

Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual

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I. Introduction

MatchmakerTwo-Hybrid System 3 is an advanced GAL4-based two-hybrid system that provides a transcriptional assay for detecting protein interactions *in vivo* in yeast. You can use this system to screen a library for novel proteins that interact with a known bait protein, or to test two previously cloned proteins for an interaction. MatchmakerTwo-Hybrid System 3 incorporates many features that reduce the incidence of false positive results and allow you to quickly identify and confirm protein interactions. Key features of System 3 are detailed in Figure 1.

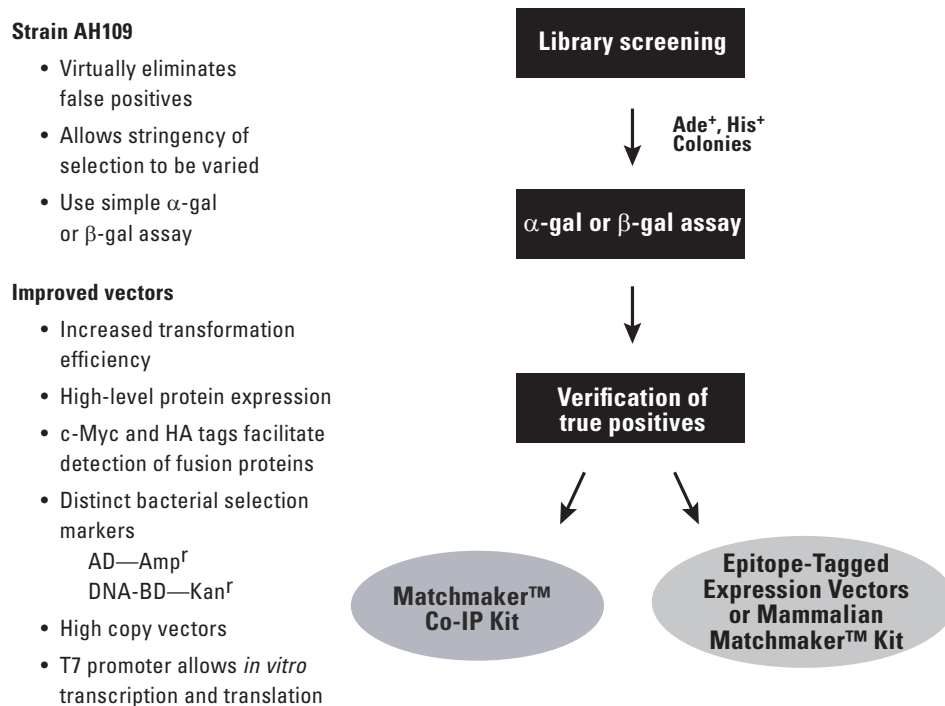


Figure 1. Overview of Matchmaker™ System 3 advances and screening process.

Principle of the two-hybrid assay

In Matchmaker System 3 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, the DNA-BD and AD are brought into proximity, thus activating transcription of four reporter genes (Figure 2). This technology can be used to identify novel protein interactions, confirm suspected interactions, and define interacting domains. Moreover, two-hybrid technology provides immediate access to the genes encoding the interacting proteins.

Sensitive *in vivo* assay

Yeast two-hybrid systems provide a sensitive method for detecting relatively weak and transient protein interactions. Such interactions may not be biochemically detectable, but may be critical for proper functioning of complex biological systems (Guarente, 1993; Estojak *et al.*, 1995). The sensitivity of Matchmaker GAL4 Two-Hybrid System 3 is primarily attributable to high-fold amplification of positive signals *in vivo* (i.e., transcriptional, translational, and enzymatic). In addition, because the two-hybrid assay is performed *in vivo*, the proteins are more likely to be in their native conformations, which may lead to increased sensitivity and accuracy of detection.

I. Introduction *continued*

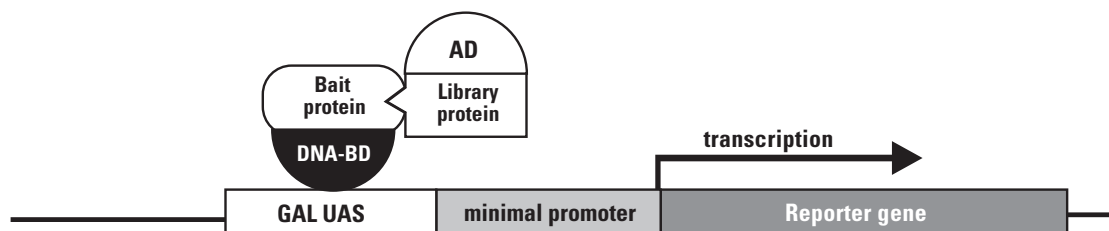


Figure 2. The two-hybrid principle. The DNA-BD is amino acids 1–147 of the yeast GAL4 protein, which binds to the GAL UAS upstream of the reporter genes. The AD is amino acids 768–881 of the GAL4 protein and functions as a transcriptional activator.

The sensitivity of the two-hybrid assay means that it can be used to pinpoint specific residues critical for protein interactions and to evaluate protein variants for the relative strength of their interactions (Yang, *et al.*, 1995). The binding data reported by Yang *et al.* (*ibid.*) lead them to suggest that protein interactions with dissociation constants (K_d) above $\sim 70 \mu\text{M}$ can be detected using a GAL4-based two-hybrid assay.

New yeast strain reduces false positives

System 3 features the yeast strain AH109, which virtually eliminates false positives by using three reporters—*ADE2*, *HIS3*, and *MEL1* (or *lacZ*)—under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Figure 3). These promoters yield strong and specific responses to GAL4. As a result, two major classes of false positives are eliminated: those that interact directly with the sequences flanking the GAL4 binding site and those that interact with transcription factors bound to specific TATA boxes.

The *ADE2* reporter alone provides strong nutritional selection: the option of using *HIS3* selection reduces the incidence of false positives and allows you to control the stringency of selection (James *et al.*, 1996). Furthermore, you have the option of using either *MEL1* or *lacZ*, which encode α -galactosidase and β -galactosidase, respectively. *MEL1* is an endogenous gene found in several yeast strains. Because α -galactosidase is a secreted enzyme, it can be assayed directly on X- α -Gal (Cat. No. 630407) indicator plates, which employ blue/white screening.

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 3. Reporter constructs in yeast strains AH109 and Y187. Strain AH109 is a derivative of strain PJ69-2A and includes the *ADE2* and *HIS3* markers (James *et al.*, 1996). *MEL1* is an endogenous GAL4-responsive gene. The *lacZ* reporter gene was introduced into PJ69-2A to create AH109 (A. Holtz, unpublished). 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. Strain Y187 contains the *lacZ* reporter gene under control of the GAL1 UAS.

I. Introduction *continued*

Optimized vectors facilitate downstream confirmation

The Matchmaker System 3 DNA-BD and AD fusion vectors, pGBKT7 and pGADT7, were designed for high-level protein expression and to facilitate confirmation of protein interactions. Bait and library inserts are expressed as GAL4 fusions with c-Myc and hemagglutinin (HA) epitope tags, respectively. The epitope tags eliminate the need to generate specific antibodies to each new protein and allow convenient identification of the fusion proteins.

System 3 vectors also include T7 promoters downstream of the GAL4 coding sequences. These promoters allow you to transcribe and translate the epitope-tagged fusion proteins *in vitro*. The Matchmaker Co-IP Kit (Cat. No. 630449) allows you to confirm protein interactions through an *in vitro* coimmunoprecipitation. The T7 promoter is also a priming site for DNA sequencing.

Finally, pGBKT7 and pGADT7 were designed to express different bacterial selection markers—kanamycin and ampicillin, respectively—to simplify their independent isolation in *E. coli*. Purifying the plasmids from *E. coli* allows the isolation of AD vectors after a library screening. Each vector also contains the high-copy pUC origin of replication to ensure high yields from plasmid preparations. These features provide the high quality and quantity of DNA necessary for sequencing and characterizing inserts by PCR or restriction digests.

Increased transformation efficiency

Another benefit of System 3 is that yeast strains carrying pGBKT7 are transformed more efficiently than strains containing other DNA-BD vectors (Louret *et al.*, 1997; *Clontechiques*, January 1999). This higher transformation efficiency facilitates the introduction of AD fusion libraries into yeast, which maintains the complexity of the library and increases the probability of detecting novel two-hybrid protein interactions.

Considerations

The Matchmaker Systems have been used to identify many different types of protein interactions, including those from prokaryotes, yeast, plants, *Drosophila*, and mammals. However, all yeast two-hybrid systems have potential limitations:

- Some bait proteins may have intrinsic DNA-binding and/or transcriptional activating properties; hence, deletion of certain portions of bait proteins may be required to eliminate unwanted activity before the proteins can be used in a two-hybrid screen (Bartel *et al.*, 1993b).
- Some hybrid proteins may not be stably expressed in yeast or localized to the yeast nucleus. For protein interactions that normally occur on the cell surface, a phage display system may be a more appropriate choice. However, the two-hybrid system has been used to identify extracellular protein interactions (Ozenberger & Young, 1995; Kuo *et al.*, 1992).
- In some cases, the DNA-BD or AD fusion moiety may occlude the normal site of interaction, or may impair the proper folding of the hybrid protein, and thus interfere with the ability of the two hybrid proteins to interact (van Aelst *et al.*, 1993).
- The conditions in yeast cells may not allow the proper folding or posttranslational modifications (such as glycosylation) required for interaction of some mammalian proteins. Conversely, the detection of a specific interaction between mammalian proteins in a yeast system does not *necessarily* indicate that there is a corresponding interaction in the proteins' native environment (Fields & Sternglanz, 1994).
- Some protein interactions may not be detectable in a GAL4-based two-hybrid system, but may be detectable using a LexA-based system such as the Matchmaker LexA Two-Hybrid System (Cat. No. K1609-1; Gyuris *et al.*, 1993; reviewed in Golemis *et al.*, 1996; and Mendelsohn & Brent, 1994), and vice versa. We cannot predict which system will give the best results for particular protein interactions.

I. Introduction *continued*

Compatible Matchmaker™ products

- **Pretransformed Matchmaker™ cDNA Libraries** provide a high-quality library previously transformed into Y187. Simply mate to AH109 that has been transformed with your bait.
- **Matchmaker™ GAL4 cDNA Libraries** provide a convenient means to quickly screen a high-quality premade cDNA library.
- **pBridge™ Three-Hybrid Vector** (Cat. No. 630404) allows you to investigate ternary protein complexes (Tirode *et al.*, 1997). pBridge allows expression of the DNA-BD/bait and a third protein. The third protein is only expressed in the absence of methionine.
- The **Matchmaker™ Co-IP Kit** (Cat. No. 630449) provides reagents for quickly and independently confirming protein interactions through an *in vitro* coimmunoprecipitation.
- **Matchmaker™ Antibodies** allow you to easily detect fusion proteins. See Related Products for details.
- The **pCMV-Myc & pCMV-HA Vector Set** (Cat. No. 631604) allows *in vivo* coimmunoprecipitations in mammalian cells.
- The **Mammalian Matchmaker™ Two-Hybrid Assay Kit** (Cat. No. 630301) is ideal for confirming protein interactions in mammalian cells.
- **X- α -Gal** (Cat. No. 630407) allows you to detect α -galactosidase activity.

