Matchmaker™ Pretransformed Libraries User Manual

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I. Introduction & Protocol Overview

Matchmaker™ Pretransformed Libraries are high-complexity cDNA libraries cloned into a yeast GAL4 activation domain (AD) vector and pretransformed into *Saccharomyces cerevisiae* host strain Y187.

Matchmaker Pretransformed Normalized Libraries are premium libraries that have an equalized gene representation, by specifically lowering the proportion of highly abundant transcripts prior to library construction.

Principle of the two-hybrid assay

In a Matchmaker GAL4-based two-hybrid assay, a **bait** protein is expressed as a fusion to the Gal4 DNA-binding domain (DNA-BD), while libraries of **prey** proteins are expressed as fusions to the Gal4 activation domain (AD; Fields & Song, 1989; Chien et al., 1991). When bait and library (prey) fusion proteins interact, the DNA-BD and AD are brought into proximity to activate transcription of four reporter genes (*ADE2*, *HIS3*, *MEL1*, and *LacZ*) (Figure 1).

This technology can be used to:

- identify novel protein interactions
- confirm suspected interactions
- define interacting domains

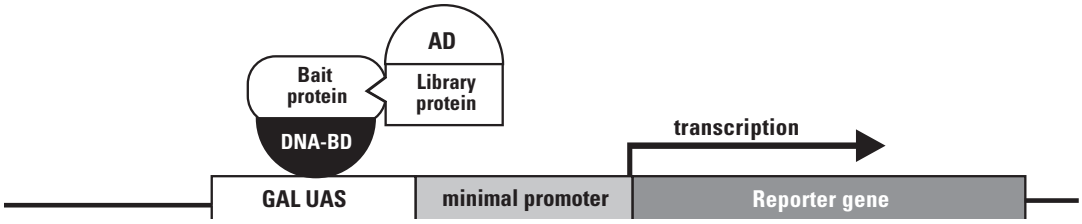

Figure 1. The two-hybrid principle. Two proteins are expressed separately, one fused to the Gal4 DNA binding domain (BD) and the other fused to the Gal4 transciptional activation domain (AD). In yeast strain AH109, activation of the reporters (*HIS3, ADE2, MEL1*, and *LacZ*) only occurs when the proteins interact.

The Bait

To make your *GAL4* DNA-BD/bait construct, we recommend using pGBKT7, which is available separately (Cat. No. 630443) or as a component of our complete Matchmaker Two-Hybrid System 3 (Cat. No. 630303). To investigate ternary protein complexes, we suggest you use pBridge (Cat. No. 630404), a three-hybrid vector that contains two MCS regions so that you can express a Gal4 DNA-BD fusion and a second protein of interest that may act as a "bridge" between bait and prey.

Four Reporter Genes to Detect Protein Interactions

In response to two-hybrid interactions, Clontech's Matchmaker Yeast Strain AH109 expresses four integrated reporter genes under the control of distinct Gal4-responsive upstream activating sequences (UASs) and TATA boxes (Figure 2).

HIS3. AH109 is unable to synthesize histidine and is therefore unable to grow on media that lack this essential amino acid. When bait and prey proteins interact, Gal4-responsive His3 expression permits the cell to biosynthesize histidine and grow on a his- minimal medium.

ADE2. AH109 is also unable to grow on minimal media that does not contain adenine. However, when two proteins interact, Ade2 expression is activated, allowing these cells to grow on ade- minimal medium.

MEL1. *MEL-1* encodes α-galactosidase, an enzyme occurring naturally in many yeast strains. As a result of two-hybrid interactions, α-galactosidase (*MEL1*) is expressed and secreted by the yeast cells. Yeast colonies that express Mel1 turn blue in the presence of the chromagenic substrate X-α-Gal (Cat. No. 630407).

LacZ. *Lac Z* encodes ß-galactosidase (ß-Gal), an *E.coli* enzyme which is integrated into the AH109 chromosome. As a result of two-hybrid interactions, ß-galactosidase is expressed, but not secreted. Thus, only if the cells are lysed, as in a colony lift assay, can a blue color be detected in the presence of X-Gal. In contrast, X-α-Gal detection allows blue colonies to be visualized directly on the growth medium.

I. Introduction & Protocol Overview continued

The promoters controlling His3, Ade2, and Mel1/LacZ expression in AH109 are unrelated except for the short protein binding sites in the UAS region that are specifically bound by the Gal4 DNA-BD. Thus, library proteins that interact with unrelated sequences flanking or within the UAS (i.e., false positives) are automatically screened out.

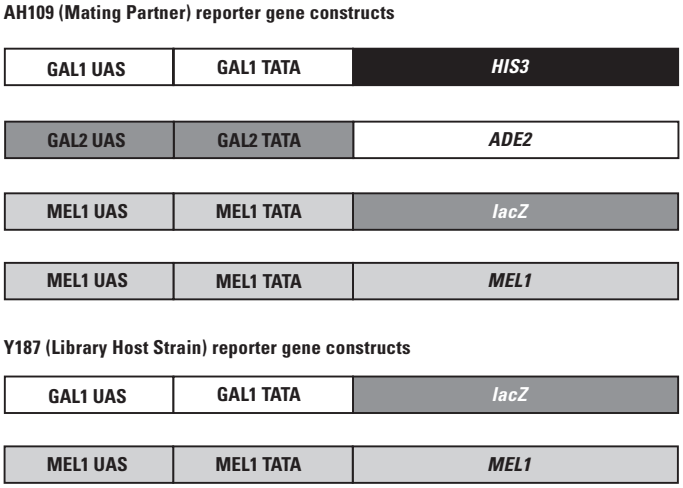

Figure 2. Reporter gene constructs in yeast strains AH109 and Y187. In AH109, the *HIS3, ADE2*, and *MEL1*/*LacZ* reporter genes are under the control of three completely heterologous Gal4-responsive UAS and promoter elements—*GAL1*, *GAL2*, and *MEL1*, respectively. The protein-binding sites within the UASs are different, although each is related to the 17-mer consensus sequence recognized by Gal4 (Giniger et al., 1985; Giniger & Ptashne, 1988).

I. Introduction & Protocol Overview continued

Matchmaker Screening Protocol Overview

The entire Matchmaker screening process consists of the following steps:

- Step 1. Perform control experiments
- Step 2. Clone and test bait for autoactivation and toxicity
- Step 3. Screen pretransformed library by mating (Figure 3)
- Step 4. Confirm and interpret results

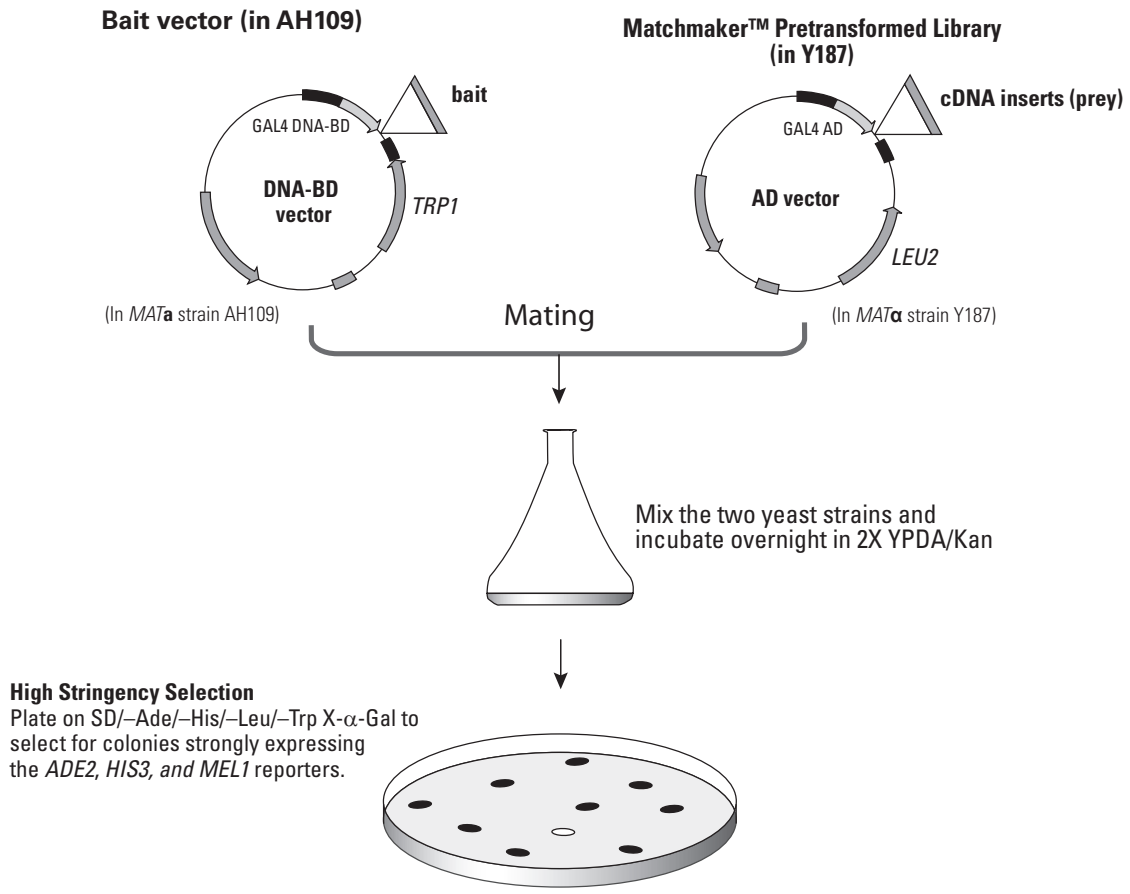

Figure 3. Two-hybrid screening using Matchmaker™ Pretransformed cDNA Libraries. Your bait protein is expressed as a fusion with the Gal4 DNA-BD in yeast strain AH109. The high-complexity pretransformed cDNA library, which expresses fusions with the Gal4 AD, is provided in yeast strain Y187. When cultures of the two transformed strains are mixed together overnight, they mate to create diploids. Diploid cells contain four reporter genes: *HIS3*, *ADE2*, *MEL1*, and *LacZ*, that are activated in response to two-hybrid interactions. Expression from the *LacZ* reporter can only be detected if the cells are lysed in a colony lift assay.

II. List of Components

Each 1 ml library aliquot is sufficient for a complete library screening of >1 x 10^6 independent clones. See Section III and Appendix A for information on library construction. See Section V for host strain genotypes and phenotypes; Appendix B for a library titering protocol; and Appendix C for Control Plasmid information. Pretransformed control strains must be recovered from the frozen stock (Section VII) and grown in liquid culture prior to use in mating.

NOTE: Freezing Medium consists of YPD liquid medium + 25% glycerol (see Section VI, Table III).

Store all components at –70°C.

Library

- 5 x 1.0 ml **Matchmaker Pretransformed or Matchmaker Pretransformed Normalized Library Culture**

  In *Saccharomyces cerevisiae* strain Y187 in freezing medium. Depending on which library you purchased, this may be cloned in pGADT7-Rec2, pGADT7-RecAB, or pACT2. (See Certificate of Analysis for details.)

  Once a library aliquot has been thawed, **do not refreeze it**. With every freeze/thaw cycle, there is a ~10% loss in viability, which could affect the quality of the library.

Control Plasmids (pretransformed in yeast)

- 1.0 ml **pGBKT7-53 in AH109** (MATa)

  Positive control plasmid pretransformed into *S. cerevisiae* strain AH109 in freezing medium. pGBKT7-53 encodes a DNA-BD/murine p53 fusion protein.

