible colonies.
- f. Score for growth. Calculate the mating efficiency.
- g. Confirm nutritional and reporter phenotypes of diploids (Table X).
- h. Pick representative colonies from selection plates. Streak onto fresh medium.
- i. After colonies have grown, seal plates with Parafilm and store at 4°C. For long term storage (>2 weeks), prepare glycerol stock cultures and freeze at –70°C. These diploids are useful as reference strains when you wish to check a new batch of SD selection medium.

TABLE X. SET-UP FOR CONTROL TWO-HYBRID MATINGS

Cross	Plate on SD Minimal Media ^a (100-mm plates)	Phenotype	
		Mel1	His/Ade
Positive Control			
AH109[pGADT7-RecT] x Y187[pGBKT7-53]	-Leu ^b -Trp ^b -Leu/-Trp ^b -Ade/-His/-Leu/-Trp/X-α-Gal	Blue	+
Negative Control			
AH109[pGADT7-RecT] x Y187[pGBKT7-Lam]	-Leu ^b -Trp ^b -Leu/-Trp ^b -Ade/-His/-Leu/-Trp/X-α-Gal	no colonies	-

^a Spread 100-µl aliquots of 1:10 and 1:100 dilutions of the mating culture

^b Use these plates to calculate the mating efficiency.

XII. Constructing & Screening a Two-Hybrid Library: Protocol B

Protocol B: Screen by Cotransformation

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Before starting:

- Pour SD agar plates.

SD/-Leu	~5-10	100-mm plates
SD/-Trp	~5-10	100-mm plates
SD/-Leu/-Trp	~5-10	100-mm plates
SD/-His/-Leu/-Trp	~50	150-mm plates
SD/-Ade/-His/-Leu/-Trp/X- α -Gal	~50	150-mm plates

Allow SD agar plates to dry at room temperature for 2-3 days or at 30°C for 3 hr before plating any transformation mixtures.

- Plan controls (Step 3) Controls should be done in parallel with experimental work.
- Prepare Freezing Medium: YPD medium with 25% (v/v) glycerol.
- Prepare PEG/LiAc Solution (Section III).

1. Cotransform yeast strain AH109 with ds cDNA, pGADT7-Rec, and pGBKT7/bait.

Important: Note that you must prepare competent yeast cells before starting library construction. Please take some time to review the procedure in Appendix B, and plan your work accordingly.

- a. Prepare competent yeast cells (Appendix B).
- b. In a sterile, 15-ml tube combine the following:
 - 20 μ l ds cDNA (from Section IX.I, Step 16)
 - 6 μ l pGADT7-Rec (0.5 μ g/ μ l)
 - 5 μ g pGBKT7/bait plasmid DNA (\leq 10 μ l)
 - 20 μ l Herring Testes Carrier DNA, denatured*

*Transfer ~50 μ l of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 15-ml reaction tube.
- c. Add 600 μ l of competent cells to the DNA.
- d. Gently mix by vortexing.
- e. Add 2.5 ml PEG/LiAc Solution.
- f. Gently mix by vortexing.
- g. Incubate at 30°C for 45 min. Mix cells every 15 min.
- h. Add 160 μ l DMSO, mix, and then place the tube in a 42°C water bath for 20 min. Mix cells every 10 min.
- i. Centrifuge at 700 x g for 5 min.
- j. Discard the supernatant and resuspend in 3 ml of **YPD Plus Liquid Medium**.

Note: YPD Plus is specially formulated to promote transformation. Do not use standard YPD medium for this step.
- k. Incubate at 30°C with shaking for 90 min.
- l. Centrifuge at 700 x g for 5 min.
- m. Discard the supernatant and resuspend in 6 ml of NaCl Solution (0.9%).

2. Select for transformants expressing interacting proteins

- a. Spread the cotransformation mixture on TDO or QDO plates (150 μ l cells/ 150-mm plate).
 - TDO stands for Triple Dropout Medium: SD/-His/-Leu/-Trp
 - QDO stands for Quadruple Dropout Medium: SD/-Ade/-His/-Leu/-Trp

See Figure 11.

Note: Determine the transformation efficiency as follows:

- i. Remove a 30- μ l aliquot from the 6-ml suspension and dilute with 720 μ l of NaCl Solution (for a final volume of 750 μ l).
- ii. Spread 150- μ l aliquots of this dilution on two separate 150-mm plates: SD/-Leu/-Trp and SD/-Leu.

XII. Constructing & Screening a Two-Hybrid Library: Protocol B *continued*

- b. Incubate at 30°C until colonies appear.
After 2–3 days, some colonies will be visible, but plates should be incubated for at least 5 days to allow slower growing colonies to appear. Ignore the small, pale colonies that may appear after 2 days but never grow to >1 mm in diameter. True Ade⁺, His⁺ colonies are robust and can grow to >2 mm. Also, Ade⁺ colonies are white or light pink, whereas Ade⁻ colonies will slowly turn red on adenine-limited medium.
- c. Score for growth on the SD/-Leu and SD/-Leu/-Trp dilution plates.
- Count the colonies growing on SD/-Leu.
#colonies x 1,000 = #transformants / 3 µg pGADT7-Rec.
Expected results: ≥1 x 10⁶ transformants / 3 µg pGADT7-Rec
 - Count the colonies growing on SD/-Leu/-Trp.
#colonies x 1,000 = #clones screened
Expected results: ≥5 x 10⁵ clones / library
- d. For colonies growing on TDO medium: Replica plate colonies onto QDO medium. Incubate at 30°C for 3–8 days.
- e. Choose Ade⁺/His⁺ colonies for further analysis.
Not all of the colonies surviving this selection will be true two-hybrid positives. However, the most common class of false positives will be eliminated by screening for expression of *ADE2* and *HIS3* (James *et al.*, 1996). Other types of false positives can be eliminated as described in Section XIII.
- f. Streak out Ade⁺/His⁺ colonies on fresh SD/-Ade/-His/-Leu/-Trp/X-α-Gal master plates and grow for 2–4 days at 30°C. Having X-α-Gal present in the medium enables you to test Ade⁺/His⁺ colonies for the activation of a third reporter: *MEL1*, which encodes α-galactosidase, a secreted enzyme that hydrolyzes X-α-Gal to produce a blue end product.

3. Controls for Protocol B: Small-Scale Yeast Cotransformation

Use this small-scale cotransformation protocol to produce the following two control strains.

Two-Hybrid Cotransformation Controls

	Cotransform AH109 with:		Control Strain[plasmids]
1. Positive Control	<ul style="list-style-type: none"> SV40 Large T PCR Fragment pGADT7-Rec pGBKT7-53 	Recombination <i>in vivo</i> →	AH109[pGADT7-RecT + pGBKT7-53]
2. Negative Control	<ul style="list-style-type: none"> SV40 Large T PCR Fragment pGADT7-Rec pGBKT7-Lam 	Recombination <i>in vivo</i> →	AH109[pGADT7-RecT + pGBKT7-Lam]

TABLE XI. SET-UP FOR CONTROL TWO-HYBRID COTRANSFORMATIONS

Component	#1 Positive Control (µl)	#2 Negative Control (µl)
SV40 Large T PCR Fragment (25 ng/µl)	5.0	5.0
pGADT7-Rec (<i>Sma</i> I-linearized; 500 ng/µl)	0.5	0.5
pGBKT7-53 (500 ng/µl)	0.5	—
pGBKT7-Lam (500 ng/µl)	—	0.5
Herring Testes Carrier DNA (10 mg/ml), denatured*	5	5
AH109 Competent Yeast Cells	50	50
PEG/LiAc Solution	500	500

* Transfer ~50 µl of Herring DNA to a microcentrifuge tube and heat at 100°C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 1.5-ml reaction tube.