TABLE I. LIST OF ABBREVIATIONS

Two-Hybrid Terminology

AD/library	A fusion of the GAL4 AD with a library cDNA/protein.
DNA-BD/bait	A fusion of the GAL4 DNA-BD with a bait cDNA/protein.

Yeast Phenotypes

Ade ⁻ , His ⁻ , Leu ⁻ , or Trp ⁻	Requires adenine (Ade), histidine (His), leucine (Leu), or tryptophan (Trp) in the medium to grow; is auxotrophic for at least one of these specific nutrients.
Ade ⁺	Expresses the <i>ADE2</i> reporter gene; i.e., does not require Ade in the medium to grow.
His ⁺	Expresses the <i>HIS3</i> reporter gene; i.e., does not require His in the medium to grow.
LacZ ⁺	Expresses the <i>lacZ</i> reporter gene; i.e., is positive for β -galactosidase activity.
Mel1 ⁺	Expresses the <i>MEL1</i> reporter gene; i.e., is positive for α -galactosidase activity.

Miscellaneous

Ade2p	Protein encoded by the yeast <i>ADE2</i> gene.
3-AT	3-amino-1,2,4-triazole; a competitive inhibitor of the His3 protein.
CHX	Cycloheximide
DO	Dropout (supplement or solution); a mixture of specific amino acids and nucleosides used to supplement SD base to make SD medium; DO solutions are missing one or more of the nutrients required by untransformed yeast to grow on SD medium.
His3p	Protein encoded by the yeast <i>HIS3</i> gene.
SD medium	Minimal Synthetic Dropout medium; comprised of a nitrogen base, a carbon source (glucose or galactose), and a DO supplement.
YPH	Yeast Protocols Handbook (PT3024-1)

II. Overview: A Yeast Two-Hybrid Screen

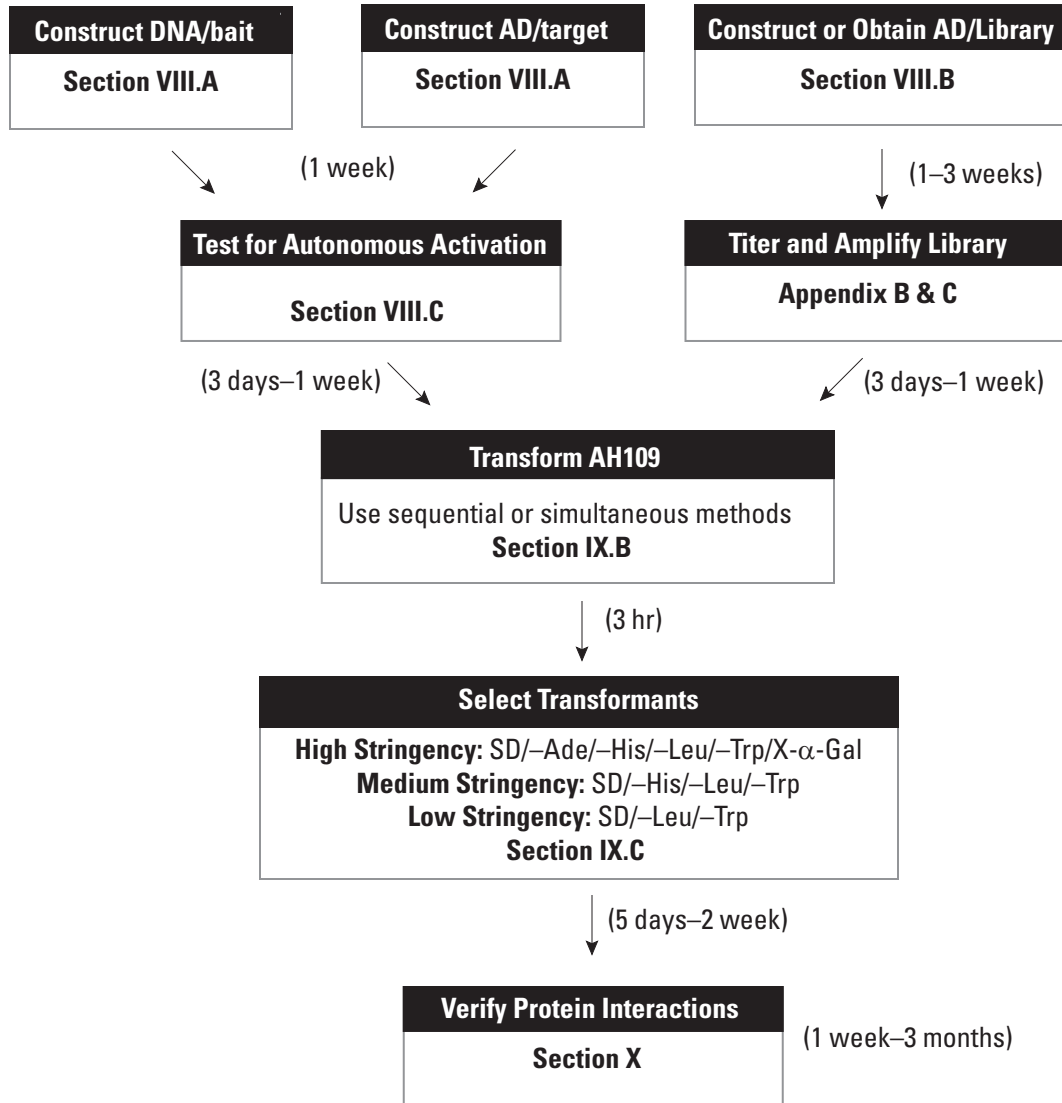


Figure 4. Overview of performing a yeast two-hybrid screen. The appropriate User Manual sections are indicated.

III. Lists of Components

A. Matchmaker™ Two-Hybrid System 3

Store all yeast strains at –70°C.

Store sequencing primers and plasmid DNA at –20°C.

- 50 µl **pGBKT7 Cloning Vector** (0.1 µg/µl)
- 50 µl **pGADT7 Cloning Vector** (0.1 µg/µl)
- 50 µl **pGBKT7-53 Control Vector** (0.1 µg/µl)
- 50 µl **pGBKT7-Lam Control Vector** (0.1 µg/µl)
- 50 µl **pGADT7-T Control Vector** (0.1 µg/µl)
- 50 µl **pCL1 Control Vector** (0.1 µg/µl)
- 0.5 ml **AH109** *Saccharomyces cerevisiae* in YPDA medium/25% glycerol.
- 0.5 ml **Y187** *Saccharomyces cerevisiae* in YPD medium/25% glycerol.
- 40 µl **T7 Sequencing Primer** (0.1 µg/µl)
5'-TAATACGACTCACTATAGGGC-3' (21-mer)
- 40 µl **3' DNA-BD Sequencing Primer** (0.1 µg/µl)
5'-ATCATAAATCATAAGAAATTCGCC-3' (24-mer)
- 40 µl **3' AD Sequencing Primer** (0.1 µg/µl)
5'-AGATGGTGACGATGCACAG-3' (20-mer)
- 10 g **-Leu/-Trp Dropout Supplement**
- 10 g **-Ade/-His/-Leu/-Trp Dropout Supplement**
- 1 ml **Herring Testes Carrier DNA, denatured**
- **Vector Information Packets** (PT3248-5 & PT3249-5)

B. Matchmaker™ GAL4 cDNA & Genomic Libraries

Store all components at –70°C.

Divide the library cultures into 100 µl aliquots, and store at –70°C. Avoid multiple freeze/thaw cycles.

Retiter and amplify the library before use (Appendices B and C).

The appropriate vector information is provided in the accompanying vector information packet.

- 2 x 1.0 ml **Plasmid Library Culture** in LB broth/25% glycerol
- 0.5 ml **AH109** *Saccharomyces cerevisiae* in YPDA medium/25% glycerol.
- 0.5 ml **CG-1945** *Saccharomyces cerevisiae* in YPD medium/25% glycerol.

IV. Additional Materials Required

The following reagents and materials are not supplied but are required. Recipes for these materials are provided in Appendix A and the YPH.

- **YPDA or the appropriate SD liquid medium**
- **Sterile 1X TE/LiAc** (Prepare immediately prior to use from 10X stocks)
- **TE buffer or sterile, distilled H₂O**
- **Appropriate sterile tubes or flasks** for transformations.
- **Appropriate SD agar plates**
 - Notes:**
 - Prepare the selection media and pour the required number of agar plates in advance.
 - Allow SD agar plates to dry at room temperature for 2–3 days or at 30°C for 3 hr prior to plating any transformation mixtures. Moisture droplets on the agar surface can cause uneven spreading.
- **Yeastmaker™ Yeast Transformation System** (Cat. No. 630439) provides all the necessary reagents for yeast transformations.
 - Note:**
 - Boil the carrier DNA for 20 min and quickly cool it on ice just prior to use.
- **Sterile PEG/LiAc solution** (Prepare immediately prior to use from 10X stocks)
- **100% DMSO** (Dimethyl sulfoxide; Sigma Cat. No. D-8779)
- **1X TE buffer**
- **Sterile glass rod, bent pasteur pipette, or 5-mm glass beads** for spreading cells on plates.
- **65% Glycerol/MgSO₄ solution** for the low-stringency procedure.
- **X-α-Gal** (Cat. No. 630407)

V. Yeast Strains & Phenotypes

This section provides detailed phenotypes of the yeast strains included with Matchmaker System 3 and Matchmaker GAL4 cDNA and Genomic Libraries. For additional information on the growth and maintenance of yeast, see the YPH, Chapter III. We also recommend Guthrie & Fink's *Guide to Yeast Genetics and Molecular Biology* (1991) and Heslot & Gailardin's *Molecular Biology and Genetic Engineering of Yeasts* (1992).

A. Yeast Host Strains

The complete genotypes of AH109, Y187, and CG-1945 are provided in Table II. All strains are *gal4⁻* and *gal80⁻*; this prevents interference of native regulatory proteins with the regulatory elements in the two-hybrid system.

1. Use **AH109** as the host strain if you plan to screen an AD/library using *HIS3*, *ADE2*, and *MEL1*.
2. **System 3 Users Only:** Use **Y187** as the host strain if you plan to test for an interaction between two known proteins using the *lacZ* reporter only. In addition, use Y187 as a mating partner to verify protein interactions.
3. **Library Users Only:** Use **CG-1945** as the host strain if you plan to separate DNA/bait and AD/library plasmids by cycloheximide counterselection. Alternatively, use pGBKT7 to construct your bait. This DNA-BD vector contains a kanamycin resistance marker; therefore, vectors can be separated in *E. coli* without cycloheximide counterselection.