- 1.0 ml **pTD1-1 in Y187** (MATα) or

  0.5 ml **pGADT7-T**

  Positive control plasmid pretransformed into *S. cerevisiae* strain Y187 in freezing medium. pTD1-1 and pGADT7-T each encode an AD/SV40 large T antigen fusion protein.

Yeast Strain

- 1.0 ml *S. cerevisiae* **AH109** (MATa)

  Reporter host strain in freezing medium. AH109 is recommended for use as the host strain for your bait plasmid construct.

III. Pretransformed Library Information

Library Preparation

Matchmaker Libraries may be cloned into one of several commonly used *GAL4* AD vectors. Three of these are described below. To find out which vector your library was constructed in, refer to the Certificate of Analysis (CofA) included with your library. Vector maps are available at **www.clontech.com**.

- **Libraries constructed in pGADT7-Rec:**

  Pretransformed Libraries cloned in pGADT7-Rec are constructed using our Matchmaker Library Construction & Screening Kit (Cat. No. 630445), pGADT7-Rec offers a simple and efficient method for constructing two-hybrid libraries via recombination-mediated cloning directly in *S. cerevisiae*. See PT3529-1 for a description of the procedure.

- **Libraries constructed in pGADT7-RecAB:**

  Pretransformed normalized libraries are constructed in pGADT7-RecAB. cDNA synthesized using our SMART technology is normalized to reduce the proportion of highly abundant transcripts. Normalized cDNA is SfiI-digested, and cloned into the SfiI A/B sites of pGADT7-RecAB.

  Once cloned, the library is amplified in *E. coli*, rescued, and used to transform yeast strain Y187. The resulting colonies are harvested at high density in freezing medium and immediately aliquoted and frozen at –70°C.

- **Libraries Constructed in pACT2:**

  Matchmaker Pretransformed Libraries cloned in pACT2 are prepared using a modified Gubler & Hoffman procedure (1983). Once cloned, the library is amplified in *E. coli*. The library plasmid is then isolated and used to transform yeast strain Y187. The resulting colonies are harvested, aliquoted, and frozen at –70°C.

See **Appendix A** for additional information regarding the library construction process.

IV. List of Abbreviations

AD fusion library [or AD library]	A cDNA library (such as a Matchmaker Pretransformed Library) constructed in an activation domain (AD) vector such that the proteins encoded by the inserts are fused to the 3' end of the Gal4 AD
AD/library plasmid	Plasmid encoding a fusion of the Gal4 activation domain and a library cDNA
AD/library protein	A protein fusion comprised of the Gal4 activation domain and a polypeptide encoded by a library cDNA
AD vector	Plasmid encoding the yeast Gal4 activation domain
DNA-BD vector	Plasmid encoding the Gal4 DNA-binding domain
DNA-BD/bait plasmid	Plasmid encoding a fusion of the Gal4 DNA binding domain and a bait cDNA
DNA-BD/bait protein [or "bait"]	A protein fusion comprised of the Gal4 DNA binding domain and a polypeptide encoded by a bait cDNA

Yeast Phenotypes

Ade–, or His–, or Leu–, or Trp–	Requires adenine (Ade), or histidine (His) or leucine (Leu), or tryptophan (Trp) in the medium to grow; i.e., is auxotrophic for one (or more) of these specific nutrients
LacZ$^+$	Expresses the *LacZ* reporter gene; i.e., is positive for ß-galactosidase (ß-gal) activity.
Mel1$^+$	Expresses the *MEL1* reporter gene; i.e., is positive for α-galactosidase (α-gal) activity

Miscellaneous

SD	Minimal, synthetically defined medium for yeast; is comprised of a nitrogen base, a carbon source (glucose unless stated otherwise), and a DO supplement
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
TDO	Triple dropout medium: SD/–His/–Leu/–Trp or SD/–Ade/–Leu/–Trp
QDO	Quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp.
YPD	A blend of yeast extract, peptone, and dextrose in optimal proportions for growth of most strains of *S. cerevisiae*
YPDA	YPD medium supplemented with adenine (0.003% final concentration)

V. Host Strain Information

The phenotypes and complete genotypes of AH109 and Y187 (the library strain) are shown in Tables I and II.For additional information on the growth and maintenance of yeast, see the Yeast Protocols Handbook (YPH). We also recommend the Guide to Yeast Genetics and Molecular Biology (Guthrie & Fink, 1991).

Table I: Yeast Host Strain Genotypes

Strain	Genotype[a]	Reporters	Transformation Markers	Reference
AH109[b, c]	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2 URA3 : : MEL1$_{UAS}$-MEL1$_{TATA}$-LacZ MEL1*	*HIS3, ADE2, MEL1, LacZ*	*trp1, leu2*	Holtz, unpublished
Y187[c]	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3 : : GAL1$_{UAS}$-GAL1$_{TATA}$-LacZ MEL1*	*MEL1, LacZ*	*trp1, leu2*	Harper et al., 1993

[a] The *GAL1*, *GAL2*, and *MEL1* upstream activating sequences (UASs) are recognized and bound by the Gal4 BD. 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 (James et al., 1996). The *ade2-101* mutation in the precursor strain, PJ69-2A, was replaced (by recombination) with the *GAL2-ADE2* reporter construct. In the absence of *GAL4*, AH109 displays the Ade– phenotype.

[c] The *LacZ* reporter construct was integrated into the yeast genome by homologous recombination at the *ura3-52* mutation (A. Holtz, unpublished). Recombinants were selected on SD/–Ura. The met– phenotype in this strain is unstable.

Table II: Phenotype Testing on Various SD Media

Strain	SD/–Ade	SD/–His	SD/–Leu	SD/–Trp	SD/–Ura
AH109	–	–	–	–	+
Y187	–	–	–	–	+
AH109[pGBKT7-53]	–	–	–	+	+
Y187[pGADT7-T or pTD1-1]	–	–	+	–	+
Control Diploid[1]	+	+	+	+	+

[1] Diploid strain derived from mating AH109[pGBKT7-53] with Y187[pGADT7-T or pTD1-1].

VI. Yeast Media & Additional Materials Required

Table III contains a list of yeast media, components, and corresponding Clontech catalog numbers required for the protocols described in this user manual, while Table IV lists additional media supplements. **Recipes for the media are located in Appendix D.** The following considerations should be taken into account when culturing yeast for a two-hybrid screen.

- Minimal media that is routinely used for culturing *S. cerevisiae* is called "synthetically defined" medium or SD. **SD base** supplies everything that a yeast cell needs to survive (including carbon and nitrogen sources) with the exception of essential amino acids, which are added separately as a **dropout (DO) supplement**. The particular DO supplement that is chosen will determine which plasmids and/or activated reporters are selected for.
- For example, SD base mixed with -Leu/-Trp dropout supplement (SD/-Leu/-Trp) is used to select for the bait and prey plasmids. Cells harboring these plasmids are able to grow because the vectors encode tryptophan and leucine biosynthesis genes, respectively, that are otherwise absent from the cell. **We often refer to SD/-Leu/-Trp as Double Dropout (DDO) in this user manual.**
- Similarly, SD/-Ade/-His/-Leu/-Trp selects for the presence of bait and prey plasmids, but also selects for the activation of the Gal-responsive *HIS3* and *ADE2* genes as part of the two-hybrid assay. Colonies that grow on this **Quadruple Dropout (QDO)** contain both bait and prey plasmids and also express proteins that interact with each other to activate *HIS3* and *ADE2*.
- Tools for plating yeast include a sterile glass rod, bent Pasteur pipette, or 5 mm glass beads for spreading cells on plates. (Use 5–7 beads per 100 mm plate).

Table III: Yeast Media and Supplements Required for a Two-Hybrid Screen

Yeast Media	Clontech Cat. No.
Rich Media (for routine culturing of untransformed yeast)	
YPDA	Appendix D
YPD Medium (500 g)	630409
YPD Agar Medium (700 g)	630410
Minimal Media	
Minimal SD Base Medium (267 g)	630411
Minimal SD Agar Base (467 g)	630412
Dropout Supplements	
-Trp DO Supplement (10 g)	630413
-Leu DO Supplement (10 g)	630414
-His DO Supplement (10 g)	630415
-Ura DO Supplement (10 g)	630416
-Leu/-Trp DO Supplement (10 g)	630417
-His/-Leu DO Supplement (10 g)	630418
-His/-Leu/-Trp DO Supplement (10 g)	630419
-Ade/-His/-Leu/-Trp DO Supplement (10 g)	630428
Freezing Medium	
YPD Medium & 25% glycerol	

VI. Yeast Media & Additional Materials Required continued

Table IV: Additional Media Supplements

Supplement Name	Clontech Cat. No. [1]	Stock Solution Concentration
X-α-Gal (25 mg)	630407	20 mg/ml in dimethyl formamide
L-Adenine Hemisulphate	Sigma A9129	0.2% stock solution
L-Leucine	Sigma L8000	–
Kanamycin Sulfate	–	50 mg/ml stock solution
Dimethyl Formamide	–	–

[1] Unless otherwise specified

Recipes for each of these media are found in Appendix D.

Rich Media

YPDA liquid

0.5X YPDA liquid

2X YPDA liquid

YPDA agar

Single DO Media

SD/-Trp liquid

SD/-Trp agar

SD/-Leu liquid

SD/-Leu agar

Double DO Media

SD/-Leu/-Trp liquid

SD/-Leu/-Trp agar

Triple DO Media

SD-/-His/-Leu/-Trp liquid

SD-/-His/-Leu/-Trp agar

SD/-Ade/-His/-Trp agar

Quadruple DO Media

SD/-Ade/-His/-Leu/-Trp liquid

SD/-Ade/-His/-Leu/-Trp agar

VII. Control Experiments

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Use this procedure to perform a control mating before screening a two-hybrid library.

A. General Considerations

AH109 pretransformed with pGBKT7-53, and Y187 pretransformed with pTD1-1 or pGADT7-T, are provided with every Matchmaker Pretransformed Library as a positive control. To familiarize yourself with the procedures and expected results of a two-hybrid assay, use these strains to perform a control mating before you begin screening the library. Select for diploids and for two-hybrid interactors as described below.

- pGBKT7-53 encodes the Gal4 DNA-BD fused with murine p53; pGADT7-T and pTD1-1 each encode the Gal4 AD fused with SV40 large T-antigen. Since p53 and large T-antigen are known to interact in a yeast two-hybrid assay (Li & Fields, 1993; Iwabuchi et al, 1993), mating AH109 pretransformed with pGBKT7-53 to Y187 pretransformed with pGADT7-T (or pTD1-1) will result in diploid cells containing both plasmids. Thus, all Leu^+, Trp^+ diploids should also be Ade^+, His^+, $Mel1^+$ because all reporter genes will be transcriptionally active (Table V).
- A negative control can also be performed using pGBKT7 and pTD1-1 or pGADT7-T. Diploid yeast containing pGBKT7 and pGADT7-T (or pTD1-1) will grow on SD/-Leu, SD/-Trp and SD/-Leu/-Trp minimal media, but no colonies should grow on QDO (-Ade/-His/-Leu/-Trp).
- Table V indicates the selection media required for strains containing a DNA-BD vector, AD vector or both, as well as the selection for diploids expressing interacting proteins.