XII. Constructing & Screening a Two-Hybrid Library: Protocol B *continued*

- a. Prepare competent yeast cells (Appendix B).
- b. Set up two 1.5-ml microcentrifuge tubes. Add DNA, competent yeast cells, and PEG/LiAc Solution using the volumes and in the order indicated (Table XI).
- c. Mix thoroughly by gently vortexing.
- d. Incubate in a water bath at 30°C for 30 min. Vortex gently every 15 min.
- e. Add 20 µl of DMSO to each tube, mix, and then place the tube in a 42°C waterbath for 20 min. Vortex gently every 5 min.
- f. Centrifuge at high speed for 15 sec.
- g. Remove supernatant and resuspend in 1 ml of **YPD Plus Liquid Medium**.
- h. Incubate in a water bath at 30°C for 90 min. Mix every 15 min by gently vortexing.
- i. Centrifuge at high speed for 15 sec.
- j. Discard the supernatant and resuspend in 1 ml of NaCl Solution by gently pipetting up and down.
- k. Spread 100 µl of a 1:10, 1:100, and 1:1,000 dilution onto 100-mm SD agar plates:
 - SD/–Leu/–Trp
To check the cotransformation efficiency
 - SD/–Ade/–His/–Leu/–Trp/X–α-Gal
To select for cotransformants expressing interacting proteins
- l. Incubate the plates at 30°C (face down) for 2–4 days, until colonies appear.
- m. Compare your results with those shown in Table XII.
- n. Pick the largest colonies and restreak them on the same selection medium.
- o. After colonies have grown, seal these master plates with Parafilm and store at 4°C. For long term storage (>2 weeks), prepare glycerol stock cultures and freeze at –70°C. These strains are useful references for checking new batches of SD selection medium.

TABLE XII. CONTROL TWO-HYBRID COTRANSFORMATIONS: EXPECTED RESULTS

Control Strain	Plate on SD Minimal Media	Phenotype	
		MeI1	His/Ade
Positive Control			
AH109[pGADT7-RecT + pGBKT7-53]	–Ade/–His/–Leu/–Trp/X–α-Gal	Blue	+
Negative Control			
AH109[pGADT7-RecT + pGBKT7-Lam]	–Ade/–His/–Leu/–Trp/X–α-Gal	no colonies	–

XIII. Analyzing Positive Interactions

This section presents strategies for verifying and analyzing protein-protein and protein-DNA interactions. Figure 12 provides a detailed overview.

A. Retest the Phenotype

- Library transformants may contain more than one AD/library plasmid, which can complicate the analysis of putative positive clones. Thus, it is a good idea to restreak the positive colonies on SD dropout plates 2–3 times to segregate the AD/library plasmids. You should restreak on an SD dropout medium that selects for both the library and bait plasmids as well as for the one-hybrid or two-hybrid interaction:
 - Restreak positive one-hybrid clones on SD/–His/–Leu/–Trp + optimal [3-AT].
Restreak positive two-hybrid clones on either TDO or QDO medium. Keep in mind that QDO is a more stringent selection. Reminder:
 - TDO stands for triple dropout medium = SD/–Ade/–Leu/–Trp or SD/–His/–Leu/–Trp
 - QDO stands for quadruple dropout medium = SD/–Ade/–His/–Leu/–Trp
 - Incubate plates at 30°C for 4–6 days.
- [Optional] Test the phenotype further by assaying for a third reporter gene or by selecting for the interaction under different concentrations of 3-AT.
 - Two-hybrid colonies: Replica plate or transfer well-isolated colonies to SD/–Ade/–His/–Leu/–Trp plates containing X- α -Gal to verify that they maintain the correct phenotype and to test for the expression of *MEL1*.
 - One-hybrid colonies: Replica plate or transfer well-isolated colonies to SD/–His/–Leu/–Trp plates containing different concentrations of 3-AT to verify that they maintain the correct phenotype and to test the strength of the interaction.
- Collect the restreaked and retested positive colonies in a grid fashion on fresh master plates.
- Incubate plates at 30°C for 4–6 days.
- After colonies have grown, seal plates with Parafilm, and store at 4°C for up to 4 weeks.

B. Rescue the Library cDNA Insert

To identify the gene (and thus protein) responsible for a positive one-hybrid or two-hybrid interaction, first rescue the gene by Plasmid Isolation or by PCR Colony-Screening:

Plasmid Isolation:

- Isolate plasmid DNA from yeast using the Yeastmaker Yeast Plasmid Isolation Kit (Cat. No. 630441) or other suitable method.
- Because the plasmid DNA isolated from each yeast colony will be a mixture of the bait plasmid and at least one type of AD/library plasmid, you will need to separate the plasmids by selection in *E. coli*.

Note: Bait plasmids used with previous versions of our two-hybrid and one-hybrid systems contained the gene for ampicillin resistance. Bait plasmids used with newer versions of our kits, however, contain the gene for kanamycin resistance.

If you used bait and library plasmids that contain different antibiotic selection markers:

- Transform the isolated plasmid extract from yeast into a general purpose *E. coli* strain.
- Plate the transformants on LB medium containing ampicillin to select for the AD/library plasmid only.

If you used bait and library plasmids that contain the same antibiotic selection marker:

- Transform the yeast-purified plasmid into KC8 *E. coli* cells.
- Plate the transformants on M9 medium lacking leucine to select for the AD/library plasmid only.

Note: KC8 cells have a defect in *leuB* that can be complemented by yeast *LEU2*.

- Purify the AD/library plasmid using any suitable method.
- Amplify the cDNA insert by PCR using the Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433) and the Advantage 2 PCR Kit (Cat. No. 639206).
- Analyze the PCR product as described in Part C.