TABLE II. MATCHMAKER™ YEAST STRAIN GENOTYPES

Strain	Genotype	References
AH109	<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>	James <i>et al.</i> , 1996; A. Holtz, unpublished
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>	Harper <i>et al.</i> , 1993
CG-1945	<i>MAT_a</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>ade2-101</i> , <i>lys2-801</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>gal4-542</i> , <i>gal80-538</i> , <i>cyh'2</i> , <i>LYS2 :: GAL1_{UAS}-GAL1_{TATA}-HIS3</i> , <i>URA3 :: GAL4_{17-mers(x3)}-CYC1_{TATA}-lacZ</i>	Feilotter <i>et al.</i> , 1994; C. Giroux, pers. comm.

B. Phenotypes

1. Nutritional Requirements

To verify phenotypes and to become familiar with the yeast strains, test them for the phenotypes shown in Table III.

- a. Streak each strain onto adenine-supplemented YPD (YPDA) plates. Incubate at 30°C for 3–5 days. Propagate additional cultures only from isolated colonies.
Note: The stock may be refrozen several times without significantly decreasing viability.
- b. Using a sterile loop or toothpick, streak 3–4 colonies onto the indicated SD selection plates.
- c. Incubate plates at 30°C for 4–6 days; yeast strains grow more slowly on SD selection media than on YPDA.
- d. Seal stock plate with Parafilm, and store at 4°C.

TABLE III. MATCHMAKER™ YEAST STRAIN PHENOTYPES

Strain	SD/–Ade	SD/–Met	SD/–Trp	SD/–Leu	SD/–His	SD/–Ura	YPDA	YPD/CHX
AH109	–	+	–	–	–	+	+	–
Y187	–	–	–	–	–	+	+	–
CG-1945	–	+	–	–	–	+	+	+

V. Yeast Strains & Phenotypes *continued*

2. Colony Color and Size

- a. **Y187** and **CG-1945** carry the *ade2-101* mutation. On medium with low amounts of adenine, the colonies will turn pink after a few days and may turn darker as the colony ages. 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.

In the absence of GAL4, **AH109** also exhibits the *ade2-101* phenotype. However, in the presence of protein interactions, the *ADE2* marker complements in *cis* the AH109 *ade2-101* phenotype.

- b. When transformed with pAS2-1, or any pAS2-derived plasmid, **CG-1945** grows more slowly and forms noticeably smaller colonies than untransformed CG-1945.

3. Antibiotic Resistance

CG-1945 is cycloheximide resistant. When making competent CG-1945 cells, use liquid YPD medium without cycloheximide.

4. *MEL1* and *lacZ* Reporter Gene Expression Levels

- a. In response to GAL4 activation, AH109 and Y187 secrete α -galactosidase, which can be detected on medium containing X- α -Gal (Aho *et al.*, 1997).
- b. In response to GAL4 activation, Y187 exhibits a higher level of induced β -galactosidase activity than both AH109 and CG-1945. This is because of differences in the strengths of the *lacZ* promoter constructs. In Y187, *lacZ* is under control of the intact *GAL1* UAS; in AH109 and CG-1945, *lacZ* is under control of the weaker *MEL1* UAS and UAS_G 17-mer consensus sequence, respectively. Therefore, use liquid cultures of Y187 for quantitative β -galactosidase assays. For further information on β -galactosidase assays, see the YPH.

5. Leaky *HIS3* Expression

- a. 3-AT is a competitive inhibitor of the yeast *HIS3* protein (His3p). 3-AT is used to inhibit low levels of His3p expression, and thus, to suppress background growth on SD medium lacking His (Fields, 1993; Durfee *et al.*, 1993).
- b. CG-1945 transformants are suppressed by the addition of 5–15 mM 3-AT.
In general, AH109 does not require 3-AT. However, if your DNA/bait produces background growth on SD/–His/–Trp plates, you will need to optimize the concentration of 3-AT.
To optimize the 3-AT concentration, plate cells transformed with your DNA-BD/bait plasmid on SD/–His/–Trp plates containing 0, 2.5, 5, 7.5, 10, 12.5, and 15 mM 3-AT. Use the lowest concentration of 3-AT which, after one week, allows only small (<1 mm) colonies to grow.
- c. A high concentration of 3-AT in the medium can kill freshly transformed cells. Thus, if you wish to use excess 3-AT to select only very strong two-hybrid interactions, we recommend using the low-stringency selection protocol.

6. Clumping

For unknown reasons, strain CG-1945 often clumps in liquid culture. Disperse clumps by vortexing vigorously.

VI. Control Vectors

Matchmaker Two-Hybrid System 3 provides positive and negative control vectors. Vector information is provided in Table IV.

A. Positive Controls

pCL1 encodes the full-length, wild-type GAL4 protein and provides a positive control for α -galactosidase and β -galactosidase assays.

pGBKT7-53 and pGADT7-T encode fusions between the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively. p53 and large T-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 DNA-BD with human lamin C and provides a control for a fortuitous interaction between an unrelated protein and either the pGADT7-T 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. MATCHMAKER™ TWO-HYBRID SYSTEM 3 VECTORS

	Fusion	Epitope ^a	Yeast selection	Bacterial selection
Cloning vectors				
pGBKT7	DNA/bait	c-Myc	<i>TRP1</i>	kanamycin
pGADT7	AD/library	HA	<i>LEU2</i>	ampicillin
Control vectors				
pCL1	GAL4		<i>LEU2</i>	ampicillin
pGADT7-T	AD/T-antigen	HA	<i>LEU2</i>	ampicillin
pGBKT7-53	DNA-BD/p53	c-Myc	<i>TRP1</i>	kanamycin
pGBKT7-Lam	DNA-BD/lamin C	c-Myc	<i>TRP1</i>	kanamycin

^a HA = hemagglutinin

VII. Matchmaker™ GAL4 cDNA & Genomic Libraries

You can use premade Matchmaker GAL4 cDNA and Genomic Libraries with all Matchmaker GAL4-based systems.

A. Library Construction

cDNA libraries are prepared using a modified Gubler & Hoffman procedure (1983).

cDNA Priming Methods

- **Oligo(dT) priming** eliminates the synthesis of lengthy poly (dT) regions and ensures that full-length clones and 3' ends will be well-represented in the library (Chenchik *et al.*, 1994; Borson, *et al.*, 1992; Moqadam & Siebert, 1994).
- **Oligo(dT) + random-priming** may lead to a greater representation of all portions of the gene, including amino-terminal and internal domains, regardless of mRNA secondary structure; random priming also generates a wider size-range of cDNA.
- **Unidirectional libraries** are made with oligo(dT) primers that have one vector-compatible restriction enzyme site. The other site is added (with sticky ends) by the adaptor that is ligated to the cDNA. Thus, digestion with one restriction enzyme ensures the cDNA's proper orientation when ligated to a vector that has been digested with the appropriate two enzymes.

Adaptors and Linkers

Please refer to the Product Analysis Certificate (PAC) for information on the specific adaptor or linker used in the construction of your Matchmaker Library.

Notes:

- The open reading frame of the insert starts at the codon immediately following the C-terminal codon (a.a. 881) of the GAL4 AD, not within the adaptor.
- If an *EcoR* I linker is used, the cDNA is methylated to protect any internal *EcoR* I sites.
- If an adaptor is used in the construction of nondirectionally cloned libraries, no methylation or restriction enzyme digestion of the cDNA is required; therefore, any internal *EcoR* I sites present in the cDNA will not be cut.
- If an adaptor is used in the construction of unidirectionally cloned libraries, the cDNA is methylated to protect the alternative site.
- If the library is synthesized using *EcoR* I/*Not* I/*Sa* I adaptors, you may excise the inserts from the vector using sites within the adaptor.

cDNA Size Fractionation

The adaptor-ligated double-stranded cDNA is size-fractionated to remove unincorporated primers, unligated adaptors, and adaptor dimers; this process also removes low-molecular weight (<400 bp) incomplete cDNAs.

Insert Size Range and Average Insert Size

Sizes are determined by running the cDNA on a gel prior to cloning, and comparing the profile to MW size markers.

Library Amplification

Unless otherwise stated on the PAC, all libraries are amplified once.

VII. Matchmaker™ GAL4 cDNA & Genomic Libraries *continued*

B. Library Quality Control Information

The following information is provided on the PAC. These data were obtained at the time of library construction.

Number of Independent Clones

The number of independent clones is estimated before amplification. Most libraries are guaranteed to have at least 1×10^6 independent clones.

Library Titer

Library titer is determined after amplification and must be $>10^8$ cfu/ml for plasmid libraries.

Insert Size Analysis

The insert size of 15 randomly selected clones is determined by PCR amplification using insert screening primers.

Sequence Representation

Sequence representation is evaluated by colony hybridization using a gene-specific probe. All Human Matchmaker cDNA Libraries must show a minimum β -actin frequency of 0.1%, and all other mammalian Matchmaker cDNA Libraries must show a minimum β -actin frequency of 0.05%. Nonmammalian cDNA libraries are screened with a ubiquitously expressed species-specific probe.

Note: The frequency of β -actin positive clones varies among libraries made with RNA from different tissues and species. A frequency of $>0.1\%$ in a human cDNA library suggests a reasonably high probability of finding a rare transcript (Hagen *et al.*, 1988). For nonhuman mammalian cDNA libraries, a frequency of 0.05% suggests a reasonably high probability of finding a rare message (Clontech observations, unpublished).

Presence of Genomic DNA or rRNA Sequences in cDNA Libraries

The purified poly A⁺ RNA used to construct Matchmaker cDNA libraries is not treated with DNase, due to potential degradation by contaminating RNase activity. Therefore, the poly A⁺ preparation may have $<1\%$ of genomic DNA and $<5\%$ of rRNA.

PCR-based Sequence Screening

- **Human cDNA libraries**

A representative sample (up to 10^7 cfu or $>10^7$ pfu) of the total library is used as a template in a PCR reaction with human β -actin PCR primers. These primers amplify a 1.1-kb fragment located at the 5' end of the gene. A sample of the library may also be used as a template for PCR amplification of G3PDH and transferrin receptor cDNA fragments.

- **Nonhuman mammalian cDNA libraries**

A representative sample (up to 10^7 cfu or $>10^7$ pfu) of the total library is used as a template in a PCR reaction with β -actin PCR primers. Species-specific PCR primers are used for Mouse and Rat Matchmaker cDNA Libraries; human primers are used for other nonhuman mammalian libraries. Mouse and rat libraries may also be used as templates in PCR reactions using species-specific G3PDH primers.

- **Nonmammalian cDNA libraries**

A representative sample of the total library (containing up to 10^7 cfu or $>10^7$ pfu) is used as a template in a PCR reaction using the appropriate species-specific primers for a ubiquitously expressed gene.

VIII. Preparing for a Yeast Two-Hybrid Screen

A. Construct Fusion Genes

The following is a brief protocol on constructing gene fusions. For more detailed information, see Sambrook *et al.* (1989). The orientation and reading frame of each fusion must be maintained in order to express fusion proteins.