Table V: Mating the Pretransformed Control Strains

Mating Strain [plasmid]	Plate on SD Minimal Agar Medium	Selects for	MEL1 Phenotype
AH109[pGBKT7-53]	–Leu	pGADT7-T or pTD1-1	White
	–Trp	pGBKT7-53	White
x	–Leu/–Trp[1] (DDO)	Diploids containing pGBKT7-53 and pGADT7-T or pTD1-1	Blue
Y187[pGADT7 or pTD1-1]	–Ade/–His/–Leu/–Trp[2] (QDO)	Diploids expressing the *ADE2* and *HIS3* reporters	Blue

[1] Controls for mating efficiency.
[2] Selects for diploids expressing interacting proteins.

Protocol 7–10 days

B. Protocol: Control Mating Protocol

1. Materials:
   - SD-/Trp Agar plates (Appendix D)
   - SD-/Leu Agar plates (Appendix D)
   - SD/-Ade/-His/-Leu/-Trp/X-α-Gal agar plates (Appendix D)
   - 2xYPDA medium (Appendix D)
   - YPD liquid medium + 25% glycerol (Freezing Medium)
   - AH109[pGBKT7-53] (supplied)
   - Y187[pTD1-1 or pGADT7-T] (supplied)
   - Y187[pGBKT7]

NOTES:

- Use the Transformation Protocol, Section XII, to transform pGBKT7 into Y187.
- X-α-Gal is not the same as X-Gal.

VII. Control Experiments continued

2. Streak the provided control strains from the glycerol stocks on the selection media indicated below:
   - AH109[pGBKT7-53] SD/-Trp Agar
   - Y187[pTD1-1 or pGADT7-T] SD/-Leu Agar
   - Negative control: AH109[pGADT7] SD/-Trp Agar
3. Grow at 30°C for 3 days.

Note: If you wish, you may stop the experiment at this step and resume work later. The plates can be stored at 4°C in subdued lighting for up to one month.

4. Pick one 2–3 mm colony of each type for use with this small scale mating procedure and mate the following.
   - Positive Control: AH109[pGADT7-53] and Y187[pTD1-1 or pGADT7-T]
   - Negative Control: AH109[pGBKT7] and Y187[pGADT7]
5. Place both colonies in a single 1.5 ml centrifuge tube containing 500 µl of 2xYPDA and vortex to mix.
6. Incubate with shaking at 200 rpm at 30°C overnight [20-24 hr].
7. From the mated culture (0.5ml) spread 100 µl of 1/10, 1/100 and 1/1,000 dilutions on each of the following agar plates. Incubate plates (colony side down) at 30°C for 3–5 days.
   - SD/-Trp
   - SD/-Leu
   - SD/-Leu/-Trp (DDO)
   - SD/-Leu/-Trp/-Ade/-His/X-α-Gal (QDO + X-α-Gal):
8. Expected results after 3–5 days:

   Positive controls:
   - Similar number of colonies on DDO and QDO agar plates
   - Colonies on QDO + X-α-Gal are blue

Notes:
- For positive interactions, theoretically, the number of colonies should be the same on both media. DDO selects for both plasmids and QDO selects for the plasmids as well as for the interactions of the hybrid proteins encoded by them. However, a difference (approximately 10–20% lower on QDO) is usually observed.
- If you see no colonies on DDO, compare to colony counts on SD/–Trp and SD–/Leu single dropout media to determine if there was a problem with the bait or the prey cultures, respectively.

   Negative control: colonies on DDO, no colonies on QDO agar plates.
9. Pick healthy 2 mm colonies from DDO plates, restreak onto fresh DDO plates, and incubate at 30°C for 3–4 days.
   - Short-term storage (< 4 weeks): Seal with Parafilm and store at 4°C.
   - Long-term storage: Scoop a large healthy colony and fully resuspend in 500 µl of YPD + 25% glycerol. Store at –80°C.

Notes:
- These diploids are useful as reference strains for checking new batches of growth media, and for comparisons in future experiments.
- When reviving frozen stocks, remember to restreak onto DDO selective medium.

VIII. Cloning and Testing Bait for Autoactivation and Toxicity

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided to test your bait for autoactivation (Section C) and toxicity (Section D).

A. Generate a Bait Clone

Generate a *GAL4* DNA-BD fusion by cloning your gene of interest in frame with the *GAL4* DNA binding domain of pGBKT7 (see Appendix C and **www.clontech.com** for map).

For a simple cloning procedure, we recommend using one of Clontech's In-Fusion PCR Cloning Kits (see **www.clontech.com** for details).

- Alternatively, you may construct the fusion using standard restriction/ligation protocols (Sambrook and Russell, 2001).
- If you have already cloned your gene into a Creator donor vector, transfer your gene to our pLP-GBKT7 acceptor vector using Cre recombinase. Refer to the Creator DNA Cloning Kits User Manual (PT3460-1) for details.
- If you wish to find proteins that interact with a membrane-bound or secreted protein, it may be necessary to first modify the protein (van Aelst et al., 1993) or to use only selected domains as the bait (as in Kuo et al., 1997).
- In order to confirm that the fusion construct is in-frame, the fusion junction may be sequenced using T7 primer.

B. Detecting Bait Expression

If you wish to determine whether or not your bait is expressed well in yeast, both of the following antibodies will detect bait proteins in yeast containing pGBKT7-based bait plasmids (via Western blot). In order to make yeast protein extracts, we strongly recommend that you use one of the protocols supplied in the Yeast Protocols Handbook.

- GAL4 DNA-BD Monoclonal Antibody (Cat. No. 630403)
- c-Myc Monoclonal Antibody (Cat. No. 631206)

Protocol 4–6 days.

C. Protocol: Testing Your Bait for Autoactivation

As a first step for any two-hybrid screen, it is imperative to confirm that your bait does not autonomously activate the reporter genes in AH109, in the absence of a prey protein.

1. Materials:
   - pGBKT7 containing your gene of interest cloned in frame with the *GAL4* DNA-BD (pGBKT7).
   - Competent AH109 yeast cells (Section XII.A)
   - SD/-Trp/ X-α-Gal agar plates (Appendix D)
   - SD/-Ade/-His/-Trp/X-α-Gal agar plates (TDO, triple dropout; Appendix D)

NOTE: X-α-Gal is required, not X-Gal (Appendix D)

2. Transform 100 ng of your pGBKT7+Bait construct using the small-scale transformation protocol (Section XII.B).

NOTE: (POSITIVE CONTROL) For comparison, we recommend that you also plate the positive control (AH109/Y187 diploid with pGBKT7-53 and pGADT7-T or pTD1-1) on SD-/Ade/-His/-Leu/-Trp/X-α-Gal plates (Appendix D).

VIII. Cloning and Testing Bait for Autoactivation and Toxicity continued

3. Spread 100 µl of a 1/10 dilution and a 1/100 dilution of your transformation mixture onto separate:
   - SD/-Trp/ X-α-Gal plates
   - SD/-Ade/-His/-Trp/X-α-Gal plates
4. Expected results after 3–5 days:

Sample	Selective Agar Plate	Distinct 2 mm Colonies	Color
Bait autoactivation test	SD/-Trp/ X-α-Gal	Yes	White
Bait autoactivation test	SD/-Ade/-His/-Trp/X-α-Gal	No	N/A
Positive control (Section VII)	SD/-Ade/-His/-Trp/X-α-Gal	Yes	Blue

NOTE: See Section XIV (Troubleshooting) if your bait activates the reporters, i.e., if distinct blue colonies grow on SD/-Ade/-His/-Trp/x-α-gal.

Protocol 4–6 days.

D. Protocol: Testing Your Bait for Toxicity

You should demonstrate that your bait protein is not toxic when expressed in yeast. If your bait is toxic to the yeast cells, both solid and liquid cultures will grow more slowly.

If expression of your bait protein does have toxic effects, you may wish to switch to a vector (such as pGBT9) that has a lower level of expression.

NOTE: pGBT9 is supplied as a control vector in Clontech's Yeastmaker™ Transformation System 2 (Cat. No. 630439).

1. Materials:
   - AH109 competent cells (Section XII.A)
   - SD/-Trp agar plates (Appendix D)
   - SD/-Trp liquid medium (Appendix D)
2. Transform 100 ng of the following vectors using the small-scale transformation protocol (Section XII.B)
   - pGBKT7 (empty)
   - pGBKT7 + cloned bait gene
3. Spread 100 µl of 1/10 and 1/100 dilutions of your transformation mixtures onto SD/-Trp.
4. Grow at 30°C for 3–5 days:

NOTE: If your bait is toxic, you may notice that colonies containing your bait vector are significantly smaller than colonies containing the empty pGBKT7 vector.

IX. Two-Hybrid Library Screening Using Yeast Mating

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for performing a yeast two-hybrid library screening.

Matchmaker Pretransformed and Pretransformed Normalized Libraries are screened by a simple yeast mating procedure —not by transformation. Pretransformed libraries can either be purchased premade from Clontech (**www.clontech.com**) or can be made yourself using the Matchmaker 2 Hybrid Library Construction and Screening Kit (Cat. No. 630445).

- You will note that the mating procedure is very easy, you simply mix a concentrated bait culture with 1 ml of your pretransformed library and incubate overnight before plating on QDO selective media (see Figure 3).
- In addition to plating on QDO media, it is imperative that you determine the number of clones that you screened (as judged by cfu/ml on SD/-Leu/-Trp agar). Screening fewer than 1 million clones, particularly for a standard library that has not been normalized, may result in an inability to detect positive interactions.

With a normalized pretransformed Matchmaker library, up to threefold fewer clones need to be screened, because the representation has been equalized. Each individual transcript is more likely to be represented in a smaller number of independent clones.

1. Materials:
   - Pretransformed Library (supplied)
   - Bait construct transformed into AH109 on SD/-Trp (Section XII and Appendix D)
   - SD/-Trp liquid medium (Appendix D)
   - The following SD agar plates (Appendix D)
     - SD/-Trp 5–10 100 mm plates)
     - SD/-Leu (5–10 100 mm plates)
     - SD/-Leu/-Trp (5–10 100 mm plates)
     - SD/-Ade/-His/-Leu/-Trp/X-α-Gal (50–55 150 mm plates)
   - 2xYPDA liquid medium (Appendix D)
   - 0.5xYPDA liquid medium (Appendix D)
   - Kanamycin sulfate (50 mg/ml)
   - YPD + 25% glycerol liquid (freezing) medium

NOTE: For this procedure it does not matter which strain contains the bait or prey, but they must be transformed into strains of opposite mating types.

- AH109 = MATa
- Y187= MATα

Clontech's Pretransformed libraries are supplied in strain Y187, so your bait must be in strain AH109.

2. Construct your bait, test for auto-activation and toxicity (Section VIII).

IX. Two-Hybrid Library Screening Using Yeast Mating continued

3. Perform the control experiments (Section VII).

NOTE: Control experiments are strongly recommended; the control strains will aid interpretation of results when you screen your library.

4. Prepare a concentrated overnight culture of the bait strain (AH109 [pGBKT7+Bait]) as follows:
   a. Inoculate one fresh, large (2–3 mm) colony of your bait strain into 50 ml of SD/-Trp liquid medium.
   b. Incubate shaking (250–270 rpm) at 30°C until the OD_{600} reaches 0.8 (16–20 hr).
   c. Centrifuge to pellet the cells (1,000 g for 5 min), discard the supernatant.
   d. Resuspend the pellet to a cell density of >1x10^8 cells per ml in SD/-Trp (4–5 ml). [The cells can be counted using a hemocytometer.]
5. Combine the Library Strain with the Bait Strain as follows:
   a. Thaw a 1 ml aliquot of your library strain in a room temperature water bath. Remove 10 µl for titering on 100 mm SD/-Leu agar plates (see Appendix B, Section B for library titering instructions).