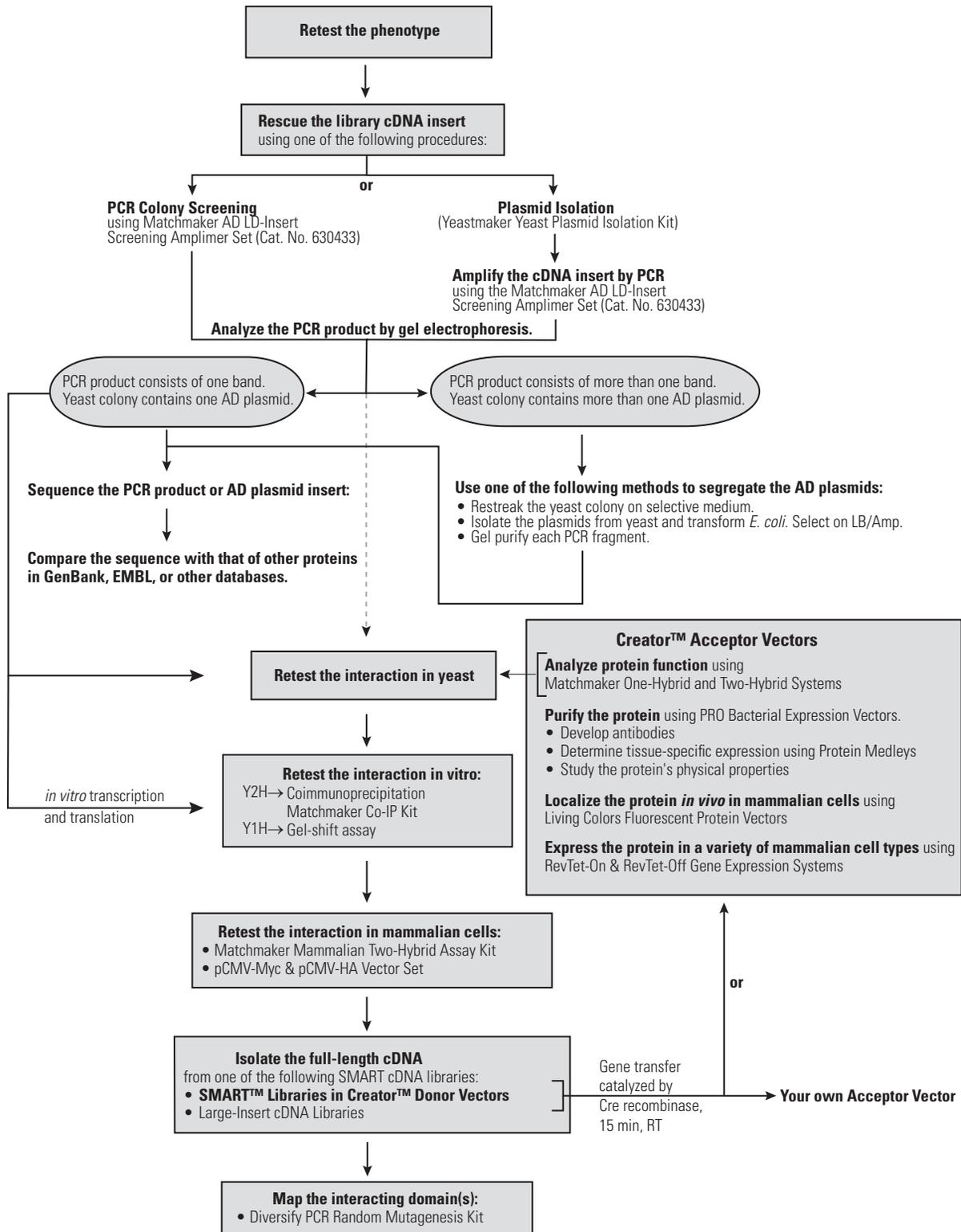
XIII. Analyzing Positive Interactions *continued*

Figure 12. Strategies for analyzing and verifying putative positive one-hybrid and two-hybrid interactions.

XIII. Analyzing Positive Interactions *continued*

PCR Colony Screening:

This procedure uses the Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433) and Advantage 2 PCR Polymerase Mix. We recommend using the Advantage 2 Polymerase Mix, rather than any other DNA polymerase formulation, because we find that it performs well in yeast cell samples, and because it is optimized for applications that involve longer templates and require high fidelity.

1. Preheat a PCR thermal cycler to 94°C.
2. Place the Advantage 2 PCR Kit components and GAL4ADLD-Insert Screening Amplimers on ice and allow them to thaw completely. Mix each component thoroughly before use.
3. Prepare a Master Mix by combining the components as specified in Table XIII.

TABLE XIII: ASSEMBLING MASTER MIXES FOR PCR COLONY SCREENING

Reagent	1 rxn	10 rxns + 1 extra	25 rxns +1 extra
PCR-grade deionized H ₂ O	41 µl	451 µl	1066 µl
10X Advantage 2 PCR Buffer	5 µl	55 µl	130 µl
5' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
3' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
50X dNTP Mix (10 mM ea.)	1 µl	11 µl	26 µl
50 X Advantage 2 Polymerase Mix	1 µl	11 µl	26 µl
Total	50 µl	550 µl	1300 µl

4. Using a sterile pipette tip, scrape a few cells from a colony that you wish to analyze. Place the cells in the bottom of a clean 250-µl PCR tube.
5. Add 50 µl of Master Mix to the tube. Gently pipette up and down to disperse the cells.
6. Overlay the reaction mixture with 2 drops of mineral oil if necessary. Cap the tube and place it in a preheated thermal cycler.
7. Begin thermal cycling. If you have a hot-lid thermal cycler, use the following program:

Note: These cycling parameters may not be optimal for non hot-lid thermal cyclers.

- 94°C 3 min
 - 25–30 cycles:
 - 94°C 30 sec
 - 68°C 3 min
 - 68°C 3 min
 - Soak at 15°C
8. Analyze the PCR product as described in Part C.

C. Analyze the cDNA Insert by Agarose/EtBr Gel Electrophoresis

Analyze a 5-µl aliquot of the PCR product (from Part B) alongside DNA size markers on a 0.8% 1XTAE agarose/EtBr gel.

Tip: To distinguish the PCR product from similar size inserts in other AD/library plasmids, digest the PCR product with a frequent-cutter restriction enzyme such as Alu I or Hae III. Run a small sample on a 2% agarose/EtBr gel. Compare the digestion pattern with that of other inserts.

- If a high percentage of the colonies appear to contain the same AD/library insert, expand your PCR analysis to another batch of 50 colonies. Alternatively, eliminate the abundant clones by performing yeast colony hybridization on each master plate. Refer to the YPH for this procedure. Use a vector-free oligonucleotide probe designed from the sequence of the most abundant insert. Transfer a representative of each type of insert to a new master plate.
- If the PCR product consists of more than one band, see Part E, below.
- If the PCR product consists of a single band:

XIII. Analyzing Positive Interactions *continued*

1. Prepare a new master plate with a representative clone from each group.
2. If you are satisfied with the number of unique clones, prepare a glycerol stock of each unique type. Store at -80°C .
3. Purify the PCR product using any suitable method. We normally use a NucleoSpin Extraction Kit (Cat. No. 635961)
4. Proceed with Part D.

D. Sequence the cDNA Insert

AD/library cDNA inserts can be sequenced using the Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433), a T7 Sequencing Primer, or the 3' AD Sequencing Primer provided with MatchmakerTwo-Hybrid System 3 (Cat. No. 630303). **Always check the vector sequence against the primer you wish to use. Be aware that some Matchmaker AD plasmids (e.g., pACT2) do not contain a T7 Promoter.**

Verify the presence of an open reading frame (ORF) fused to the GAL4 AD sequence, and compare the sequence to those in GenBank, EMBL, or other databases.

- If your sequencing results reveal a very short peptide (<10 amino acids) fused to the AD—or no fusion peptide at all—keep sequencing beyond the stop codon. You may find another (larger) open reading frame (ORF). Such gaps can occur when a portion of the 5' untranslated region of an mRNA is cloned along with the coding region. A Western blot will reveal the presence and size of an AD fusion protein.
- In some cases, two different ORFs may be expressed as a fusion with the AD even though a nontranslated gap comes between them due, for example, to occasional translational read-through.
- If your sequencing results fail to reveal any ORF in frame with the AD coding region, it could be that the positive library clone is transcribed in the reverse orientation from a cryptic promoter within the ADH1 terminator (Chien *et al.*, 1991). Yeast also allow translational frameshifts. A large ORF in the wrong reading frame may actually correspond to the expressed protein.