- You can generate a fusion gene if compatible restriction sites are present in the test genes and the corresponding vector. If not, generate the gene fragment by PCR with useful restriction sites incorporated into the primers (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 using a PCR primer that incorporates the desired mutation.
- If you are investigating two known genes, use either vector—unless one has an activation or DNA-binding activity that would interfere with the proper functioning of the two-hybrid system.
 1. Purify the gene fragment, whether generated by PCR or cut out of a plasmid.
Note: We recommend the NucleoSpin Extraction Kit (Cat. No. 635961) for rapid isolation of DNA fragments.
 2. Digest the DNA-BD or AD vector with the appropriate restriction enzyme(s), treat with phosphatase, and purify.
 3. Ligate the appropriate vector and insert. Transform ligation mixtures into *E. coli*.
 4. Identify insert-containing plasmids by restriction analysis or PCR using the Matchmaker Insert Screening Amplimer Set (Cat. No. 630433).
 5. Use the Sequencing Primers included with Matchmaker System 3 to check the orientation and reading frame of the junctions.

B. Obtain or Construct an AD Fusion Library

Premade Matchmaker cDNA Libraries and Pretransformed cDNA Libraries from a variety of tissues and species are available from Clontech. Alternatively, construct an AD fusion library in pGADT7 using either intronless genomic DNA or cDNA such that at least 10⁶ different hybrid proteins will be expressed (Ausubel *et al.*, 1995).

Notes:

- Two-hybrid libraries are usually constructed in the AD vector rather than the DNA-BD vector. Fusing random proteins to a DNA-BD will produce a much larger percentage of fusions that function as autonomous transcriptional activators (Ma & Ptashne, 1987).
- Other GAL4 AD vectors are compatible with Matchmaker Two-Hybrid System 3, provided they carry the *LEU2* nutritional marker.

1. Amplification of premade libraries

Obtain premade libraries as *E. coli* transformants, not as purified DNA. Amplify the library to produce enough plasmid DNA to screen the library in yeast. If you have obtained a Matchmaker cDNA Library, follow the amplification protocol provided in Appendix C. If you have obtained a library from another commercial source, follow the manufacturer's instructions.

2. Construction of cDNA libraries

Use any standard method for generating cDNA (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995). For detailed information regarding construction of two-hybrid cDNA libraries, see Vojtek *et al.* (1993), Durfee *et al.* (1993), Dalton & Triesman (1992), and Luban *et al.* (1992).

Be sure to reserve a 1.0-ml aliquot of your library, frozen in 25% glycerol, so that you can go back and amplify it later.

You can also construct genomic DNA libraries from an organism whose genome contains few or no introns, such as bacteria or yeast. pGADT7 contains a unique *Bam* HI site for constructing a *Sau* 3AI-digested genomic DNA library and a unique *Cla* I site for constructing a library according to James *et al.* (1996).

Note: A procedure for titrating a plasmid library in *E. coli* is in Appendix B.

VIII. Preparing for a Yeast Two-Hybrid Screen *continued*

C. Verify that Constructs Do Not Activate Reporter Genes

1. Independently transform your DNA-BD and AD fusion constructs into strain AH109. Assay the transformants for *MEL1* activation by selecting for transformants on SD/-Trp/X- α -Gal and SD/-Leu/X- α -Gal, respectively. Perform positive and negative controls in parallel (See Section IX, Table VI).

If a partner is known for your DNA-BD/bait, use it to check whether an interaction is detectable before investing in a library search.

2. If the transformant colonies are *white*, prepare stock plates and liquid cultures for freezing. If transformant colonies are *blue*, see Section XI for possible solutions.

D. Verify Protein Expression

1. Independently transform the DNA-BD and AD fusion constructs into strain AH109.
2. Prepare Western blots from the transformants and probe the blots with antibodies to the c-Myc and HA epitope tags (Cat. Nos. 631206, 631207) or the GAL4 DNA-BD and AD Monoclonal Antibodies (Cat. Nos. 630403, 630402). Use untransformed yeast as a control. See the YPH (Section IV) for protocols on preparing protein extracts from yeast.

Note: Using polyclonal antibodies may result in multiple cross-reacting bands.

Alternatively, use the T7 promoter to transcribe and translate the epitope-tagged fusion proteins *in vitro*. Confirm protein transcription and translation by coimmunoprecipitation using the Matchmaker Co-IP Kit (Cat. No. 630449).

IX. Library Transformation & Screening Protocols

In this section, we provide detailed protocols for polyethylene glycol/lithium acetate (PEG/LiAc)-mediated transformation of yeast (Ito *et al.*, 1983; Schiestl & Gietz, 1989; Hill *et al.*, 1991; Gietz *et al.*, 1992).

The procedures described here are for library screens using strain AH109 only. If you choose to use strain CG-1945, follow the same general procedures and plate on SD/-His/-Leu/-Trp. DO NOT PLATE STRAIN CG-1945 ON MEDIA LACKING ADENINE; IT WILL NOT GROW.

A. Transformation Scales

We provide protocols for small-, large-, and library-scale transformations. Table V compares the transformation methods.

- **Use small-scale transformations to:**
 - Verify that the DNA-BD/bait does not autonomously activate reporter genes
 - Look for toxicity effects of DNA-BD/bait on host
 - Perform control experiments
 - Transform the DNA-BD/bait for sequential transformations
- **Use large-scale transformations** when you are learning two-hybrid screening, or when you do not have enough DNA for a library-scale transformation (Table V). You can perform either sequential or simultaneous transformations.
- **In a sequential transformation**, the DNA-BD/bait plasmid is introduced through a small-scale transformation; selected transformants are then grown up and transformed with the AD fusion library through a large-scale transformation. A sequential transformation may be preferred because it uses significantly less plasmid DNA than a simultaneous cotransformation (Table V).
- **A simultaneous cotransformation** is generally preferred because it is easier to perform than a sequential transformation—and because of the risk that expression of the DNA-BD/bait protein may be toxic to the cells. If the DNA-BD/bait protein is toxic, clones arising from spontaneous deletions in the DNA-BD/bait plasmid will have a growth advantage and will accumulate at the expense of clones containing intact plasmids.
- **Library-scale transformations** are preferred when screening an AD/library.

In the case of large and library scale simultaneous cotransformations, you must determine the transformation efficiencies of both plasmids *together*, as well as of each type of plasmid *independently*. Example calculations are shown in Section IX.D.

TABLE V. COMPARISON OF TWO-HYBRID TRANSFORMATION METHODS

Transformation	Amount of Limiting Plasmid	Amount of Cells	Transformation Efficiency	No. of Indep. Clones Amplified	No. of Plates
Library-scale	100–500 µg	8 ml	10 ³ –10 ⁴	1 x 10 ⁶	50 x 150-mm
Large-scale					
Simultaneous	10–50 µg	1.5 ml	10 ³ –10 ⁴	1 x 10 ⁵	5 x 150-mm
Sequential	10–50 µg	1.5 ml	10 ⁴ –10 ⁵	1 x 10 ⁶	50 x 150-mm
Small-scale	0.1 µg	1.5 ml	10 ⁵	na	1 x 100-mm

na = not applicable

^a cfu per µg of the AD library.

^b Total approximate number of transformants expected on SD/-Leu/-Trp selection plates assuming that: 1) the minimal amount of plasmid was used; 2) the transformed cells were resuspended in the volumes recommended in the protocol; 3) 200 µl of transformed cells were spread on each plate; and 4) the transformation efficiencies were optimal.

IX. Library Transformation & Screening Protocols *continued*

B. Yeast Transformation Protocols

Tips for a successful transformation

- Use a 1–3 week-old colony (2–3 mm) to inoculate each liquid culture. If colonies on the stock plate are <2 mm, use several colonies.
Note: To aid in resuspending the cells, place the colony in a 1.5-ml tube containing 0.5 ml of medium and vortex vigorously. Then transfer the cell suspension to the complete volume of culture medium.
- If the overnight or 3-hr cultures are visibly clumped, disperse the clumps by vortexing.
- When you are collecting cells by centrifugation, a swinging bucket rotor results in better recovery of the cell pellet.
- Titer the optimal concentration of 3-AT needed to eliminate background growth on –His selection plates.
- For the highest transformation efficiency, **use competent cells within 1 hr preparing them.** If necessary, you can store competent cells after Step 11 at room temperature for several hours with only a minor reduction in competency.
- When performing simultaneous cotransformations, the bait plasmid must be used in excess, and the AD plasmid or library must be limiting.
- To obtain an even growth of colonies, spread the transformation mixture over the agar surface until all liquid has been absorbed. Alternatively, use 5-mm sterile glass beads (5–7 beads per 100-mm plate; 7–9 beads per 150-mm diameter plate) to promote even spreading. To spread the colonies, shake the plate back and forth—not round and round.

Below are procedures for preparing competent cells and transforming yeast. Set up the control and experimental transformations listed in Table V.

	Transformation Scale		
	<u>SMALL</u>	<u>LARGE</u>	<u>LIBRARY</u>
1. Inoculate 1 ml of YPDA or SD ^a with several 2–3 mm colonies.			
2. Vortex vigorously to disperse any clumps.			
3. Transfer cells to a flask containing this volume of YPDA or SD ^a :	50 ml	50 ml	150 ml
4. Incubate at 30°C for 16–18 hr with shaking (250 rpm) to stationary phase (OD ₆₀₀ >1.5).			
5. Transfer overnight culture (enough to produce an OD ₆₀₀ = 0.2–0.3) into this volume of YPDA:	300 ml	300 ml	1 L
6. Incubate at 30°C for 3 hr with shaking (230–270 rpm). The OD ₆₀₀ will be 0.5 ± 0.1.			
7. Place cells in 50-ml tubes and centrifuge at 1,000 x g for 5 min at room temperature.			
8. Discard the supernatant and resuspend cell pellets by vortexing in this volume of sterile TE or H ₂ O:	25–50 ml	25–50 ml	500 ml
9. Pool cells centrifuge at 1,000 x g for 5 min at room temperature.			
10. Decant the supernatant.			
11. Resuspend the cell pellet in this volume of freshly prepared, sterile 1XTE/LiAc:	1.5 ml	1.5 ml	8 ml ^b
12. Prepare PEG/LiAc solution.	10 ml	10 ml	100 ml

^a Use SD/–Trp when performing the second transformation in a sequential transformation protocol.

^b For library cotransformations only: remove two 100-μl aliquots of competent cells to perform control transformations with pCL1, and pGBKT7-53 + pGADT7-T.

IX. Library Transformation & Screening Protocols *continued*

	Transformation Scale		
	<u>SMALL</u>	<u>LARGE</u>	<u>LIBRARY</u>
13. In the indicated tube, add and mix the following:	1.5 ml	50 ml	500 ml
• DNA-BD/bait ^a	0.1 µg	20–100 µg	0.2–1.0 mg
• AD/library	0.1 µg	10–50 µg	0.1–0.5 mg
• Herring testes carrier DNA	0.1 mg	2 mg	20 mg
14. Add this volume of yeast competent cells: and mix well by vortexing.	0.1 ml	1 ml	8 ml
15. Add this volume of sterile PEG/LiAc solution: and vortex at high speed to mix.	0.6 ml	6 ml	60 ml
16. Incubate at 30°C for 30 min with shaking (200 rpm).			
17. Add this volume of DMSO: Mix well by gentle inversion or swirling. Do not vortex.	70 µl	700 µl	7.0 ml
18. Heat shock for 15 min in a 42°C water bath. For large- and library-scale, swirl occasionally to mix.			
19. Chill cells on ice for 1–2 min.			
20. Centrifuge cells for: at room temperature at:	5 sec 14 K rpm	5 min 1,000 x g	5 min 1,000 x g
21. Remove the supernatant.			
22. Resuspend cells in this volume of 1XTE ^b :	0.5 ml	1.0 ml or 10 ml ^c	10 ml
23. Proceed to Section IX.C for plating.			