NOTE: Use a hemocytometer to count the cells. Your 1 ml aliquot should contain >2x10^7 cells. To check the titer, spread 100 µl of 1/100, 1/1,000, 1/10,000 dilutions on SD/-Leu agar plates. If your titer is 2x10^7 cells/ml you will have 200 colonies on the 1/10,000 dilution plate.

   b. Combine the 1 ml of Library Strain with the 5 ml Bait Strain in a sterile 2 L flask.
   c. Add 45 ml of 2xYPDA liquid medium (with 50 µg/ml kanamycin).
   d. Rinse cells from the library vial twice with 1 ml 2xYPDA and add to the 2 L flask.
6. Incubate at 30°C for 20–24 hr, slowly shaking (30–50 rpm).

NOTE: Use the lowest shaking speed possible that prevents the cells from settling on the base of the flask. Vigorous shaking can reduce the mating efficiency.

7. After 20 hr, check a drop of the culture under a phase contrast microscope (40X). If zygotes are present, continue to Step 8, if not, allow mating to continue, incubate for an additional 4 hr.

NOTE: A zygote typically has a 3-lobed structure, the lobes represent the two haploid parental cells and the budding diploid cell.

8. Centrifuge to pellet the cells (1,000 g for 10 min).
9. Meanwhile rinse the 2L flask twice with 50 ml 0.5xYPDA (with 50 µg/ml kanamycin), combine the rinses and use this to resuspend the pelleted cells.
10. Centrifuge to pellet the cells (1,000 g for 10 min) and discard the supernatant.
11. Resuspend all pelleteted cells in 10 ml of 0.5xYPDA/Kan liquid medium. Measure the total volume of cells + medium.

NOTE: e.g., 10 ml medium + 1.5 ml cells = 11.5 ml

IX. Two-Hybrid Library Screening Using Yeast Mating continued

12. From the mated culture spread 100 µl of 1/10, 1/100, 1/1,000, 1/10,000 dilutions on each of the following 100mm agar plates and incubate at 30°C for 3–5 days.

    - SD/-Trp
    - SD/-Leu
    - SD/-Leu/-Trp (DDO)

    NOTE: This step is essential to calculate the number of clones screened (see Step 14).

13. Plate the remainder of the culture, 200 µl per 150 mm on SD/-Ade/-His/-Leu/-Trp/X-α-Gal (QDO+X-α-Gal) agar plate (50–55 plates). Incubate at 30°C for 3–8 days.

NOTES:

- Using the highest stringency possible to detect activation of the three reporters *ADE2*, *HIS3* and *MEL1*, results in fewer false positives.
- Ignore small pale colonies that may appear after two days but never grow to > 1 mm in diameter. True Ade+, His+ colonies are robust and can grow to > 2 mm.

14. **Calculate the number of screened clones** (diploids) by counting the colonies from the SD/-Leu/-Trp (DDO) plates after 3–5 days.

    - Number of Screened Clones = cfu/ml of diploids x resuspension volume (ml)
    - It is imperative that at least 1 million diploids are screened, using less than this will result in less chance of detecting genuine interactions on QDO.

Example Calculation

- Resuspension volume (Step 11) = 11.5 ml
- Plating Volume = 100 µl
- 50 colonies grew on the 1/1,000 dilution on DDO plates.

Therefore Number of Clones screened = 50 x 11.5 x 10 x 1,000 = 5.75 million

IX. Two-Hybrid Library Screening Using Yeast Mating continued

15. **Determine the Mating Efficiency.**

Mating efficiencies of 2-5% are readily achieved using this procedure. If your mating efficiency is less than 2% and you cannot screen 1 million diploids (Step 14), refer to the Troubleshooting Guide (Section XIV) for tips on improving the mating efficiency, and screen more clones.

a. Measure viabilities

- No. of cfu/ml on SD/–Leu = viability of the Prey Library
- No. of cfu/ml on SD/–Trp = viability of Bait
- No. of cfu/ml on SD/–Leu/–Trp = viability of diploids

NOTE: The strain (bait or prey) with the lower viability is the "limiting partner."

b. Calculate Mating Efficiency (percentage of diploids):

$$\frac{\text{No. of cfu/ml of diploids}}{\text{No. of cfu/ml of limiting partner}} \times 100 = \%\ \text{Diploids}$$

Example Calculation

- Resuspension volume (Step 11) = 11.5 ml
- Plating Volume = 100 µl
- 5,000 colonies grew on the 1/10,000 on SD/-Trp
- 100 colonies grew on the 1/10,000 dilution on SD/-Leu
- 50 colonies grew on the 1/1,000 dilution on DDO plates

Therefore (in cfu/ml),

- Viability of Prey Library = 1×10^7 (
- Viability of Bait = 5×10^8
- Viability of Diploid = 5×10^5

Since the Prey Library is the limiting partner in this example, mating efficiency is calculated as follows:

$$\frac{5 \times 10^5}{1 \times 10^7} \times 100 = 5\%\ \text{Mating Efficiency}$$

16. All positive interactions must be further analyzed (Section XI) to identify duplicates and to verify that the interactions are genuine.

- It is recommended that you compare the Blue colonies that grow on QDO +X-α-Gal colonies to the positive controls (Section VII) to verify the strength of the interaction based on the intensity of the blue color.
- In particular, you need to confirm that the clones are not false positives. A false positive is a clone that can activate all the reporters even in the absence of your bait (i.e., when cotransformed with empty pGBKT7). The use of AH109 significantly reduces the incidence of false positives (Section XI.D), as does the use of a Pretransformed Normalized Library.

X. Analysis of Results

After a high-stringency screen to identify potential binding partners for your protein of interest, you may have very few positives, or too many positives to analyze. In these scenarios, we recommend checking the following:

A. Too Few Positives

Have you screened >1million independent clones? Refer to Section IX, Step 14 to determine if you screened 1 million independent clones. Optimize the mating/transformation procedure (see Section XIV. Troubleshooting Guide) and repeat the screening procedure.

- Check that your QDO growth media performs as expected with the positive and negative controls.
- If you screened >1 million independent clones and detected no positive colonies on high stringency QDO/X-α-Gal , repeat the screen with medium stringency plates:

  TDO/X-α-Gal (SD/-His/-Leu/-Trp/X-α-Gal).

- If you choose a lower stringency, you will also need to add and optimize the concentation of 3-AT (see Yeast Protocols Handbook).

B. Too Many Positives

Have you determined that your bait does not autoactivate the reporters (Section VIII.B)?

- Check that your QDO growth media performs as expected with the positive and negative controls.
- Your bait may interact with a partner that is abundant in the library. Sort duplicates by Yeast Colony PCR (Section XI.B). After the clones have been sorted into groups, a representative of each unique type can then be analyzed for false positive interactions (Section XI.D).
- Alternatively, you may wish to try a Matchmaker Pretransformed Normalized Library.

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for confirmation of phenotype (Section A), yeast colony PCR to eliminate duplicates (Section B), rescue and isolation of library plasmids responsible for activation of reporters (Section C), and distinguishing genuine positive from false positive interactions (Section D).

The following represents the recommended order of events to confirm that the positive interactions are genuine. The strategy is summarized in Figure 4. Note, however, that your preferred order of events may be somewhat determined by the number of positives obtained from your assay. For instance, if your bait protein interacts with a protein that is abundant in the library you may have a large number of potential positives to sort, many of which may be the same. In this case you may choose to perform colony PCR (Section XI.B) to sort the duplicate clones before segregating and rescuing the plasmid. If you have a low number of positive clones you may choose to omit the colony PCR screening step altogether.

We recommend performing the following steps prior to sequencing your positive clones:

- Confirmation of phenotype by restreaking
- Yeast Colony PCR
- Rescue and isolation of the library plasmid responsible for activation of reporters
- Distinguishing genuine positive from false positive interactions

Protocol 3–5 days

A. Confirmation of Phenotype by Restreaking

1. Materials:
   - Single colonies of yeast obtained from the library screen growing on QDO/X-α-Gal.
   - SD/-Ade/His/-Leu/-Trp/X-α-Gal agar plates (Appendix D)
2. Restreak positive clones to single colonies on QDO/+X-α-Gal plates (Appendix D).

   Always compare blue color to positive and negative control colonies grown on DDO/+X-α-Gal plates (Appendix D).
3. Expected results:

   Positive colonies will grow to single colonies and turn blue on QDO/-X-α-Gal upon restreaking after 2–4 days.

TIP: Be careful not to patch too many cells (Section XIII.A).

4. If you have many potential positives to test, continue to Section B for yeast colony PCR to eliminate duplicates. Otherwise, proceed to Section C for rescue and isolation of library plasmids responsible for activation of reporters.

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

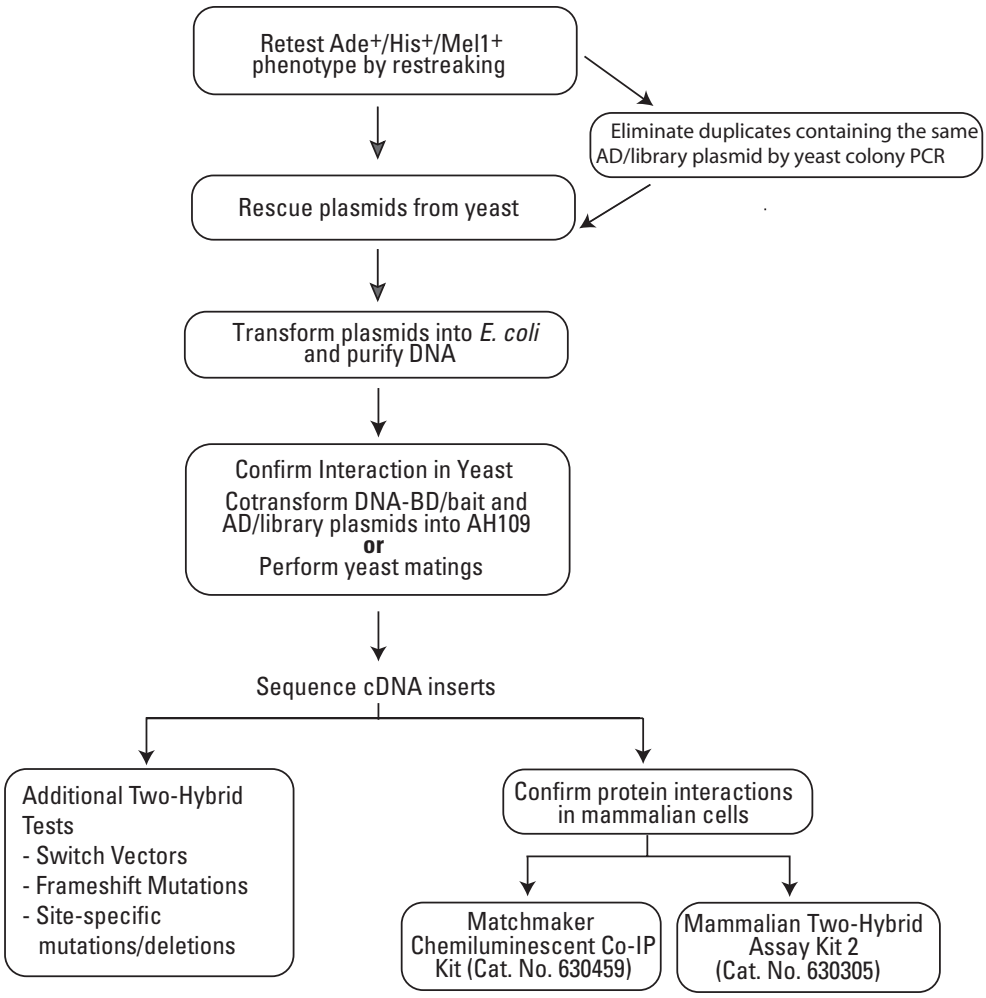

Figure 4. Strategies for analyzing and verifying putative positive interactions

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

Protocol
4 hr–1 day

B. Protocol: Yeast Colony PCR to Eliminate Duplicates

This procedure uses the Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No 630433) and Advantage 2 PCR Polymerase Mix (Cat. No. 639201). **We strongly 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.