E. Segregation of AD/Library Plasmids

Use one of the following procedures when a positive one-hybrid or two-hybrid colony contains multiple, non-identical AD/library plasmids.

- **Segregation in Yeast:**
 1. Restreak positive colonies on SD dropout plates 2–3 times to segregate the AD/library plasmids, as described in Part A, Step 1.
 2. Replica plate or transfer well-isolated colonies to the appropriate SD dropout plates to verify that they maintain the correct phenotype.
- **Segregation in Bacteria:**
 1. Isolate the plasmids from yeast using any standard method.
Note: The Yeastmaker Yeast Plasmid Isolation Kit (Cat. No. 630441) provides a complete system for isolating plasmid DNA from yeast.
 2. Transform *E. coli* DH5 α cells with the plasmid preparation and select on LB/amp plates. Pick individual colonies.
- **PCR Fragment Purification** using agarose gel electrophoresis.

F. Retest the Interaction in Yeast

Retest protein-protein and protein-DNA interactions in yeast by either cotransformation or yeast mating.

1. Cotransformation

- a. Transform competent yeast cells* with the bait and AD/library plasmids. Alternatively, transform yeast cells* with bait plasmid, pGADT7-Rec(2), and the PCR product of interest, generated in Part B.

* For one-hybrid analysis, use yeast strain Y187. For two-hybrid analysis, use yeast strain AH109.

Be sure to include the appropriate negative and positive controls. For examples, refer to the controls used in Sections XI and XII. One negative control should consist of yeast cells transformed with your candidate AD/library plasmid and an empty bait plasmid—either pHis2 or pGBKT7, depending on the system you are using.

XIII. Analyzing Positive Interactions *continued*

If you are retesting a one-hybrid interaction, and a non-binding mutant of your target element is available, consider using it as an additional negative control. Prepare a mutant-type construct otherwise identical to your original target-reporter construct and transform Y187 with the mutant reporter plasmid, the candidate cDNA insert, and pGADT7-Rec2. Colonies should result from transcriptional activation using the wild-type but not the mutant-type target. For an example, see Li & Herskowitz, 1993.

- b. Plate on the appropriate SD dropout medium.
- c. Incubate plates at 30°C until colonies appear.

2. Yeast Mating

The following procedure describes how yeast mating can be used to retest positive **two-hybrid interactions**. If you have access to a *MAT α* strain such as AH109, a similar approach could be used for the analysis of one-hybrid interactions, in which case the DNA-BD/bait plasmid is replaced by the pHis2 reporter plasmid.

If you have many positive clones to analyze, it will be more convenient to handle the clones in batches of 10 or so each.

- a. Transform AH109 with the AD/library plasmid(s), and select on SD/–Leu.
- b. Transform Y187 (or a suitable *MAT α* strain) with the following three plasmids, and select on SD/–Trp plates:
 - i. DNA-BD
 - ii. DNA-BD/bait
 - iii. pGBKT7-Lam
- c. For each candidate AD/library plasmid to be tested, set up the yeast matings indicated in Figure 13 using the Trp⁺ and Leu⁺ transformants obtained in Steps a & b above. Refer to Section XII.A.8 for a small-scale yeast mating procedure.
- d. Select for diploids by spreading mating mixtures on SD/–Leu/–Trp plates as directed.
- e. Streak or replica-plate to SD/–Ade/–His/–Leu/–Trp/X- α -gal. True positives are AD/library clones exhibiting reporter gene expression only when the AD/library plasmid is introduced by mating with the plasmid encoding the DNA-BD/bait protein.

Additional Two-Hybrid Tests:

- Transfer the library insert from the AD to the DNA-BD vector and vice versa, and then repeat the two-hybrid assay (Chien *et al.*, 1991; van Aelst *et al.*, 1993). You should still be able to detect the interaction.
- Create a frameshift mutation just upstream of the library insert in the AD plasmid by cutting at the *Mlu* I site, filling in the overhangs, and then religating (Bendixen *et al.*, 1994). Repeat the two-hybrid assay; you should not be able to detect the interaction.
- Generate site-specific deletion or substitution mutants and repeat the two-hybrid assay.

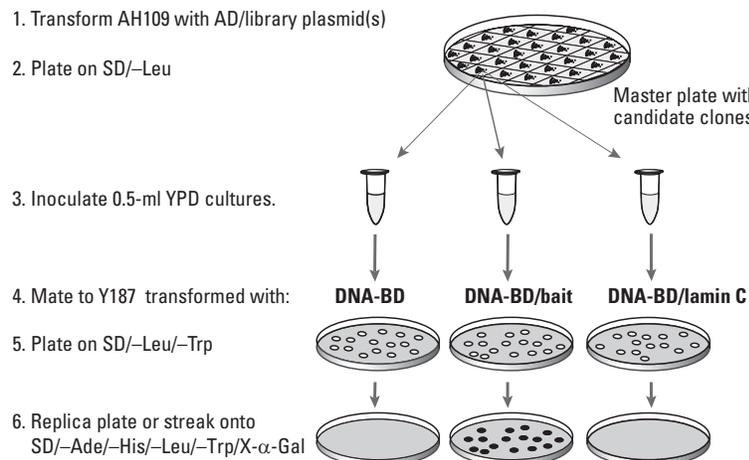


Figure 13. Yeast mating to verify protein-protein (two-hybrid) interactions.

XIII. Analyzing Positive Interactions *continued*

G. Retest the Interaction *In Vitro*

- **Two-hybrid** (Protein-protein) interactions can often be confirmed *in vitro* using coimmunoprecipitation (Co-IP). The **Matchmaker™ Co-IP Kit (Cat. No. 630449)** includes all the essential reagents (including antibodies, Protein A Beads, and a detailed step-by-step protocol) needed to perform *in vitro* Co-IP. Following is a general Co-IP protocol suitable for Matchmaker vectors.
 - a. Transcribe and translate the epitope-tagged fusion proteins *in vitro* using the T7 promoters in the AD and DNA-BD vectors.

Note: Matchmaker System 3 vectors—pGADT7, pGADT7-Rec, pGADT7-Rec2, pLP-GADT7, pGBKT7, and pLP-GBKT7—contain a T7 RNA polymerase promoter and either a c-Myc or hemagglutinin (HA) epitope tag so that you can use them directly for *in vitro* transcription and translation. (For all other GAL4-based vectors, you must incorporate a T7 promoter and epitope tag using PCR and an appropriate pair of primers.) Because the T7 promoter in System 3 vectors is located downstream of the GAL4 coding sequence, the GAL4 domains are not transcribed. Thus, *in vitro* Co-IP specifically detects interactions between bait and library proteins.
 - b. Coimmunoprecipitate the epitope-tagged fusion proteins using c-Myc and HA antibodies (Durfee *et al.*, 1993; Zhang *et al.*, 1993).