^a For simultaneous cotransformation, we recommend a molar ratio of 2:1 (DNA-BD vector:AD vector) for optimal efficiency. For sequential transformations, add either the DNA-BD vector construct or the AD vector construct (not both).

^b If using the high stringency selection method, resuspend cells in YPDA. The media will aid the yeast in recovery from the shock of transformation, but will not adversely affect screening.

^c Use 1.0 ml for simultaneous cotransformation. Use 10 ml for the second transformation in a sequential transformation protocol.

IX. Library Transformation & Screening Protocols *continued*

C. Plating and Screening Transformation Mixtures

You can select AH109 transformants using high-, medium-, or low-stringency media (Figure 5). Less stringent screens increase the number of false positives, while more stringent screens may result in false negatives.

- **High-stringency:** Plate transformations on SD/–Ade/–His/ –Leu/–Trp/X- α -Gal medium to screen for *ADE2*, *HIS3*, and *MEL1* expression. This screen virtually eliminates false positive interactions; however, low-affinity protein interactions may be missed.
- **Medium-stringency:** Plate library transformations on SD/–His/–Leu/–Trp medium to screen for expression of *HIS3*. Plan to screen at least 1.5–3 times the number of independent clones in the library. Subsequently, replica plate His⁺ colonies onto SD/–Ade/–His/ –Leu/–Trp/X- α -Gal medium to screen for *ADE2* and *MEL1* expression.
- **Low-stringency:** Perform this screen if you are having trouble picking up positive clones or if you suspect that your bait protein interacts very weakly or transiently with other proteins. Plate out library transformations on SD/–Leu/–Trp medium to select the DNA-BD and AD vectors. This selection step provides an initial phase of growth that maximizes plasmid copy number, which results in higher levels of fusion protein. This, in turn, improves the chances of detecting AD fusion proteins that interact weakly or transiently with the bait. This screen typically results in up to 1,000 candidate colonies. Therefore, you must optimize the 3-AT concentration needed to control background growth. Furthermore, a low stringency screen may result in a population preference for clones exhibiting stronger activation of the *HIS3* reporter, and extra steps may be required to sort the clones into groups before you proceed with further analysis.

TABLE VI. SET UP FOR A TWO-HYBRID LIBRARY SCREEN

Vectors	Scale ^a	SD Minimal Medium	Amount to Plate (μ l)	Phenotype	
				Mel1/LacZ	His/Ade
Control					
pCL1	S	–Leu	100	Blue	+
pGADT7-T	S	–Leu	100	White	–
+ pGBKT7-53	S	–Trp	100	White	–
	S	–Leu/–Trp	200	Blue	+
	S	–Ade/–His/–Leu/–Trp/X- α -Gal ^b	200	Blue	+
pGADT7-T	S	–Leu	100	White	–
+ pGBKT7-Lam	S	–Trp	100	White	–
	S	–Leu/–Trp	200	White	–
	S	–Ade/–His/–Leu/–Trp/X- α -Gal ^b	200	No Growth	–
Experimental					
DNA-BD/bait	L/Lib	–Leu ^c	100	White	–
+ AD library	L/Lib	–Trp ^c	100	White	–
	L/Lib	Low: –Leu/–Trp ^{d,e,f}	100	White	–
	L/Lib	Medium: –His/–Leu/–Trp ^{b,e}	200	White/Blue	–/+
	L/Lib	High: –Ade/–His/–Leu/–Trp/X- α -Gal ^b	200	White/Blue	–/+

^a S = small scale; L = large scale; Lib = library scale.

^b If necessary, see Section V.B.5 for guidelines on how much 3-AT to add.

^c To test the transformation efficiency of each plasmid, dilute 1 μ l of the transformation with 100 μ l of H₂O. Spread 1 μ l onto 100-mm SD/–Leu and SD/–Trp plates.

^d To test the cotransformation efficiency spread 100 μ l of a 1:1,000, 1:100, and 1:10 dilution onto 100-mm SD/–Leu/–Trp plates.

^e Plate at least 1.5–3 times the number of independent colonies.

^f In a low-stringency library screen, use three –His selection plates: one with the optimal concentration of 3-AT; one with a 10–15-mM higher 3-AT concentration to control for background growth due; and one with a 5-mM lower 3-AT concentration for improved growth of weak positives.

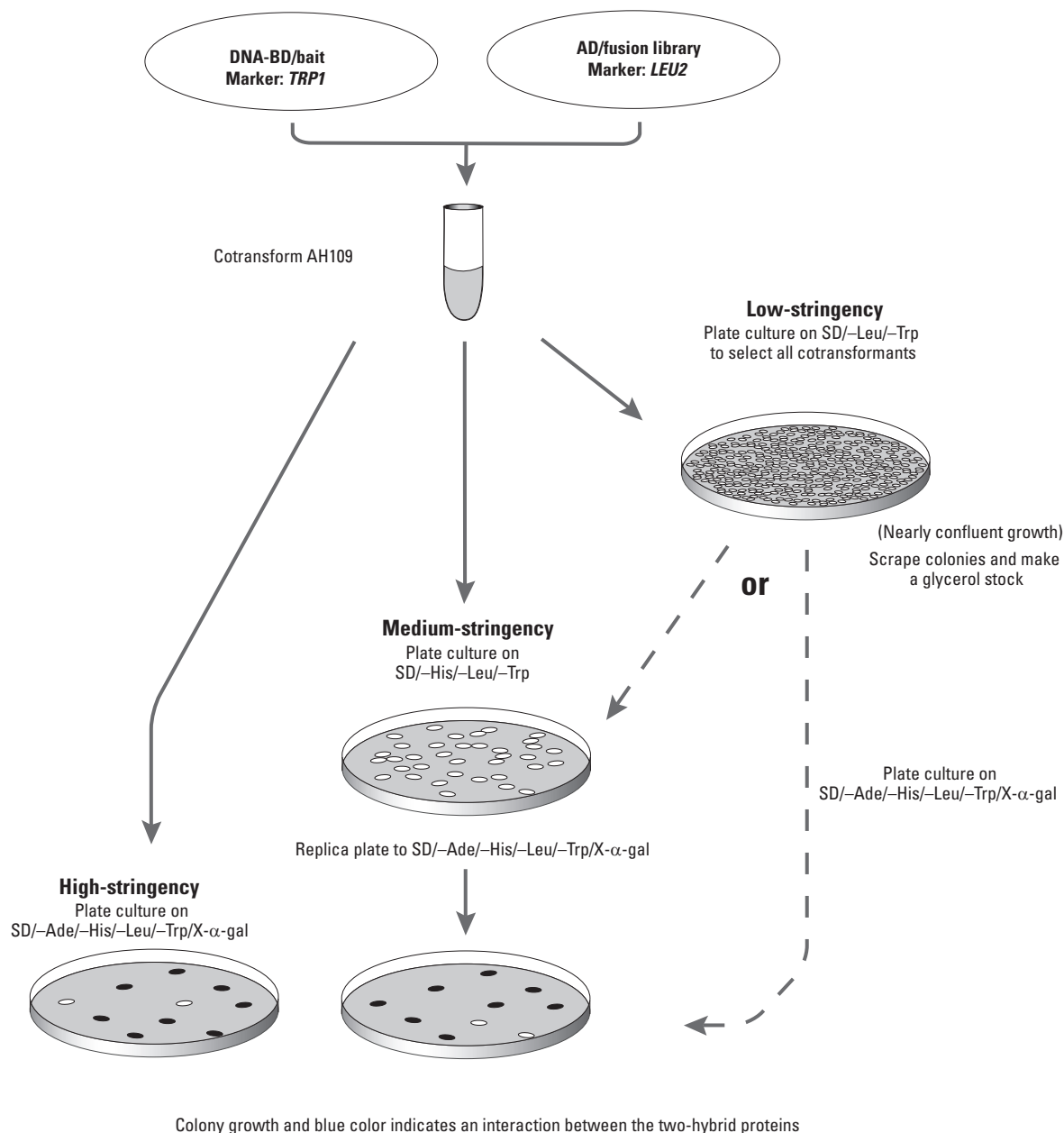
IX. Library Transformation & Screening Protocols *continued*

Figure 5. Screening an AD fusion library using strain AH109. 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.

1. Plate transformation mixtures as indicated in Table V. Plate small-scale transformations on 100-mm plates and large- and library-scale transformations on 150-mm plates. With a new bait and library combination, predicting the optimal method is difficult. Therefore, plate a third of the transformation on low-, medium-, and high-stringency plates.
2. Incubate plates upside-down at 30°C until colonies appear.
3. If screening an AD/library, calculate the transformation efficiency and estimate the number of clones screened, as described in Section IX.D.

IX. Library Transformation & Screening Protocols *continued*

4. LOW-STRINGENCY PROTOCOL ONLY. Harvest the library transformants as follows:

- Chill plates at 4°C for 3–4 hr.
- Add 1–5 ml of TE buffer (pH 7.0) to each plate. Carefully scrape the colonies into the liquid using a heat-bent, sterile Pasteur pipette. Combine all liquids in a sterile 50-ml tube, and vortex to resuspend the cells.

Note: If the combined volume is too large, reduce it as follows: centrifuge for 5 min at 1,000 x g, remove all but 25–50 ml of the supernatant, and vortex to resuspend the cells.

- Create a glycerol stock by adding an equal volume of sterile 65% glycerol/MgSO₄ solution.
- Divide into 1-ml aliquots, and store at 4°C for a week or at –80°C up to 1 yr.
- Titer the glycerol stock on SD/–Leu/–Trp (Appendix B). Incubate plates at 30°C for 3–4 days or until colonies are easy to count. Calculate the cfu/μl of library.
- Plate the amplified yeast cotransformants at high density on either:

- **High-stringency plates:** SD/–Ade/–His/–Leu/–Trp/X-α-gal

- **Medium-stringency plates:** SD/–His/–Leu/–Trp

To compensate for possible errors in the amplified library titer, plate 0.5 x 10⁶ cfu on some plates and 2 x 10⁶ cfu on others. Also, plate appropriate controls for comparison.

Note: If you plate on medium-stringency plates, you must replica plate to high-stringency plates to eliminate false positives.

- Incubate plates upside-down at 30°C until colonies appear.

5. Choose Ade⁺/His⁺/Mel1⁺ colonies for further analysis.

Note: After 2–3 days, some Ade⁺/His⁺ colonies will be visible on the high-stringency plates; however, incubate plates for 5–10 days to allow weak positives to grow. Ignore small, pale colonies that appear after 2 days but never grow to >2 mm in diameter. True His⁺ colonies are robust and can grow to >2 mm. Ade⁺ colonies will remain white to pale pink; Ade[–] colonies will gradually turn reddish-brown and stop growing. Stronger ADE2 expression will be white, while weaker expression will be progressively more red.