1. Materials
   - Single colonies of yeast from two-hybrid screen, growing on QDO or QDO/X-α-Gal (Appendix D)
   - 0.8% TAE Agarose/EtBr gel
   - Master mix using Advantage 2 Polymerase, as described below.
   - LD insert, screening amplimer (Cat. No. 630433), Advantage 2 (Cat. No. 639201)
2. Prepare a PCR master mix by combining the components specified in Table VI.

Table VI: Recommended PCR Master Mixes for PCR Amplification of Library Vector Insert

Reagent	1 rxn	10 rxns + 1 extra	25 rxns + 1 extra
PCR-grade deionized H_2O	41 µl	451 µl	1,066 µl
10X Advantage 2 PCR Buffer	5 µl	55 µl	130 µl
5' AD LD Amplimer Primer (20 µM)	1 µl	11 µl	26 µl
3' AD LD Amplimer Primer (20 µM)	1 µl	11 µl	26 µl
50X dNTP Mix (10 mM each)	1 µl	11 µl	26 µl
50X Advantage 2 Polymerase Mix	1 µl	11 µl	26 µl
Total	50 µl	550 µl	1,300 µl

3. Prealiquot 50 µl of PCR mix into tubes or wells. Then, using a pipette tip, scrape a few cells from a colony into an individual tube or well and pipette up and down to mix the cells. Test 50 colonies.

Attention

TIP: Using too many cells can inhibit the PCR reaction. Simply touching the colony with the tip should provide a sufficient quantity of cells. If your PCR mix turns turbid, you may be using too many cells.

4. Follow the following PCR cycling parameters:
   - 94°C for 3 min
   - 25-30 cycles
     - 94°C for 30 sec
     - 68°C for 3 min
5. Analyze PCR products by electrophoresis on a 0.8% TAE Agarose/EtBr gel.
   - Load 5 µl per lane.
   - The presence of more than a single band is common, indicating the presence of more than one prey plasmid in a cell (see Section XI.C).

NOTE

NOTE: To confirm that similar sized bands contain the same insert, you may choose to digest the PCR product with AluI or HaeIII or other frequently cutting enzymes and electrophorese on a 2% agarose/EtBr gel.

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

6. 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.

   NOTE: Alternatively, eliminate the abundant clones by performing yeast colony hybridization on each master plate. Refer to the Yeast Protocols Handbook for this procedure (Section IX.A). Use a vector free oligonucleotide probe designed from the sequence of the most abundant insert.

7. At this stage, to quickly identify the clones, the PCR products (observed as a single band on gel) can be spin column-purified and sequenced using T7 primer.

Protocol 2–7 days

C. Protocol: Rescue and Isolation of Library Plasmid Responsible for Activation of Reporters

1. Segregation of Library Plasmid in Yeast

Transformed yeast cells (unlike transformed *E.coli* cells) can harbor more than one version of a related plasmid. This means that in addition to containing a prey vector that expresses a protein responsible for activating the reporters, it may also contain one or more prey plasmids that do not express an interacting protein.

- If you rescue the plasmid via *E.coli* transformation without first segregating the non-interacting prey, there is a chance that you will rescue a non-interacting prey plasmid.
- To increase the chance of rescuing the positive prey plasmid we recommend that you streak 2–3 times on DDO/X-α-Gal, each time picking a single blue colony for restreaking. After the first streaking you may see a mixture of blue and white colonies indicating segregation of positive interactors (blue) from non-interactors (white). After streaking one or two more times you should only see blue colonies. The plasmid should be rescued from one of these clones (see Step 2).

2. Rescuing the Library Plasmid from Yeast

The following methods are recommended for rescuing your plasmid from yeast:

- To identify the gene responsible for the positive interaction, rescue the plasmid from yeast cells grown on QDO using the Yeastmaker Yeast Plasmid Isolation Kit (Cat. No. 630441) or other suitable method. A procedure is also described in the Yeast Protocols Handbook.
- The nucleic acid rescued directly from yeast will be a mixture of Bait Plasmid, Prey Plasmid and Yeast Genomic DNA, so you will need to isolate the prey plasmid by transformation and selection in *E.coli,* followed by any standard plasmid preparation procedure (see Step 3).

3. Transformation of *E.coli* and Isolation of the Library Prey Plasmid

- **If your bait is cloned in pGBKT7** (which contains a kanamycin resistance gene), you can select for the prey plasmid simply by selection on LB plus 100 µg/ml ampicillin using any commonly used cloning strain of *E.coli* (e.g. DH5α, or Fusion-Blue™ from Clontech).
- **If your bait is cloned into an older Matchmaker bait vector** such as pGBT9, pAS2 or pAS2-1, both bait and prey vectors confer ampicillin resistance. To select for colonies that contain only the prey vector, you can transform KC8 *E.coli* cells (Cat. No. 630435) and plate on M9 minimal medium lacking leucine. KC8 cells have a defect in *leuB* that can be complemented by yeast Leu2 (see Yeast Protocols Handbool, Appendix C). Note that the same dropout supplements used for yeast SD medium can be used for M9 minimal medium e.g., the -Leu DO supplement (Cat. No. 630417).

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

Protocol 4–6 days

D. Protocol: Distinguishing Genuine Positive from False Positive Interactions

Yeast Strain AH109 contains four reporters under the control of three distinct *GAL4* UAS sequences. As a result of following the high stringency screening protocols described in this user manual, the incidence of false positives is reduced to a minimum compared to other systems. The incidence of false positives is further reduced with a Pretransfomed Normalized Library due to more equal representation of each transcript. However, with every two-hybrid screen there is a chance of detecting false positives and it is important to confirm that your interactions are genuine using the following criteria (see Figure 5):

- **Genuine Positive**: Both Bait and Prey are required to activate the Gal4-responsive reporters
- **False Positive**: Prey can activate the Gal4-responsive reporters in the absence of your bait.

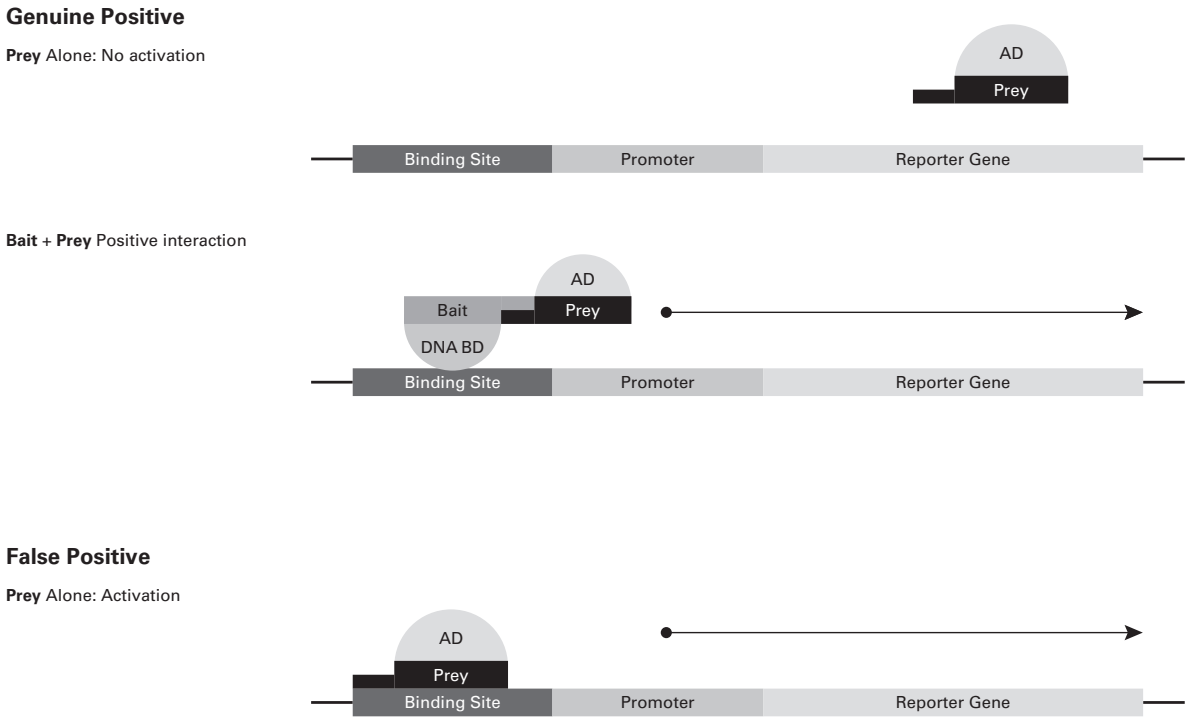

Figure 5. Illustration of the activation of reporter gene expression in genuine and false positives.

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

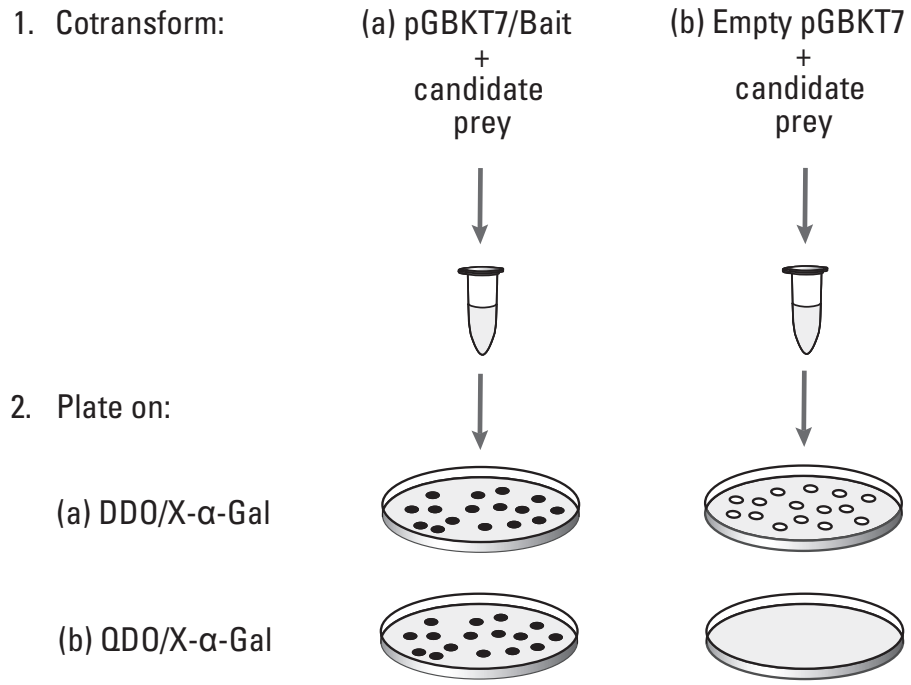

Figure 6. Using cotransformation on selective media to verify protein interactions. Expected results from genuine interactions.

You can confirm protein interactions in yeast on selective media (see Appendix D for recipes) using the following cotransformation procedure (Figure 6). This can also be done by yeast mating (see Section VII.B).