Note: If the fusion proteins *do not* coimmunoprecipitate, use other means to confirm the interaction. Protein interactions with weak affinities may escape detection by coimmunoprecipitation. See Phizichy & Fields (1995) for details on more sensitive detection methods. Furthermore, the AD fusion proteins may potentially not be in-frame with the epitope tag.
- **One-Hybrid** (Protein-DNA) interactions can often be confirmed and studied *in vitro* using an electrophoretic mobility-shift assay (EMSA; Wu *et al.*, 1994).
 - a. Transcribe and translate the HA epitope-tagged fusion protein *in vitro* using the T7 promoter in the AD vector pGADT7-Rec2.

Note: The T7 promoter in pGADT7-Rec2 is located downstream of the GAL4 coding sequence; thus, the GAL4 activation domain is not transcribed *in vitro*.
 - b. Perform an EMSA assay with your wild-type and mutant DNA targets to verify and, if desired, to map the DNA-binding activity of the protein.

H. Retest the Interaction in Mammalian Cells

In mammalian cells, proteins are more likely to be in their native conformations and to have the appropriate post-translational modifications; therefore, results are more likely to represent biologically significant interactions.

- **Two-hybrid interactions**

Clontech offers the following products for testing two-hybrid interactions in mammalian cells:

 - The **pCMV-Myc&pCMV-HA Vector Set** (Cat. No. 631604) for *in vivo* coimmunoprecipitation in mammalian cells. The CMV promoter in these vectors allows constitutive expression of the bait and library cDNA in a wide variety of mammalian cell types.
 - The **Mammalian Two-Hybrid Assay Kit** (Cat. No. 630301) is ideal for confirming protein interactions via two-hybrid interactions in mammalian cells.
- **One-hybrid interactions**

One way to test one-hybrid interactions in mammalian cells is to clone your DNA target into a mammalian reporter vector. For example, if your DNA target is part of larger, *cis*-acting regulatory sequence—a promoter or promoter/enhancer element—the element can be cloned into one of our promoterless Living Colors® Fluorescent Protein, pSEAP, or pβgal Reporter Vectors. Similarly, the cDNA encoding the putative DNA-binding protein can be cloned into a constitutive mammalian expression vector such as pIRES2-EGFP. If the putative DNA-binding protein functions as a transcriptional activator, you may be able to detect its activity by cotransfecting cells with these vector constructs.

XIV. Troubleshooting Guide

A. Constructing DNA-BD Fusions

DNA-BD/bait activates reporter genes

- The bait protein has a transcriptional activation domain. This is especially likely if the bait protein is a transcription factor. Acidic amphipathic domains are often responsible for unwanted transcriptional activation (Ruden *et al.*, 1991; Ruden, 1992). Remove the activating domain by creating specific deletions within the gene. Retest the deletion constructs for activation. At the amino acid level, a net negative charge per 10 amino acids is a minimal AD. Note that such deletions may also eliminate a potentially interacting domain.
- If two test proteins are being assayed, switch from the DNA-BD to the AD vector and vice versa.

Bait protein is toxic to yeast cells

- In some cases, strains that do not grow well in liquid culture will grow reasonably well on agar plates. Resuspend the colony in 1 ml of SD/-Trp, then spread the cell suspension on five 100-mm SD/-Trp plates. Incubate the plates at 30°C until the colonies are confluent. Scrape the colonies from each plate, pool them in one tube, and resuspend in a total of 5 ml of 0.5XYPDA. Use the cell suspension in the normal mating procedure.

B. Generating cDNA Libraries

Low yield of ds cDNA

- One or more essential reagents may have been inadvertently omitted from the first-strand or ds cDNA synthesis steps. Repeat both steps, being careful to check off every item as you add it to the reaction.
- Low yields of ds cDNA may be due to PCR undercycling. If you suspect undercycling, incubate the PCR reaction mixture for two more cycles and recheck the product. If you already used the maximum recommended number of cycles, increase by three more cycles. If additional cycles do not improve the yield of PCR product, repeat the PCR using a fresh aliquot of the first-strand product.
- A low yield of ds cDNA may also be due to a low yield of first-strand cDNA. Possible problems with the first-strand reaction include a mistake in the procedure (such as using a suboptimal incubation temperature or omitting a component) or insufficient RNA in the reaction. It is also possible that the RNA has been partially degraded (by contaminating RNases) before the first-strand synthesis. Problems with the first-strand cDNA synthesis can be more easily diagnosed if you perform parallel reactions using the control RNA provided in the kit. If good results were obtained with the control RNA but not with your experimental RNA, then there may be a problem with your RNA.

Size distribution of ds cDNA is less than expected

- Your RNA starting material may be degraded, very impure, or too dilute. Check the quality and quantity of your RNA by running a sample on a gel. If the RNA seems too dilute, but otherwise of good quality, restart the experiment using more RNA. If the RNA seems degraded, restart the experiment using a fresh lot or preparation of RNA. Also, check the stability of your RNA by incubating a small sample at 37°C for 2 hr. Run it on a gel, parallel to a fresh (unincubated) sample. If the RNA appears to be unstable, it will yield poor results. If this is the case, reisolate the RNA using a different method (see Related Products). Problems with your RNA are easily diagnosed if you perform parallel reactions using the control RNA provided in this kit.

Presence of low molecular weight (<0.1 kb) material in the ds cDNA product

- The raw cDNA (e.g., before size fractionation) is expected to contain some low-molecular-weight DNA contaminants, including unincorporated primers, SMART oligonucleotides, and very short PCR products. However, these small fragments are generally removed from the ds cDNA preparation in the size fractionation step using the columns provided. Note that a preponderance of low-molecular-weight (<0.1 kb) material in the raw PCR product may be indicative of overcycling. If you suspect overcycling, then the PCR step must be repeated with a fresh sample of first-strand cDNA, using 2–3 fewer cycles.

XIV. Troubleshooting Guide *continued*

Presence of low molecular weight (<0.1 kb) in the size-fractionated ds cDNA

- CHROMA SPIN columns are designed to remove low-molecular-weight cDNA fragments, small DNA contaminants, and unincorporated nucleotides from the cDNA. When using these columns to purify ds cDNA, please keep the following points in mind:
 - The resolving function of the column will be diminished if the gel matrix becomes dry. In drying, the matrix body may shrink away from the inner wall of the column casing. The ds cDNA mixture can then flow down the sides of the column, allowing small contaminants to elute with the cDNA.
 - The column should be stored and used at room temperature. If it is chilled at 4°C and then warmed to room temperature for use, bubbles may form, which interfere with the proper functioning of the column.
 - Extreme, uneven deposition of the ds cDNA mixture on the surface of the column can cause inefficient separation of ds cDNA from low-molecular-weight contaminants.

C. Constructing & Screening One-Hybrid and Two-Hybrid Libraries

Low transformation efficiency

- Check the purity of the DNA and, if necessary, repurify by ethanol precipitation.
- The fusion protein encoded by the DNA-BD/bait plasmid (**Two-Hybrid System**) may be toxic. Try using a vector that expresses lower levels of the fusion, such as pGBT9.
- Improper media preparation. Remake media and test with control transformations.
- Check the efficiency using the pGBT9 Control Plasmid. Plate on SD/–Trp. The transformation should yield $\geq 1 \times 10^5$ colonies/ μg DNA.