6. [Option] Perform a β-galactosidase filter assay (YPH).

D. Calculations

1. Cotransformation Efficiency.

Count colonies (cfu) growing on the SD/–Leu/–Trp dilution plate that has between 30–300 cfu:

$$\frac{\text{cfu} \times \text{total suspension vol. } (\mu\text{l})}{\text{Vol. plated } (\mu\text{l}) \times \text{dilution factor} \times \text{μg DNA used}^*} = \text{cfu/μg DNA}$$

* In a cotransformation, this is the amount of *limiting* plasmid, not the total amount of DNA.

2. Number of Clones Screened.

cfu/μg x μg of library plasmid used = No. of clones screened

Example calculation:

- 100 colonies grew on the 1:100 dilution transformation efficiency control plate (dilution factor = 0.01)
- resuspension volume = 10 ml
- amount of library plasmid used = 100 μg

$$\frac{100 \text{ cfu}}{100 \mu\text{l} \times 0.01} \times \frac{(10 \text{ ml} \times 10^3 \mu\text{l/ml})}{100 \mu\text{g}} = 1 \times 10^4 \text{ cfu/μg}$$

- 1 x 10⁴ cfu x 100 μg = **1 x 10⁶ clones screened.**

3. Amount of DNA to Use.

If you screened <10⁶ clones, repeat the transformation using more DNA. Calculate the amount of DNA to use in the repeat transformation as follows:

$$\frac{10^6 \text{ clones}}{\text{No. of clones screened/μg DNA used}} = \mu\text{g DNA needed}$$

X. Analysis & Verification of Putative Positive Clones

This section provides protocols for verifying protein interactions. Figure 6 provides a detailed overview.

A. Retest Phenotypes

The initial library cotransformants may contain more than one AD/library plasmid, which can complicate the analysis of putative positive clones.

1. Restreak positive colonies on SD/–Leu/–Trp/X- α -Gal plates 2–3 times to allow loss of some of the AD/library plasmids while maintaining selective pressure on both the DNA-BD and AD vectors. Incubate plates at 30°C for 4–6 days. A mixture of white and blue colonies indicates segregation.
2. Replica plate or transfer well-isolated colonies to SD/–Ade/–His/–Leu/–Trp/X- α -Gal plates to verify that they maintain the correct phenotype.
3. Collect the restreaked and retested Ade⁺/His⁺/Mel1⁺ colonies on SD/–Ade/–His/–Leu/–Trp master plates in a grid fashion. 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. Isolate Plasmid DNA from Yeast

The Yeastmaker Yeast Plasmid Isolation Kit (Cat. No. 630441) provides the reagents and a protocol for isolating plasmid DNA from yeast. These procedures provide plasmid DNA suitable for PCR and *E. coli* transformations. A similar protocol is provided in the YPH. **Note: the plasmid DNA isolated from each positive yeast colony will be a mixture of the DNA-BD/bait plasmid and at least one type of AD/library plasmid.**

Alternatively, you may wish to try the direct transfer of plasmid DNA from yeast to *E. coli* by electroporation (Marcil & Higgins, 1992).

Note: For this method, the transformation efficiency of competent *E. coli* cells must be >10⁹ cfu/mg.

C. Sort Colonies to Eliminate Duplicates

1. Amplify AD/library inserts by PCR and characterize PCR products by digesting with a frequent-cutter restriction enzyme, such as *Alu* I or *Hae* III. Analyze fragment sizes by agarose gel electrophoresis; also, run a sample of the uncut amplified insert to check for multiple AD/library plasmids. Prepare a new master plate with a representative clone from each group. If you are satisfied with the number of unique clones, proceed to Step 3.

Notes: To amplify AD/library inserts, we recommend the Matchmaker AD LD Insert Screening Amplimer Set (Cat. No. 630433) and the Advantage 2 PCR Kit (Cat. Nos. 639206, 639207).

2. 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. 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.

CAUTION: Some positive colonies may contain multiple AD/library plasmids—even if the colony has been restreaked twice. Therefore, if a positive colony appears to have multiple AD/library plasmids, do not immediately eliminate those that contain the abundant insert.

3. Prepare a glycerol stock of each unique type, and store aliquots at –80°C.

D. Rescue AD/Library Plasmids via Transformation of *E. coli*.

1. Library Users:

- For strain **CG-1945**, use cycloheximide (CHX) counterselection to obtain colonies that have lost the DNA-BD and retained the AD. Refer to the YPH (Chapter IX) for this procedure.
- If you transformed strain **AH109** and did not use DNA-BD and AD vectors with different antibiotic markers, transform KC8 *E. coli* cells and plate on M9 medium lacking leucine. KC8 cells have a defect in *leuB* that can be complemented by yeast *LEU2*.

System 3 Users or Libraries User with a pGBKT7/bait: Transform the yeast-purified plasmid DNA into *E. coli*. To select for transformants containing only the AD/library plasmid, plate on LB medium containing ampicillin.

2. To verify that you have obtained the same AD/library plasmid, amplify inserts by PCR. Then digest the fragment with *Alu* I or *Hae* III, and run a small sample on a 3–4% agarose/EtBr gel. Compare the PCR product gel profiles from *E. coli* and yeast.

X. Analysis & Verification of Putative Positive Clones *continued*

E. Retest Protein Interactions in Yeast

You can retest protein interactions in yeast by either cotransformation or yeast mating. A real interaction will behave like the controls in Table VI.

1. Cotransformation

- Using the small scale transformation procedure, transform the DNA-BD/bait and AD/library plasmids into AH109.
- Plate on SD/–Ade/–His/–Leu/–Trp/X- α -gal.
- Incubate plates at 30°C until colonies appear.

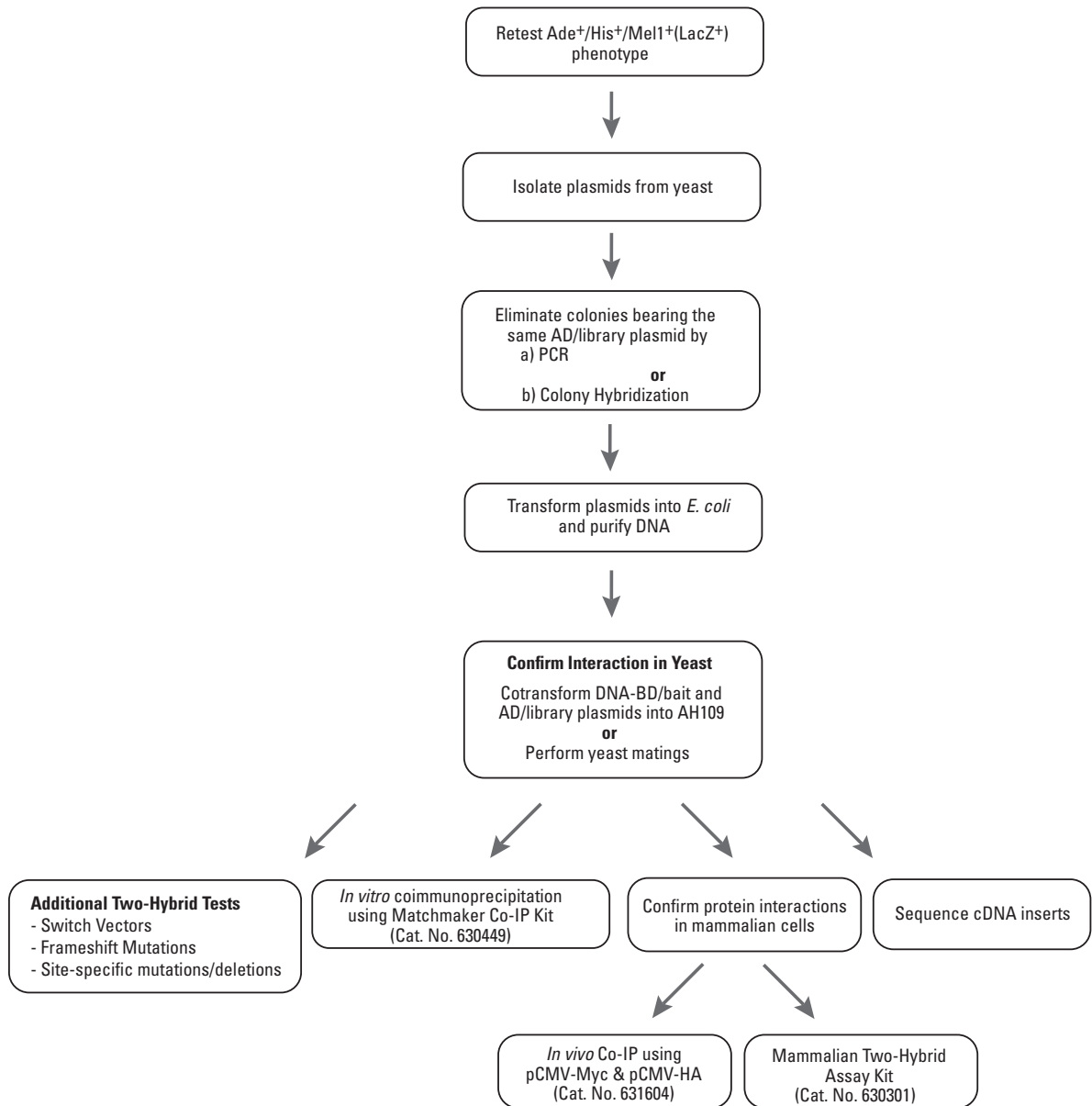


Figure 6. Strategies for analyzing and verifying putative positive clones. If the library screen was performed using strain CG-1945, see the YPH Section IX for details on how to segregate the DNA-BD/bait and AD/library plasmids.

X. Analysis & Verification of Putative Positive Clones *continued*

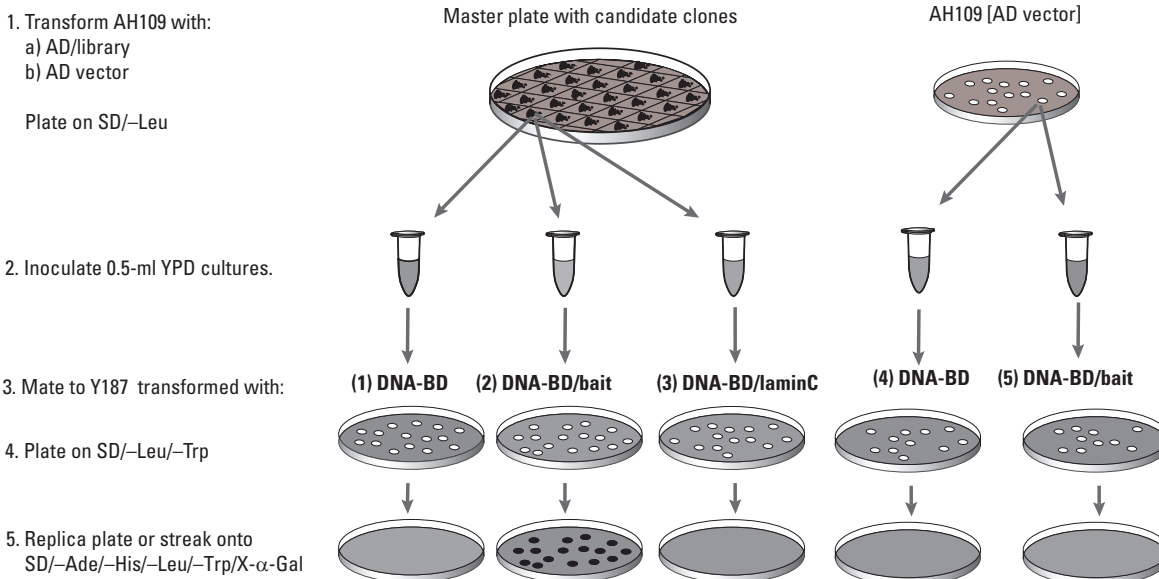


Figure 7. Yeast mating to verify protein interactions.