1. Materials:
   - Competent AH109 cells (Section XII.A)
   - SD/-Leu/-Trp/X-α-Gal Agar (Appendix D) DDO
   - SD/-Ade/-His/-Leu/-Trp/ X-α-Gal (Appendix D) QDO
2. Using the small-scale transformation procedure (Section XII.B), cotransform100 ng of each of the following pairs of vectors:
   - pGBKT7/Bait + Prey (in pGADT7-Rec or pGADT7-RecAB)
   - Empty pGBKT7 + Prey (in pGADT7-Rec or pGADT7-RecAB)

   NOTE: We recommend that you perform the experiment side by side with the positive and negative controls (Section VII).
3. Spread 100 µl of 1/10 and 1/100 dilutions of the transformation mix on the following plates:
   - DDO + X-α-Gal
   - QDO + X-α-Gal

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

4. Expected results after 3–5 days at 30°C:

a. Genuine Positive:

Sample	Selective Agar Plate	Distinct 2 mm Colonies	Color
Bait + candidate prey	DDO + X-α-Gal	Yes	Blue
	QDO + X-α-Gal	Yes	Blue
Empty pGBKT7 + candidate prey	DDO + X-α-Gal	Yes	White
	QDO + X-α-Gal	No	N/A

b. False Positive:

For false positive interactions, similar numbers of blue colonies are observed on all plates (indicating that the prey does not require your bait to activate the reporters).

Sample	Selective Agar Plate	Distinct 2 mm Colonies	Color
Bait + candidate prey	DDO + X-α-Gal	Yes	Blue
	QDO + X-α-Gal	Yes	Blue
Empty pGBKT7 + candidate prey	DDO + X-α-Gal	Yes	Blue
	QDO + X-α-Gal	Yes	Blue

NOTE: Theoretically, for positive interactions the number of colonies should be the same on both media: DDO selects for both plasmids, and QDO selects for the plasmids as well as for the interaction of the hybrid proteins encoded by them. However, a difference (approximately 10-20% lower on QDO) is usually observed.

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

E. Sequence Analysis of a Genuine Positive

Once an interaction has been verified as being genuine, the prey insert can be identified by sequencing. Use only DNA isolated from *E.coli* for this procedure. AD/library cDNA inserts can be sequenced using the following:

- Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433),
- T7 Sequencing Primer,
- or the 3' AD Sequencing Primer (provided with Matchmaker Two-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 in frame to the *GAL4* AD sequence, and compare the sequence to those in GenBank, EMBL, or other databases.

NOTES:

Before considering any of the following possibilities we recommend that you verify that your clone is not a false positive (Section XI.D).

- Most genuine positive clones will activate all reporters, however it is possible that some library clones only activate a selection of the reporters, for example the colony grows on QDO but does not turn blue in the presence of X-α-Gal. This may be due to inaccessibility of a particular prey fusion protein to a specific UAS. You can reduce the stringency of your screen to detect such positives (use TDO rather than QDO) but beware that lower stringency screening will also result in a greater incidence of false positives.
- Most library clones will contain some 3' untranslated region, be sure to scan the entire sequence to find any portion of coding region fused in-frame to the *GAL4* AD (see Appendix A, Section A).
- Yeast tolerate translational frameshifts. A large ORF in the wrong reading frame may correspond to the protein responsible for the interaction. To verify this, re-clone the insert in-frame (this can be easily done using Clontech's In-Fusion PCR Cloning System (see www.clontech.com) and determine if the *ADE2*, *HIS3*, and *MEL1* reporters are still active if your bait is also present.
- 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 non-translated gap comes between them. This is due 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).

XI. Confirmation of Positive Interactions & Rescue of the Prey Plasmid continued

F. Biochemical Methods to Confirm Positive Interactions

We recommend confirming positive interactions using the following methods:

- After sequencing the positive clones, most researchers choose to confirm each protein-protein interaction using independent, biochemical methods, such as affinity chromatography and/or immunoprecipitation (Fields & Sternglanz, 1994). For this type of analysis, we recommend the Matchmaker Chemiluminescent Co-IP Kit (Cat. No. 630459) which allows you to identify true physical protein interactions by a co-immunoprecipitation assay from mammalian cell extracts.
- You can also test protein-protein interactions in mammalian cells using either the Matchmaker Mammalian Two-Hybrid Assay Kit 2(Cat. No. 630305) or the pCMV-Myc & pCMV-HA Vector Set (Cat. No. 631604) The Vector Set includes c-Myc and HA-Tag antibodies for the isolation and identification of protein-protein complexes.

G. Downstream Analysis

You may wish to compare the strengths of two different interactions—for example, between a bait and two different prey proteins; or analyze the effects of point mutations on the strength of interaction, using the following methods:

- **Quantitative test for interactions**: The Gal-responsive *LacZ* gene (β-galactosidase) integrated in AH109 and Y187 is not secreted (in contrast to α-galactosidase encoded by *MEL1*) and it cannot be used for blue/white screening on agar plates. However, *LacZ* is an ideal reporter for quantitative studies of protein-protein interactions. We recommend the use of strain Y187 for such quantitative studies because the *LacZ* promoter in this strain expresses strongly (AH109/Y187 diploids can also be used). Quantitative *LacZ* assays are described in the Yeast Protocols Handbook.

- **Confirm interactions via immunoprecipitation assays**: Clontech offers the Matchmaker Chemiluminescent Co-IP System to perform simple immunoprecipitation assays.The Matchmaker Chemiluminescent Co-IP Vector Set (Cat. No. 630458) combines the bright fluorescence of our Living Colors AcGFPl tag with the sensitive chemiluminescent detection capability of our ProLabel technology. The vector set offers you two mammalian expression vectors—pAcGFP1-C and pProLabel-C—for generation of N-terminal-tagged AcGFP1-bait and ProLabel prey fusion proteins. After the AcGFP1-tagged bait and ProLabel-tagged prey fusion proteins are coexpressed in mammalian cells, use our Matchmaker Chemiluminescent Co-IP Kit (Cat. No. 630459) to perform immunoprecipitation.

XII. Yeast Transformation

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for preparation of competent yeast cells (Section A), transformation of competent yeast cells (Section B), and transformation plating & determination of efficiency (Section C).

The following protocol assumes that you are using Clontech's **Yeastmaker Yeast Transformation System 2** (Cat. No. 630439). When using the components from this kit, high transformation efficiencies of > $3x10^5$ are readily achieved.

A. Protocol: Preparation of Competent Yeast Cells

1. Materials:
   - Yeastmaker Yeast Transformation System 2 (Cat. No. 630439)
   - 1.1x TE/LiAc
   - YPDA agar plates (Appendix D)
   - YPDA liquid medium (Appendix D)
   - Appropriate SD selective medium
   - Frozen stock of AH109 cells
   - Sterile, deionized water

   NOTE: For 1.1x TE/LiAc, combine 1.1 ml of 10x TE with 1.1 ml of 1 M LiAc (10x). Bring the total volume to 10 ml using sterile deionized H_2O.

2. Streak a YPDA agar plate with AH109 cells from a frozen yeast stock. Incubate the plate upside down at 30°C until colonies appear (~3 days).

   NOTE: If you wish, you may stop the experiment at this step and resume work later. The plates can be stored at 4°C in subdued lighting for up to one month.

3. Inoculate one colony (diameter 2–3mm, < 4weeks old) into 3 ml YPDA medium in a sterile 15 ml culture tube.

TIP: Set up four separate 3 ml cultures from four separate colonies and choose only the fastest growing 3 ml culture to proceed. We find that faster growing cultures tend to result in higher transformation efficiencies.

4. Incubate at 30°C with shaking at 200 rpm for 8–12 hr.
5. Transfer 5 µl of the culture to a 250 ml flask containing 50 ml of YPDA.
6. Incubate shaking until the OD_{600} reaches 0.15-0.3 (16–20 hr).

   NOTE: Continue incubating until OD is reached. Do not over grow the culture.

7. Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 100 ml of fresh YPDA.

XII. Yeast Transformation continued

8. Incubate at 30°C until the OD_{600} reaches 0.4–0.5 (3–5 hr).

   NOTE: Continue incubating until OD is reached. Do not overgrow the culture.

9. Divide the culture into two 50 ml sterile Falcon conical tubes. Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend each pellet in 30 ml sterile, deionized H_20.
10. Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend each pellet in 1.5 ml of 1.1xTE/LiAc.
11. Transfer the cell suspensions to two respective 1.5 ml microcentrifuge tubes; centrifuge at high speed for 15 sec.
12. Discard the supernatant and resuspend each pellet in 600 µl of 1.1xTE/LiAc. The cells are now ready to be transformed with plasmid DNA.

    NOTE: For best results, competent cells should be used for transformation immediately, although they can be stored at room temperature for a few hours without siginificant loss in efficiency.

B. Protocol: Transformation of Competent Yeast Cells (Small-Scale)

Protocol
3 hr

The small-scale transformation volume is 50 µl.

1. Materials:
   - Yeastmaker Yeast Transformation System 2 (Cat. No. 630439)
   - Competent Yeast Cells (Section XII.A)
   - PEG/LiAc
   - 0.9% (w/v) NaCl
   - DMSO

   NOTE: For PEG/LiAc, combine 8 ml 50% PEG 3350, 1 ml 10xTE, and 1 ml 1 M LiAc (or 800 µl PEG, 100 µl 10xTE, and 100 µl 1 M LiAc).

2. Combine the following in a prechilled, sterile 1.5 ml microfuge tube:
   - 100 ng Plasmid DNA
   - 5 µl Herring Testes Carrier DNA (denatured; 10 µg/µl)

Note: To denature carrier DNA heat to 95–100°C for 5 min, then cool rapidly in an ice bath.

3. Add 50 µl competent cells and gently mix.
4. Add 0.5 ml PEG/LiAc and gently mix.
5. Incubate at 30°C for 30 min.

   NOTE: Mix cells every 10 min.

6. Add 20 µl DMSO and gently mix.
7. Incubate in a 42°C water bath for 15 min.

   NOTE: Mix cells every 5 min.

XII. Yeast Transformation continued

8. Centrifuge at high speed for 15 sec to pellet yeast cells, discard supernatant.
9. Resuspend cells in 1 ml YPD Plus liquid medium, incubate shaking for 90 min.

NOTE: YPD Plus is specially formulated to promote high transformation efficiencies and is available in the Yeastmaker Yeast Transformation System 2 (Cat. No. 630439). For small-scale procedures that do not necessarily require the highest transformation efficiencies, YPD liquid medium can be substituted for YPD Plus.

10. Centrifuge at high speed for 15 sec to pellet yeast cells, discard supernatant.
11. Resuspend cell pellet in 1 ml of 0.9% (w/v) NaCl

NOTE: This volume (1 ml) is the suspension volume (see Section C).

Protocol 3–5 days

C. Protocol: Plating and Determination of Transformation Efficiency

1. Spread 100 µl of 1/10 and 1/100 dilution onto a 100 mm plate containing the appropriate SD selection medium (Section VII.B, Table V). For example:
   - For pGBKT7, use SD/-Trp
   - For pGADT7, use SD/-Leu
   - For cotransformations, use SD/-Leu/-Trp

   NOTE: We generally do not recommend plating undiluted transformed cells for the reasons described in Section XIII.A.
2. Incubate plates upside down at 30°C until colonies appear (3–5 days).
3. Calculate transformation efficiency.

Example Calculation

$$\text{Transformation Efficiency} = \frac{\text{cfu x Suspension Volume (ml)}}{\text{Vol. plated (ml) x amount of DNA (µg)}}$$

(If 1/10 or 1/100 dilutions were plated, multiply by 10 and 100 respectively.)