Low mating efficiency (**Two-Hybrid System**)

- There may have been an insufficient number of pretransformed bait cells in the mating. When you prepare the overnight liquid culture of the bait strain, be sure to use a large, fresh colony for the inoculum. After centrifuging and resuspending, count the cells using a hemacytometer. The density should be $\geq 1 \times 10^9/\text{ml}$, an ~ 100 -fold excess over the pretransformed library cells.
- One or both of the fusion proteins is toxic to yeast. You may be able to engineer the fusion in a way that alleviates its toxicity but still allows the interaction to occur. Alternatively, use a DNA-BD or AD vector (e.g., pBridge or pGBT9) that expresses lower levels of the fusion. It may be necessary to perform the mating on agar plates (Bendixen *et al.*, 1994) or on filters (Fromont-Racine *et al.*, 1997). Be sure to set up mating controls for comparison.
- Bait proteins may interfere with mating if they share homology to yeast-mating proteins—e.g., pheromone receptors (Shultz *et al.*, 1995; Pringle *et al.*, 1992). If homology is suspected, it may be necessary to screen your library by cotransformation.

Excessive background growth on library screening medium

- Check to make sure you have prepared the selection medium correctly. Add the appropriate amount of 3-AT. Perform a 3-AT titration with the transformed reporter strain to optimize the concentration.
- If your target-pHIS2 reporter (**One-Hybrid System**) grows on SD/–His medium even in the presence of $\geq 60\text{mM}$ 3-AT, the inserted target element may be interacting with yeast endogenous transcriptional activators, or may not require trans-acting factors to activate the *HIS3* reporter. It may be necessary to redesign the target element and construct a new reporter vector.
- The DNA-BD fusion protein (**Two-Hybrid System**) may have transcriptional activating properties. Deletion of certain portions of bait proteins may be required to eliminate unwanted activity before the protein can be used in a two-hybrid screen (Bartel *et al.*, 1993b).

Failure to detect known protein-protein (**Two-Hybrid**) or DNA-protein interactions (**One-Hybrid**) interactions

- If expression of one or both of the hybrid proteins is toxic to the cell, transformants will not grow or will grow very slowly. Truncation of one of the hybrid proteins may alleviate the toxicity and still allow the interaction to occur. Try using vectors such as pGBT9 or pBridge that express lower levels of the DNA-BD fusion protein.
- The cotransformation efficiency is too low. You may not be screening a sufficient number of library cotransformants. This can be critical, especially if the interacting target protein is encoded by a rare transcript in the source tissue.

XIV. Troubleshooting Guide *continued*

- If one of the following situations is occurring, it may interfere with the ability of the two hybrid proteins to interact: (1) the hybrid proteins are not stably expressed in the host cell; (2) the fused GAL4 domains occlude the site of interaction; (3) the hybrid protein folds improperly; or (4) the hybrid protein cannot be localized to the yeast nucleus. In these cases, it may help to construct hybrids containing different domains of the bait or library protein. For example, to study proteins that normally do not localize to the nucleus, it may be necessary to generate mutant forms of the protein that can be transported across the nuclear membrane.
- Some types of protein interactions may not be detectable in a GAL4-based system. The conditions in yeast cells may not allow the proper folding or post-translational modifications required for interaction of some mammalian proteins.

AD fusion library plasmid activates the reporters independent of the DNA-BD/bait protein (**Two-Hybrid System**)

- A rare category of false positives in which an AD/library hybrid activates transcription inappropriately. Refer to Section XIII for methods to verify protein interactions; see Bartel *et al.* (1993a) for further discussion of false positives.

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XVI. Related Products

For the latest and most complete listing of all Clontech products,
please visit www.clontech.com

Products	Cat. Nos.
RNA isolation, cDNA synthesis, PCR purification	
• NucleoTrap® mRNA Kit (Mini or Midi sizes)	636022 636023
• NucleoSpin® RNA II Kit	635990 635991 635992
• NucleoSpin® Extraction Kit	635961
• Advantage® 2 PCR Kit	639206 639207
• Advantage® 2 Polymerase Mix	639201 639202
• Poly A+ RNA	many
• Total RNA	many
• Human b-Actin Control Amplimer Set	639001 639002
• Mouse b-Actin Control Amplimer Set	639007 639008
• Rat b-Actin Control Amplimer Set	639011 639012
• CHROMA SPIN™ +TE-400 Columns	636076
• SMART™ PCR cDNA Synthesis Kit	634902
Yeast culture & plasmid isolation	
• Yeastmaker™ Yeast Transformation System 2	630439
• Yeastmaker™ Yeast Plasmid Isolation Kit	630441
• YPD Medium	630409
• YPD Agar Medium	630410
• Minimal SD Base (contains glucose)	630411
• Minimal SD Agar Base (contains glucose)	630412
• Dropout (DO) Supplements for use with any SD Base	many
Two-Hybrid screening & analysis	
• Matchmaker™ Two-Hybrid System 3	630303
• pCMV-Myc & pCMV-HA Vector Set	631604
• Mammalian Matchmaker™ Two-Hybrid Assay Kit	630301
• Matchmaker™ cDNA & Genomic Libraries	many
• Matchmaker™ Pretransformed Libraries	many
Note: Clontech offers premade cDNA and genomic AD fusion libraries from a broad range of tissues for use in two-hybrid assays. For added convenience, you can also choose from a variety of Pretransformed Libraries. These libraries are not recommended for use in Matchmaker one-hybrid screens.	
• Matchmaker™ AD LD-Insert Screening Amplimer Set	630433
• GAL4 DNA-BD Monoclonal Antibody	630403
• GAL4 AD Monoclonal Antibody	630402
• HA-Tag Polyclonal Antibody	631207
• c-Myc Monoclonal Antibody	631206
• pGBKT7 DNA-BD Vector	630443
• pGADT7 AD Vector	630442
• pBridge Vector	630404
• pLP-GBKT7 DNA-BD Acceptor Vector	630406

XVI. Related Products

	Cat. Nos.
Two-Hybrid screening & analysis (continued)	
• pLP-GADT7 AD Acceptor Vector	630405
• X- α -gal	630407
• Matchmaker™ Co-IP Kit	630449
• Luminescent β -gal Detection Kit II	631712
General cloning reagents	
• Creator™ pDNR Cloning Kits	many
• QUICK-Clone™ cDNA	many
• KC8 Electrocompetent Cells	630435
• Fusion-Blue™ Competent Cells	636700

Appendix A: Typical Results of ds cDNA Synthesis

Double-stranded cDNA synthesized from Control Human Placenta Poly A⁺ RNA using the protocol in this manual should appear as a moderately strong smear from ≥ 0.1 kb to 4 kb (or more) on a 1.2% agarose/EtBr gel (Figure 14).