2. Yeast Mating

Yeast mating is a convenient method of introducing two plasmids into the same host cells (Finley & Brent, 1994; Harper *et al.*, 1993). If you have many Ade⁺, His⁺, Mel1⁺/LacZ⁺ positive clones to analyze, it will be more convenient to handle the clones in batches of 10 or so each.

- Transform AH109 with the AD/library and AD plasmid, and select on SD/-Leu.
- Transform Y187 (or a suitable Mat α strain) with the following three plasmids, and select on SD/-Trp plates:
 - DNA-BD
 - DNA-BD/bait
 - pGBKT7-Lam
- For each candidate AD/library plasmid to be tested, set up the yeast matings indicated in Figure 7 using the Trp⁺ and Leu⁺ transformants obtained in Steps a & b above.
- Refer to the YPH, Chapter IX for mating procedures. To select for diploids, spread mating mixtures on SD/-Leu/-Trp plates as directed.
- 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. Discard any β -galactosidase-positive colonies containing the AD/library plasmid alone.

F. *In vitro* Analysis

The Matchmaker Co-IP Kit (Cat. No. 630449) allows you to confirm protein interactions quickly and independently via an *in vitro* coimmunoprecipitation. The Co-IP Kit works with all GAL4-based Matchmaker System and Library vectors. Because System 3 vectors already contain T7 promoters and epitope tags, you can use them directly in *in vitro* transcription/translation reactions. For all other GAL4-based vectors, you must first use the Co-IP Primers to amplify inserts in order to incorporate the T7 promoters and epitope tags. The Co-IP Kit also provides c-Myc and HA antibodies for precipitating interacting proteins.

- Transcribe and translate the epitope-tagged fusion proteins *in vitro* using the T7 promoters in the AD and DNA-BD vectors. Note: the T7 promoter is located downstream of the GAL4 coding sequence; hence, the GAL4 domains are not transcribed.

X. Analysis & Verification of Putative Positive Clones *continued*

2. Coimmunoprecipitate the epitope-tagged fusion proteins using c-Myc and HA antibodies (Durfee *et al.*, 1993; Zhang *et al.*, 1993).

If the fusion proteins *do not* coimmunoprecipitate, use other means to confirm the interaction. Note: 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. See Section X.G. 2–5 for further recommendations.

G. Sequence AD/Library Inserts

Use only DNA isolated from *E. coli*.

1. Sequence inserts in the positive AD/library plasmids using the 3' AD Sequencing Primer and T7 Sequencing Primer provided with System 3. 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.
2. If your sequencing results reveal a peptide <10-amino acids fused to the AD—or no fusion peptide at all—keep sequencing beyond the stop codon. You may find another ORF. Nontranslated gaps upstream of ORF inserts are most commonly found in yeast genomic libraries, where intercistronic regions are very short. Such gaps can also occur in cDNA libraries, due to the cloning of a portion of the 5' untranslated region of the mRNA along with the coding region in the cDNA. If the library was built in a high-level expression vector such as pGADT7, pGAD GH, or pACT2, a Western blot will reveal the presence and size of an AD fusion protein.
3. Due to occasional translational read-through, two different ORFs may occasionally be expressed as a fusion with the AD, even though a nontranslated gap comes between them.
4. If your sequencing results fail to reveal any ORF in frame with the AD coding region, the positive library clone could be transcribed in the reverse orientation from a cryptic promoter within the *ADH1* terminator (Chien *et al.*, 1991). Such proteins apparently function as transcriptional activators as well as interacting with the bait protein.
5. Yeast also allow translational frameshifts. A large ORF in the wrong reading frame may actually correspond to the expressed protein.

H. *In vivo* Analysis

If the fusion proteins coimmunoprecipitate, confirm functional analysis *in vivo* through either a coimmunoprecipitation or a Mammalian Two-Hybrid Assay (Cat. No. 630301).

1. We recommend 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. All Matchmaker GAL4-based vectors are compatible; therefore, you can easily transfer your bait and library inserts into pCMV-Myc and pCMV-HA.
2. The Mammalian Two-Hybrid Assay Kit is ideal for confirming protein interactions via two-hybrid interactions in mammalian cells. Proteins are more likely to be in their native conformations and to have the appropriate posttranslational modifications; therefore, results are more likely to represent biologically significant interactions.

I. Additional Two-Hybrid Tests

1. 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.
2. 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.
3. Generate site-specific deletion or substitution mutants and repeat the two-hybrid assay. Assay the relative strength of the interactions using a quantitative β -galactosidase assay (YPH).

XI. Troubleshooting Guide

Problem	Cause	Solution
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 (Ma & Ptashne, 1987; Ruden <i>et al.</i> , 1991; Ruden, 1992). Acidic amphipathic domains are often responsible for unwanted transcriptional activation (Ruden <i>et al.</i> , 1991; Ruden, 1992).	<p>If two test proteins are being assayed, switch from the DNA-BD to the AD vector and vice versa.</p> <p>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.</p>
Excessive background	Improper media preparation.	Remake SD/–Ade/–His/–Leu/–Trp/X- α -Gal medium. Add the appropriate amount of 3-AT (Section V.B.5).
	Resuspension of transformed cells in YPDA is too rich.	Use water or TE.
Low transformation efficiency	Improper media preparation.	Remake media, test with control transformations.
	A problem with simultaneous cotrans-formation, even though the transformation with the AD library plasmids alone gave a transformation efficiency of $\geq 5 \times 10^4$ cfu/ μ g and with the bait plasmid alone gave $\geq 10^5$ cfu/ μ g.	Switch to sequential transformation.
	The AD library vector gave a transformation efficiency of $< 5 \times 10^4$ cfu/ μ g or the bait plasmid gave a transformation efficiency of $< 10^5$ for the bait plasmid.	<p>Repeat the experiment using more of the plasmid that had the low transformation efficiency.</p> <p>Check the purity of the DNA and, if necessary, repurify it by ethanol precipitation.</p> <p>If you are not already doing so, we strongly recommend using the pretested and optimized Yeastmaker Carrier DNA, which is available separately (Cat. No. 630440), or as part of the Yeastmaker Yeast Transformation System (Cat. No. 630439).</p> <p>Repeat the transformation, this time including a “recovery” period after the heat shock. To provide a recovery period, perform the simultaneous cotransformation as described (Section IX.B), but add the following steps after Step B.21:</p> <ol style="list-style-type: none"> 22. Resuspend cells in 1.0 L of YPDA medium for a library-scale, and 100 ml for a large-scale, transformation. 23. Incubate cells for 1 hr at 30°C with shaking at 230 rpm. 24. Pellet cells by centrifuging at 1,000 x g for 5 min at room temperature. Remove supernatant. Proceed to Step B.22.

XI. Troubleshooting Guide *continued*

Problem	Cause	Solution
Failure to detect known protein interactions	In sequential transformation, AD transforms poorly; even as empty vector. Bait protein is mildly toxic or inhibiting to transformation	Compare growth curves of host strain with DNA-BD vector and DNA-BD/bait. If bait is toxic, use sequential transformations or switch to a low expressing DNA-BD vector.
	High-level expression of one or both of the hybrid proteins is toxic to the cell; therefore, transformants will not grow or will grow very slowly. For this reason, we recommend that you check for cell toxicity before performing a library screen (Section VIII.D).	Truncation of one of the hybrid proteins may alleviate the toxicity and still allow the interaction to occur. Try using vectors that express lower levels of the fusion proteins, such as pGBT9 (a DNA-BD vector), and pGAD424, pGAD GL, or pGAD10 (AD vectors) (Holtz & Zhu, 1995).
	The transformation efficiency of one or both plasmids 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.	See previous tip on improving transformation efficiency.
	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. (See van Aelst <i>et al.</i> [1993] for one example.)	Construct hybrids containing different domains of the bait 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.
AD/library plasmid activates all three reporters independent of the DNA-BD/bait	Some types of protein interactions may not be detectable in a GAL4-based system.	Use the Matchmaker LexA Two-Hybrid System (Cat. No. K1609-1).
	Some protein interactions are not detectable using any type of two-hybrid assay.	
	A rare category of false positives in which an AD/library hybrid activates transcription inappropriately.	Refer to Section X for methods to verify protein interactions; see Bartel <i>et al.</i> (1993a) for further discussion of false positives.

XII. References

General References are indicated with a bullet (•).

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XIII. Related Products

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

GAL4-based One- and Two-Hybrid Systems and Related Products:

• pGADT7 AD Vector	630442
• pGBKT7 DNA-BD Vector	630443
• Mammalian Matchmaker™ Two-Hybrid Assay Kit	630301
• Matchmaker™ Pretransformed cDNA Libraries	many
• Matchmaker™ cDNA and Genomic Libraries	many
• Matchmaker™ Random Peptide Library	638853
• pBridge Vector	630404
• pCMV-Myc & pCMV-HA Vector Set	631604
• Matchmaker™ Co-IP Kit	630449
• Matchmaker™ AD LD-Insert Screening Amplimer Set	630433

Antibodies:

• c-Myc Monoclonal Antibody	631206
• c-Myc Monoclonal Antibody-Agarose Beads	631208
• HA-Tag Polyclonal Antibody (Affinity Purified)	631207
• GAL4 AD Monoclonal Antibody	630402
• GAL4 DNA-BD Monoclonal Antibody	630403

General Reagents for Working With Yeast:

• Yeastmaker™ Yeast Transformation System 2	630439
• Yeastmaker™ Carrier DNA	630440
• Yeastmaker™ Yeast Plasmid Isolation Kit	630441
• KC8 Electrocompetent and Chemically Competent Cells	630435 630434
• YPD Medium	630409
• YPD Agar Medium	630410
• Minimal SD Base (contains glucose or galactose)	630411 630420
• Minimal SD Agar Base (contains glucose or galactose)	630412 630421
• DO Supplements	many
• X- α -Gal	630407

General Cloning Reagents:

• Advantage® 2 Polymerase Mix	639201 639202
• Advantage® 2 PCR Kit	639206 639207
• NucleoSpin® Extraction Kit	635961

Appendix A. Media & Solution Recipes

Media for Growth and Selection of Yeast

Clontech carries a full line of yeast media including YPD, SD with glucose or galactose; with or without agar, and Dropout (DO) Supplements ideal for use with Matchmaker Two-Hybrid Systems and Libraries. Please see Section XIII for ordering information. If you purchased yeast media from Clontech, follow the directions provided with the product. Alternatively, you can prepare your own media and DO Supplements using the detailed recipes provided in the YPH, Appendix C.