After transformation using 100 ng of pGBT9 (control plasmid from Yeastmaker Yeast Transformation System 2), 100 µl of a 1/10 dilution was plated (from 1 ml total) and yielded 300 colonies after 3 days on SD–Trp.

$$\text{Transformation Efficiency} = \frac{300 \times 1}{0.1 \times 0.1} \times 10 \text{ (dilution factor)} = 3\text{x}10^5 \text{ cfu/ug}$$

NOTE: After transforming 100 ng of the pGBT9 control plasmid (supplied with Cat. No. 630439), and plating 100 µl of the 1/100 dilution, at least 30 colonies should grow after 3 days on SD/-Trp.

XIII. Tips on Plating, Patching & Streaking on Nutritional Selection Media

A. Problems with Patching or Streaking Too Many Cells

Selection in the Matchmaker Yeast Two-Hybrid Systems is based on nutritional selection, detecting growth of colonies on media lacking particular amino acids. This type of selection works most effectively when the amount of cells plated on a plate is controlled.

- Dense plating or patching can result in some growth by cells obtaining nutrients from surrounding dead cells, rather than from the medium. Plating more than the recommended volumes can give the illusion of growth.
- For many protocols described in this user manual you will note that we recommend that you perform serial dilutions prior to plating. If you plate undiluted transformations, for example, you may see a lawn of background growth after 1–2 days. It may be difficult to discern the transformed colonies appearing later over this background growth.
- Be particularly aware of this when verifying phenotypes after screening (see Section XI.A). If, for instance, you patch too many cells, even untransformed yeast may give the appearance of some growth on QDO selection medium.

B. Distinguishing Genuine Blue Colonies

Similarly, when confirming the *MEL1* phenotype using X-α-Gal containing medium, we recommend streaking or serial diluting to single colonies rather than patching.

- In an area where cells are concentrated even negative controls may turn a weak blue color on DDO/X-α-Gal (see Figure 7, Panel A) (typically a much fainter blue than the positive control). This is due to a high concentration of cells expressing residual levels of *MEL1*.
- Single colonies of negative control cells will not turn blue (see Figure 7, Panel A). If in doubt, compare all results to the control positive interaction (Figure 7, Panel B).

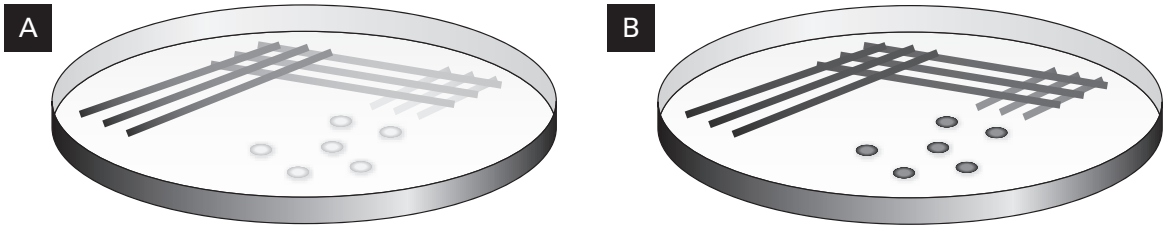

Figure 7. Positive and negative control phenotypes on SD/-Leu/-Trp/X-α-Gal agar plates. Diagrammatic representation of negative (**Panel A**) and positive (**Panel B**) control phenotypes when streaked to single colonies on DDO/X-α-Gal agar medium.

XIV. 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 et al., 1991; Ruden, 1992). 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.
Excessive background	• Improper media preparation	Remake SD/–Ade/–His/–Leu/–Trp/X-α–Gal medium.
	• 0.5X YPDA medium is too rich for the resuspension of transformed cells	Use water or TE.
Low mating efficiency	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 the culture, count the cells using a hemacytometer. The concentration should be ≥1 x 10^8 cells/ml, an ~100-fold excess over the pretransformed library cells.
	One or both of the hybrid proteins is toxic to yeast	You may be able to genetically engineer the hybrid protein in a way that will alleviate its toxicity but still allow the interaction to occur; or use a DNA-BD or AD vector that expresses lower levels of the fusion protein (e.g., pBridge or pGBT9).
	Bait protein is toxic to the 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 0.5X YPDA. Use the cell suspension in the normal mating procedure. • 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 controls that will allow you to compare the library mating efficiency with that of your bait strain mated to Y187[pGADT7-T or pTD1-1] and with that of Y187[pGADT7-T or pTD1-1] mated to AH109[pGBKT7-53]. • Bait proteins may interfere with mating if they are highly homologous to proteins involved in yeast mating (e.g., pheromone receptors). If sequence information on your bait protein is available, check it for homology to proteins known to be involved in yeast mating (Schultz et al., 1995; Pringle et al., 1997). In the rare case of homology to a pheromone receptor, it may be necessary to screen the library using a conventional library-scale yeast transformation.

Troubleshooting Guide continued

PROBLEM	CAUSE	SOLUTION
Failure to detect known protein interactions	• 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 et al. [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.
	• Some types of protein interactions may not be detectable in a GAL4-based system.	Try using a LexA-based two-hybrid system. .
	• Some protein interactions are not detectable using any type of two-hybrid assay.	
AD/library plasmid activates all three reporters independent of the DNA-BD/bait	A rare category of false positives in which an AD/library hybrid activates transcription inappropriately.	Refer to Section XI for methods to verify protein interactions; see Serebriiskii et al., 2000 and Bartel et al. (1993a) for further discussion of false positives.

XV. References

- For general reviews on yeast two-hybrid systems, see Allen *et al.,* 1995; Bartel et al., 1993a, 1993b; Bartel & Fields, (1997); Fields, 1993; Fields & Sternglanz, 1994; Fritz & Green, 1992; Guarente, 1993; Hopkin, 1996; Luban & Goff, 1995; McNabb & Guarente, 1996; Mendelsohn & Brent, 1994.
- An extensive list of Matchmaker System citations can be obtained from our website (**www.clontech.com**).
- For additional two-hybrid references, see the Golemis lab Web Site (http://www.fccc.edu:80/research/labs/golemis) or use MedLine (http://www.ncbi.nlm.nih.gov/PubMed/medline.html) and search under key words "two-hybrid."

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Appendix A: Pretransformed Yeast Library Construction

A. Library Priming

Matchmaker cDNA libraries are oligo(dT)-primed, or oligo(dT) + random-primed, as stated on the Certificate of Analysis (CofA); please refer to your CofA for the specific type of oligo(dT) primer used in the first-strand synthesis. The "lock-docking" oligo$(dT)_{25}$d(A/C/G) primer contains a degenerate nucleotide site that positions the primer at the junction of the poly-A tail and the transcript proper (Borson et al., 1992). This primer eliminates synthesis of lengthy poly(dT) regions and thereby enhances the representation of full-length clones and 3' ends in the library (Chenchik et al., 1994; Borson et al., 1992; Moqadam & Siebert, 1994). 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. For oligo(dT) + random-primed libraries, separate first-strand syntheses are performed with each type of primer; after second-strand synthesis (before ligation to the adaptor), the cDNAs are pooled in roughly equal proportions.

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.

B. Adaptors and Linkers

Please refer to the Product Certificate of Analysis (CofA) for information on the adaptor or linker used in the construction of your Matchmaker Library. pACT2-based libraries are made using adaptor ligation. pGADT7-Rec- and pGADT7-RecAB-based libraries are constructed using Clontech's SMART™ technology, as outlined in the Matchmaker Library Construction and Screening Kits User Manual (PT3529-1).

NOTES:

- The open reading frame of the insert starts at the codon immediately following the C-terminal codon (amino acid 881) of the GAL4 AD, not within the adaptor.
- If an EcoRI linker is used, the cDNA is methylated to protect any internal EcoRI 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/Sal I adaptors, you may excise the inserts from the vector using sites within the adaptor.
- For information about pGADT7-Rec, please refer to Vector Information Packet PT3530-5, supplied with all libraries constructed in this vector.

C. cDNA Size Fractionation

All ds cDNA is size-fractionated to remove unincorporated primers,unligated adaptors, and adaptor dimers; this process also removes low-molecular-weight (i.e., <400 bp) incomplete cDNAs. Matchmaker Libraries have a wide range of insert sizes (generally >400 bp), which may be an advantage in a two-hybrid library screening (Fritz & Green, 1992; Fields & Sternglanz, 1994). See the CofA for quality control information about insert size.

D. Normalization for Pretransformed Normalized Libraries

Please refer to the CofA for information on the normalization procedure used in the construction of the Pretransformed Normalized Matchmaker Libraries.

E. 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.

Appendix A: Pretransformed Yeast Library Construction continued

F. Library Amplification

Unless otherwise stated on the CofA, libraries constructed in pACT2 and pGADT7-RecAB were amplified once in *E. coli*. Libraries constructed in pGADT7-Rec are not amplified.

G. Presence of Genomic DNA or rRNA sequences in cDNA Libraries

The purified high quality poly A^+ RNA used to construct Matchmaker cDNA libraries at Clontech is not treated with DNase, due to potential degradation by contaminating RNase activity. Therefore, the poly A^+ preparation may have a small amount (<1%) of genomic DNA and <5% of rRNA.

H. Quality Control of the Original cDNA Library

- **cDNA size range and average cDNA size** are determined by running the cDNA on a gel prior to cloning and comparing the profile to MW size markers. The average insert size range is 0.5 kb to 4.0 kb.
- **Number of independent clones** is estimated before amplification. All libraries are guaranteed to have at least 1 x 10^6 independent clones and are representative of the mRNA population complexity. See Ausubel et al. (1994) for a general discussion of library size.

I. Quality Control for the Matchmaker Pretransformed & Pretransformed Normalized Libraries

Refer to the CofA included with your library.

Appendix B: Library Titering

A. General Considerations:

- Diluted libraries are always less stable than undiluted libraries. Therefore, once the library dilutions are made, plate them within the next hour, before misleading reductions in titer can 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 (i.e., kanamycin) in the medium to suppress growth of contaminating bacteria.

Protocol 4–6 days

B. Library Titering

1. Materials
   - YPDA/Kan (Appendix D)
   - SD/–Leu (100-mm plates) (Appendix D)
   - Sterile glass spreading rod, bent Pasteur pipette, or 5-mm glass beads for spreading culture on plates.
2. Transfer the 10-µl library aliquot (reserved from Section IX, Step 5) to 1 ml of YPDA/Kan in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution A** (dilution factor = 10^{-2}).
3. Remove 10 µl from Dilution A, and add it to 1 ml of YPDA/Kan in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution B** (dilution factor = 10^{-4}).
4. Add 10 µl from Dilution A to 50 µl of YPDA/Kan in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto an SD/–Leu plate.
5. Remove 50 µl aliquots from Dilution B and spread onto separate SD/-Leu plates as above.
6. Invert the plates and incubate at 30°C for 3–5 days.

   NOTE: Colony size will vary, depending on the insert.
7. Count the number of colonies on plates having 30–300 colonies.
8. Calculate the titer (cfu/ml) as follows:

$$\frac{\text{Number of colonies}}{\text{plating volume (ml)} \times \text{dilution factor}} = \text{cfu/ml}$$

NOTE: Due to slight variability in pipettes and pipetting techniques, a 2–5-fold range in titer calculations is not unusual.