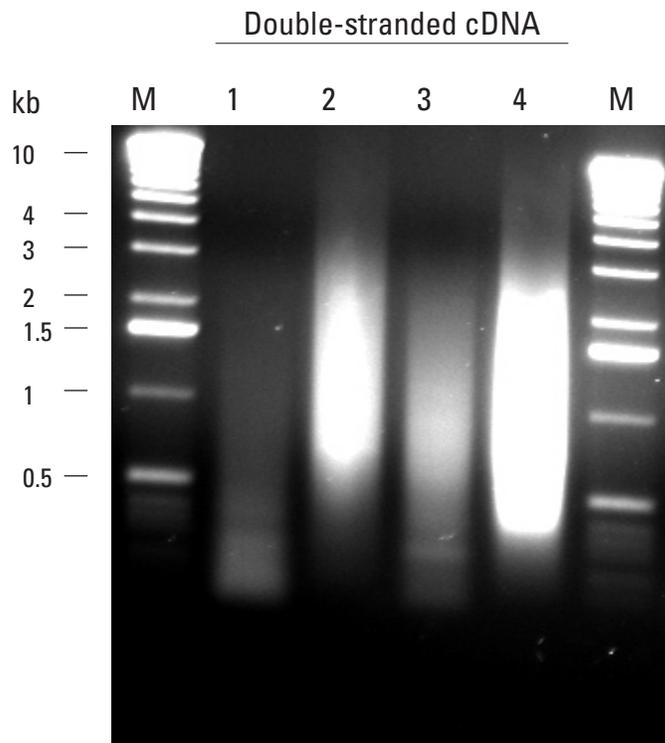


Figure 14. Double-stranded cDNA synthesized from Control Human Placenta Poly A⁺ RNA. 1 μ l (1.0 μ g) of Control Human Placenta Poly A⁺ RNA was used as the template for first-strand cDNA synthesis. Two first-strand samples were prepared: One with a random primer (our CDS III/6 Primer; **Lanes 1 & 3**), and the other with an oligo(dT) primer (our CDS III Primer; **Lanes 2 & 4**). Next, 2 μ l of the single-stranded cDNA was amplified by LD-PCR. Each ds cDNA product was then purified with a CHROMA SPIN+TE-400 Column. The ds cDNA was analyzed on a 1.2% agarose/EtBr gel before (**Lanes 1 & 2**; 7 μ l cDNA per lane) and after (**Lanes 3 & 4**; 5 μ l cDNA per lane) column purification. **Lane M** was loaded with 250 ng of a 1-kb DNA size marker.

Appendix B: Preparation of Competent Yeast Cells—LiAc Method

This protocol will yield 1.2 ml of competent cells.

Before starting:

- Streak a YPDA agar plate with a small portion of frozen yeast stock—e.g., AH109 or Y187. (If the tube has thawed prior to streaking, vortex to ensure even distribution of the yeast cells.) Incubate the plate upside down at 30°C until colonies appear (~ 3 days).
Yeast strains can be stored for up to 1 month at 4°C on YPDA medium in culture plates sealed with Parafilm.
 - Prepare 1.1XTE/LiAc Solution (Section III)
 - Prepare YPDA liquid medium (Yeast Protocols Handbook)
1. Inoculate one colony (\leq 4 weeks old, 2–3 mm in diameter) into 3 ml of YPDA medium in a sterile, 15-ml centrifuge tube.
 2. Incubate at 30°C with shaking for 8 hr.
 3. Transfer 5 μ l of the culture to a 250-ml flask containing 50 ml of YPDA.
 4. Incubate at 30°C with shaking at 230–250 rpm for 16–20 hr. The OD_{600} should reach 0.15–0.3.
 5. Centrifuge the cells at 700 x g for 5 min at room temperature.
 6. Discard the supernatant and resuspend the cell pellet in 100 ml of YPDA.
 7. Incubate at 30°C for 3–5 hr ($OD_{600} = 0.4$ – 0.5).
 8. Centrifuge the cells at 700 x g for 5 min at room temperature.
 9. Discard the supernatant and resuspend the cell pellet in 60 ml of sterile, deionized H₂O.
 10. Centrifuge the cells at 700 x g for 5 min at room temperature.
 11. Discard the supernatant and resuspend the cells in 3 ml of 1.1XTE/LiAc Solution.
 12. Split the resuspension between two 1.5-ml microcentrifuge tubes (1.5 ml per tube).
 13. Centrifuge each tube at high speed for 15 sec.
 14. Discard the supernatant and resuspend each pellet in 600 μ l of 1.1XTE/LiAc Solution.

Note: Competent cells should be used for transformation immediately following preparation; however, if necessary they can be stored at room temperature for a few hours without significantly affecting the competency.

Appendix C: One-Hybrid Control Vector Information

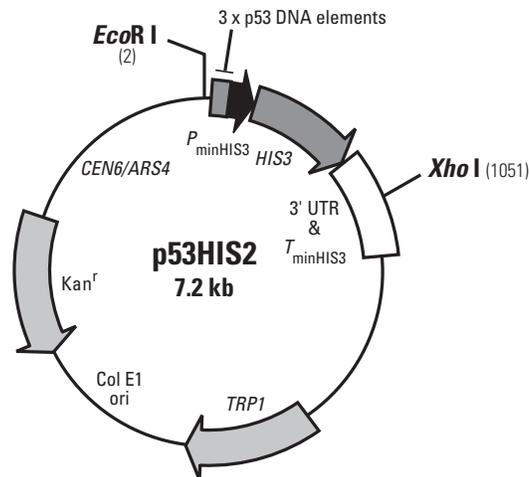


Figure 15. Map of p53HIS2 Control Vector. p53HIS2 is a yeast one-hybrid reporter vector that serves as a positive control in the Matchmaker One-Hybrid Library Construction & Screening Kit (Cat No. 630304). It contains 3 tandem copies of the consensus DNA binding site for p53. The three DNA targets are located upstream of the minimal promoter of the *HIS3* locus (P_{minHIS3}) and the *HIS3* nutritional reporter gene. p53HIS2 is designed for use with pGAD-Rec2-53, a plasmid that encodes murine p53 as a fusion to the GAL4 AD. Yeast cells that contain both of these plasmids will display the His⁺ phenotype as a result of the interaction between murine p53 and the DNA binding sites in p53HIS2. When the GAL4 AD-p53 fusion interacts with these sites, it stimulates transcription of *HIS3*, giving yeast strains such as Y187 and AH109, which are normally auxotrophic for histidine, the ability to grow on histidine dropout medium.

p53HIS2 contains an autonomous replication sequence (*ARS4*) and *TRP1* nutritional marker for replication and selection in yeast; the centromeric sequence *CEN6* ensures proper segregation of the plasmid during mitosis and meiosis. The vector also contains a Col E1 ori and kanamycin resistance gene (*Kan^r*) for propagation and selection in *E. coli*. This vector has not been completely sequenced.

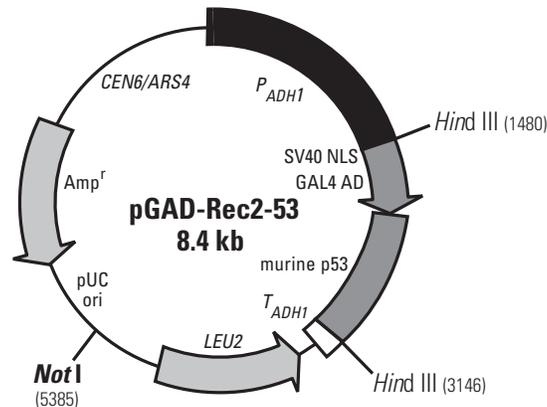


Figure 16. Map of pGAD-Rec2-53 AD Control Vector. pGAD-Rec2-53 encodes a fusion of the GAL4 AD and murine p53, a known DNA-binding protein (Thukral, S. K., *et al.*, 1994). The vector is derived from pGADT7-Rec2 and was constructed at Clontech by homologous recombination in *E. coli*. Specifically, the vector was produced by transforming competent *E. coli* cells with *EcoRI*/*Bam*H I-linearized pGADT7-Rec2 and ds cDNA encoding murine p53 (a.a. 73-391). As a result, this vector does not contain the T7 RNA polymerase promoter or hemagglutinin (HA) epitope tag, nor does it share any homology with the SMART III or CDS III oligonucleotides.