- **YPDA medium**

To 1 L of YPD Medium (Cat. No. 630409), add 15 ml of filter-sterilized 0.2% adenine hemisulfate (Sigma Cat. No. A-9126) to a final concentration of 0.003%.

Reagent for low stringency screens

- **65% glycerol/MgSO₄ solution** (sterile)

	<u>Final Conc.</u>
Glycerol	65% v/v
MgSO ₄	100 mM
Tris-HCl (pH 8.0)	25 mM

Media for Titering and Amplifying Plasmid Libraries in *E. coli*

- **LB broth**

10 g/L	Bacto-tryptone
5 g/L	Bacto-yeast extract
5 g/L	NaCl

Adjust pH to 7.0 with 5 N NaOH. Autoclave. Store at room temperature.

- **LB/amp broth**

Prepare LB broth, then autoclave and cool to 50°C. Add ampicillin to 100 µg/ml. Store at 4°C.

- **LB/amp plates**

Prepare LB broth, then add agar (18 g/L), autoclave, and cool to 50°C. Add ampicillin to 100 µg/ml. Pour plates and store at 4°C.

- **Ampicillin stock solution** (50 mg/ml in H₂O; 1000X). Store at -20°C.

X-α-Gal

Dissolve X-α-Gal at 20 mg/ml in dimethylformamide (DMF). Store X-α-Gal solutions in glass or polypropylene bottles at -20°C in the dark.

1. Pouring X-α-Gal indicator plates

- Prepare and autoclave 1.0 L of the appropriate dropout agar medium. Cool to 55°C.
- Add 1 ml of X-α-Gal (20 mg/ml).
- Pour plates and allow medium to harden at room temperature.
- Plate cells and incubate at the appropriate temperature until blue colonies form.

2. Spreading X-α-Gal onto premade plates

- Dilute X-α-Gal to 4 mg/ml in DMF.
- Pour appropriate dropout plates and allow medium to harden at room temperature.
- Spread 200 µl of X-α-Gal (4 mg/ml) onto a 15-cm plate or 100 µl onto a 10-cm plate using glass beads.
Note: To quickly (1–24 hr) determine if a yeast strain contains *MEL1*, spread X-α-Gal at 20 mg/ml as described.
- Allow plates to dry for 15 min at room temperature.
- Plate cells and incubate at the appropriate temperature until blue colonies form.

Appendix B. Library Titering

A. Important:

- Diluted libraries are always less stable than undiluted libraries. Therefore, once the library dilutions are made, use them within the next hour before drastic reductions in titer occur.
- Use proper sterile technique when aliquoting and handling libraries.
- Design and use appropriate controls to test for cross-contamination.
- Always use the recommended concentration of antibiotic in the medium to ensure plasmid stability.
- pACT and pACT2 libraries are released from λ ACT and λ ACT2 libraries, respectively. Incubating cultures of pACT and pACT2 libraries at 37°C can result in plaques on the high-density plates due to the presence of residual phage in the library. These plaques should not interfere with library titering. However, if they are numerous, retiter the library at 30–31°C and incubate 36–48 hr.

B. Plasmid Library Titering

Reagents and Materials Required:

- LB broth (Appendix A)
- LB/amp plates (100-mm plates; Appendix A)
Note: Allow the agar plates to dry unsleeved at room temperature for 2–3 days, or at 30°C for 3 hr, prior to plating cells. Moisture droplets on the agar surface can lead to uneven spreading of cells.
- Sterile glass spreading rod, bent Pasteur pipette, or 5-mm glass beads for spreading culture on plates.

1. Thaw an aliquot of the library, and place on ice.
2. Mix by gentle vortexing. Transfer 1 μ l to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution A** (1:10³).
Note: pACT and pACT2 libraries may be viscous, and repeated freeze/thaw cycles may increase viscosity. To facilitate accurate pipetting of the library, first dilute a 10- μ l sample with 10 μ l of LB broth. Then prepare further dilutions from this 1:1 dilution. Be sure to account for this extra dilution when calculating the titer.
3. Remove 1 μ l from Dilution A, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution B** (1:10⁶).
4. Add 1 μ l from Dilution A to 50 μ l of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto a prewarmed LB/amp plate.
Note: Continue spreading the inoculum over the agar surface until all visible liquid has been absorbed. This procedure is essential for even growth of the colonies.
5. Plate 50- μ l and 100- μ l aliquots of Dilution B on LB/amp plates.
6. Leave plates at room temperature for 15–20 min to allow the inoculum to soak into the agar.
7. Invert the plates, and incubate at 37°C for 18–20 hr, or at 30–31°C for 24–36 hr.
 - For pACT and pACT2 libraries: incubate plates at 30–31°C for 36–48 hr.
8. Count the number of colonies to determine the titer (cfu/ml). Calculate the titer as follows:
 - Dilution A: No. colonies \times 10³ \times 10³ = cfu/ml
 - Dilution B: (No. colonies/plating volume) \times 10³ \times 10³ \times 10³ = cfu/ml**Note:** A 2–5-fold range in titer calculations is not unusual, especially if more than one person is doing the titering.

Appendix C. Plasmid Library Amplification

You must amplify premade Matchmaker Libraries to obtain enough plasmid for library screening in yeast. You will need 100–500 µg of plasmid DNA to screen $\sim 1 \times 10^6$ independent clones (see Table IV).

Note: pACT and pACT2 library customers: If you observed plaques on your high-density library titering plates (Appendix B), you may wish to use a lower temperature (i.e., 30–31°C) when incubating the library amplification plates. The lower temperature will require a longer incubation time, as noted below. If you follow this amplification protocol exactly through Step 7, a few λ plaques on the amplification plates should not affect the quality or yield of plasmid. If you choose to include Step 8, be sure to perform it at 30°C. Lysis is more likely to occur in liquid cultures, and you risk lysing the entire culture at temperatures over 31.0°C. Growth at 30–31°C is not necessary once the pACT or pACT2 library DNA has been transferred to a new *E. coli* host.

A. Reagents and Materials Required

- LB/amp agar plates (Appendix A)

Notes:

- The exact number of plates required depends on the size of the library. Use the following calculation to determine how many plates to use. Normally, use 3 times the size of the originally library and plate at 20,000 cfu/plate.

(No. of independent clones \times 3) = No. clones to screen
 $2 \times 10^6 \times 3 = 6 \times 10^6$ clones to screen
 No. of clones to screen/ colonies per plate = No. of plates
 $6 \times 10^6 / 20,000 = 300$ plates
- If the titer is 6×10^8 , determine the amount of the library stock to spread on each plate.

No. clones to screen / library titer = µls of library to plate
 $6 \times 10^6 / 6 \times 10^8$ titer = 10 µl
- Calculate the volume of media needed to plate 150 µl on each plate.

300 plates \times 150 µl = 52.5 ml
- Add 10 µl of the library to 52.5 ml of LB amp and spread 150 µl onto each of the 300 LB amp plates
- Allow the agar plates to dry at room temperature for 2–3 days, or at 30°C for 3 hr, prior to plating cells. Moisture droplets on the agar surface can lead to uneven spreading of cells.
- LB/glycerol (1 L; LB broth containing 25% glycerol)
- Sterile glass spreading rod, bent Pasteur pipette, or 5 mm diameter sterile glass beads (~10/plate)
- [Optional, for Step 8] LB/amp broth (2 L; Appendix A) and sterile, 50–80% glycerol.

B. Plasmid Library Amplification Protocol

1. If you have not done so already, titer the plasmid library (Appendix B).
2. Plate the library directly on LB/amp plates at a high enough density so that the resulting colonies will be nearly confluent (~20,000–40,000 cfu per 150-mm plate). Plate enough cfu to obtain at least 2–3X the number of independent clones in the library.

Notes:

- The number of independent clones is the number of independent colonies present in the library before amplification. If you have purchased a library from Clontech, the size of the library is stated on the PAC.
 - To promote even growth of the colonies, continue spreading the inoculum over the agar surface until all visible liquid has been absorbed, and then allow plates to sit at room temperature for 15–20 min. If using glass beads to spread the colonies, shake the plate back and forth—not round and round.
3. Invert the plates, and incubate at 37°C for 18–20 hr.

Notes:

 - For pACT or pACT2 libraries: incubate plates at 30–31°C for 36–48 hr, or until confluent.
 - Growing the transformants on solid medium instead of in liquid culture minimizes uneven amplification of the individual clones.
 4. Add ~5 ml of LB/glycerol to each plate and scrape colonies into liquid. Pool all the resuspended colonies in one flask and mix thoroughly.

Note: To obtain higher yields, scrape the colonies into LB/amp (no glycerol) and pool the colonies in one flask. Incubate at 30–31°C for 2–4 hr with vigorous shaking (200 rpm). Add sterile glycerol to 25%, and proceed to Step 5.

Appendix C. Plasmid Library Amplification *continued*

5. Set aside one-third of the library culture (roughly equivalent to 3 L of overnight culture) for the plasmid preparation; this portion can be stored at 4°C if you plan to use it within 2 weeks. For storage >2 weeks, divide the culture into 50-ml aliquots, and store at –70°C.
 - Set aside five 1-ml aliquots of the library culture in case you wish to re-amplify the library at a later time. Store the aliquots at –70°C.
 - Divide the remainder of the library culture into 50-ml aliquots and store at –70°C.
6. Prepare plasmid DNA using any standard method that yields a large quantity of highly purified plasmid. (See Sambrook *et al.*, 1989 for CsCl gradient purification, if necessary.)

Note: The cell culture from Step 4 will be very dense ($OD_{600} \gg 1$), so adjust the plasmid preparation protocol accordingly (i.e., follow the procedure as if you were processing 3 L of overnight liquid culture) or prepare a sterile dilution from 10^{-1} to 10^{-3} . Plasmid preparation procedures are based on a culture of $OD_{600} = 1-2$. Your plasmid preparation must take into account the much higher than normal cellular concentration and be magnified accordingly. If 30 ml of culture when diluted to 10^{-2} has an $OD_{600} = 1$, treat the stock as though it were 3 L of culture and prepare plasmid DNA using a NucleoBond Mega or Giga Plasmid Kit (Cat. Nos. 635938 & 635939).
7. Expected yields of plasmid DNA per 1×10^6 cfu:
 - pACT & pACT2 Libraries: 0.25 mg
 - all other Matchmaker GAL4 Libraries: ~1 mg
8. [Optional] To obtain higher yields, scrape the colonies into LB/amp (no glycerol) and pool the colonies in one flask. Incubate at 31–30°C for 2–4 hr with at 200 rpm. Add sterile glycerol to 25%, then proceed with Step 5 above.