pGAD-Rec2-53 is designed for use as a positive control vector in Matchmaker yeast one-hybrid assays. It is not intended to serve as a cloning vector, nor is it intended to be used as a source of murine p53 cDNA. Instead, use pGAD-Rec2-53 with p53HIS2 to produce a positive control yeast strain. Yeast strains AH109 and Y187, which are normally unable to grow on histidine-deficient media, will grow on medium lacking histidine when transformed with pGAD-Rec2-53 and p53HIS2. Transformants acquire the ability to synthesize histidine as a result of the interaction between the GAL4 AD-p53 fusion, expressed by pGAD-Rec2-53, and the p53 consensus DNA-binding sequence in p53HIS2. Upon binding the consensus sequence, the GAL4 AD-p53 fusion stimulates transcription of the *HIS3* reporter gene in p53HIS2 and confers the His⁺ phenotype to the host.

pGAD-Rec2-53 contains an autonomous replication sequence (*ARS4*) and *LEU2* nutritional marker for replication and selection in yeast; the centromeric sequence *CEN6* ensures proper segregation of the plasmid during mitosis and meiosis. The vector also contains a pUC ori and ampicillin resistance gene (*Amp^r*) for propagation and selection in *E. coli*. This vector has not been completely sequenced.

Appendix D: Two-Hybrid Control Vector Information

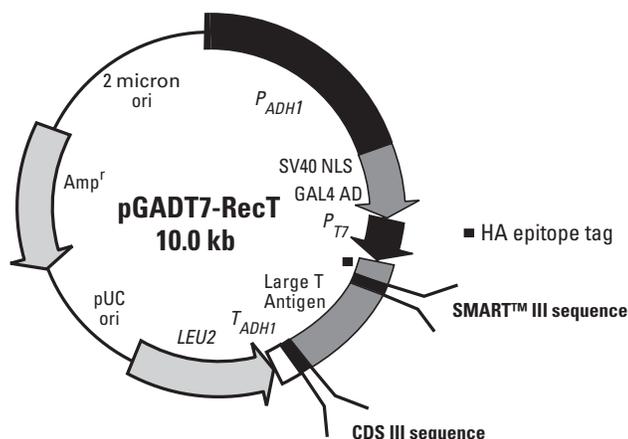


Figure 17. Map of pGADT7-RecT AD Control Vector. pGADT7-RecT is a product of recombination that encodes a fusion of the SV40 largeT antigen and the GAL4 AD. To generate this control, cotransform yeast with the SV40 LargeT PCR Fragment and pGADT7-Rec Cloning Vector (*Sma* I-linearized). Because the linearized vector shares sequence homology with the ends of the Large T PCR Fragment, these two components recombine via a double-crossover mechanism to produce the circular control plasmid pGADT7-RecT. The SV40 Large T DNA (GenBank Locus SV4CG) was derived from a plasmid referenced in Li & Fields (1993). PCR amplification was performed at Clontech.

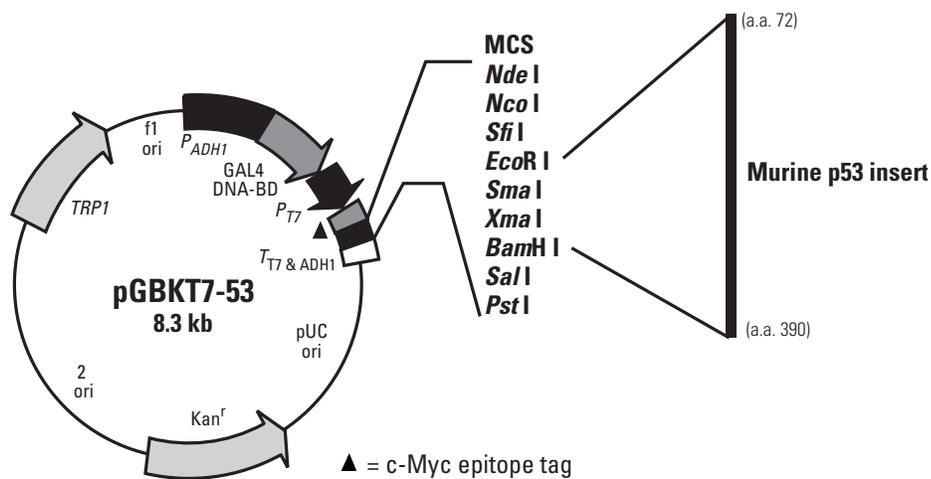


Figure 18. Map of pGBKT7-53 DNA-BD Control Vector. pGBKT7-53 is a positive control plasmid that encodes a fusion of the murine p53 protein (a.a. 72–390) and the GAL4 DNA-BD (a.a. 1–147). The murine p53 cDNA (GenBank Accession Cat. No. K01700) was cloned into pGBKT7 at the *EcoR* I and *BamH* I sites. The p53 insert was derived from the plasmid described in Iwabuchi *et al.* (1993); plasmid modification was performed at Clontech. pGBKT7-53 has not been sequenced.

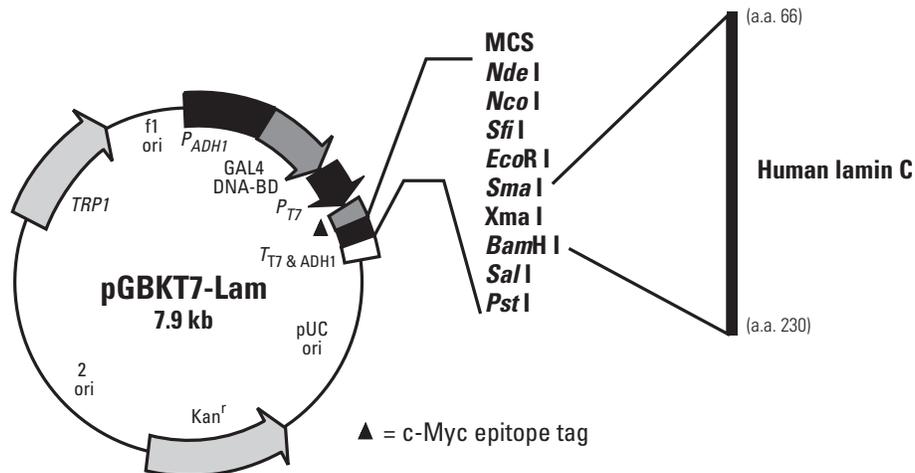
Appendix D: Two-Hybrid Control Vector Information *continued*

Figure 19. Map of pGBKT7-Lam DNA-BD Control Vector. pGBKT7-Lam is a negative control plasmid that encodes a fusion of the human lamin C protein (a.a. 66–230) and the GAL4 DNA-BD (a.a. 1–147). The lamin C cDNA insert (GenBank Accession Cat. No. M13451) was derived from the plasmid referenced in Bartel *et al.* (1993a). Plasmid modification was performed at Clontech. Yeast cotransformed with pGBKT7-Lam and pGADT7-RecT, provide a measure of the background that is due to false-positive two-hybrid interactions. pGBKT7-Lam has not been sequenced.

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