- Example calculation:
- No. of colonies on plate = 100
- Plating volume = 0.05 ml
- Dilution factor = 10^{-4}

$$\frac{100}{0.05 \text{ ml} \times 10^{-4}} = 2 \times 10^{7} \text{ cfu/ml}$$

Appendix C: Control Plasmid Information

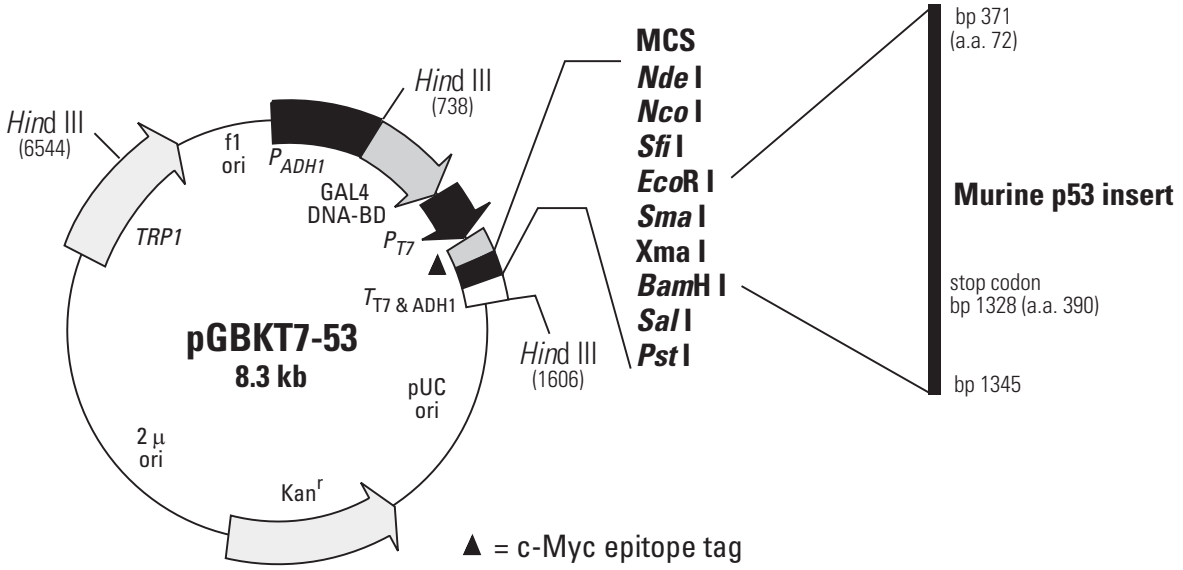

Figure 8. Map of pGBKT7-53 DNA-BD control plasmid. pGBKT7-53 is a positive control plasmid that encodes a fusion of the murine p53 protein (a.a. 72–390) and the GAL4 DNA-BD (a.a. 1–147). The murine p53 cDNA (GenBank Accession No. K01700) was cloned into pGBKT7 at the EcoR I and BamH I sites. The p53 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 C: Control Plasmid Information continued

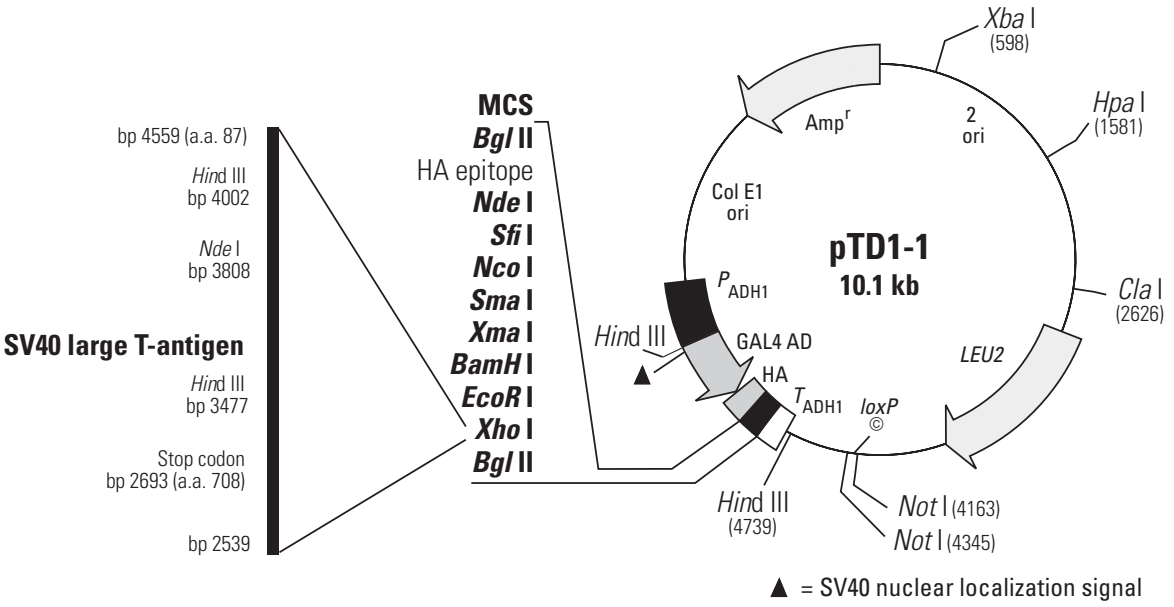

Figure 9. Map of pTD1-1 AD control plasmid. pTD1-1 is a positive control plasmid that encodes a fusion of the SV40 large T antigen (a.a. 87–708) and the GAL4 AD (a.a. 768–881). The SV40 large T antigen cDNA (GenBank Locus SV4CG) was cloned into pACT2 at the Xho I site (Li et al., 1994). The SV40 T antigen insert was derived from the plasmid referenced in Li & Fields (1993); plasmid modification was performed at Clontech. pTD1-1 has not been sequenced and it is not known whether any of the sites are unique.

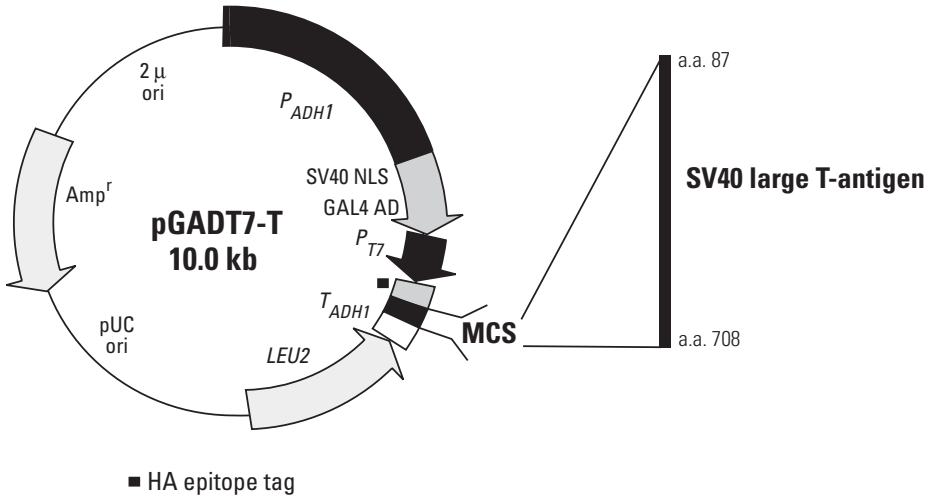

Figure 10. Map of pGADT7-T AD control plasmid. pGADT7-T is a positive control plasmid that encodes a fusion of the SV40 large T antigen (a.a. 87–708) and the GAL4 AD (a.a. 768–881). The SV40 large T antigen cDNA (GenBank Locus SV4CG), which was derived from the plasmid referenced in Li & Fields (1993), was cloned into pGADT7; plasmid modification was performed at Clontech. pGADT7-T has not been sequenced and it is not known whether any of the sites are unique.

Appendix D: Yeast Media Recipes

Agar-containing medium can be purchased separately from Clontech **(For cat. nos., see Section VI)**. Alternatively, you can add 18–20 g/L agar to media that lacks agar prior to autoclaving.

- Allow plates to harden at room temperature. Store plates in a plastic sleeve at 4°C.
- Prior to use, allow 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.
- Media should be autoclaved at 121°C for 15 min. Autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the media. Store liquid SD medium at 4°C,
- YPD and SD Base from Clontech contain Glucose.
- Ensure that the pH is adjusted appropriately; pH 5.8 for SD Minimal Media and pH 6.5 for YPD and YPDA.
- [**Optional**] To reduce the incidence of bacterial contamination the antibiotic kanamycin may be added to all media. Prepare and autoclave the media, once the medium has cooled to 55°C, add kanamycin to a final concentration of 50 µg/ml.
- To prepare YPD Agar Medium or minimal SD agar medium from YPD Medium or minimal SD base medium respectively, add agar (18–20 g/L) just prior to autoclaving.
- Continue spreading the inoculum over the agar surface until all visible liquid has been absorbed. This is essential for even growth of the colonies.

A. Addition of X–α-Gal (20 mg/ml in dimethyl formamide) to Agar Plates

Premixing into agar plates
• Prepare and autoclave 1 L of the appropriate droput agar medium
• Cool to 55°C
• Add 2 ml of X-α-Gal (20 mg/ml)
• Pour plates

Spreading onto premade agar plates
• Dilute X-α-Gal to 4 mg/ml in dimethyl formamide
• Spread 200 µl onto 15 cm plates, or 100 µl onto 10 cm plates using sterile glass beads

Appendix D: Yeast Media Recipes continued

B. Rich Media

YPDA Liquid (1 L)	
Reagent	**Amount**
YPD	50 g
L-Adenine Hemisulphate	15 ml of 0.2% stock solution
Deionized water	Up to 1 L
Adjust pH to 6.5 if necessary, then autoclave.	

YPDA Agar (1 L)	
Reagent	**Amount**
YPD agar	70 g
L-adenine hemisulphate	15 ml of 0.2% stock solution
Deionized water	Up to 1 L
Adjust pH to 6.5 if necessary, then autoclave.	

2X YPDA Liquid (1 L)	
Reagent	**Amount**
YPD	100 g
L-adenine hemisulphate	15 ml of 0.2% stock solution
Deionized water	Up to 1 L
Adjust pH to 6.5 if necessary, then autoclave.	

0.5X YPDA Liquid (1 L)	
Reagent	**Amount**
YPD	25 g
L-adenine hemisulphate	15 ml of 0.2% stock solution
Deionized water	Up to 1 L
Adjust pH to 6.5 if necessary, then autoclave.	

Appendix D: Yeast Media Recipes continued

C. Single Dropout Media

SD/-Trp Liquid (1 L)	
Reagent	**Amount**
Minimal SD Base	26.7 g
-Trp DO Supplement	0.74 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

SD/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-Trp DO supplement	0.74 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

SD/-Leu Liquid (1 L)	
Reagent	**Amount**
Minimal SD Base	26.7 g
-Leu DO Supplement	0.69 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

SD/-Leu Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-Leu DO supplement	0.69 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

Appendix D: Yeast Media Recipes continued

D. Double Dropout (DDO) Media

SD/-Leu/-Trp Liquid (1 L)	
Reagent	**Amount**
Minimal SD Base	26.7 g
-Leu/-Trp DO Supplement	0.64 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

SD/-Leu/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-Leu/-Trp DO Supplement	0.64 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

Appendix D: Yeast Media Recipes continued

E. Triple Dropout (TDO) Media

NOTE: Not all Triple Dropout supplements are available, but they can be easily made by adding back individual amino acids to quadruple dropout media. See below.

SD/-His/-Leu/-Trp Liquid(1 L)	
Reagent	**Amount**
Minimal SD Base	26.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

SD/-His/-Leu/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

SD/-Ade/-His/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-Ade/-His/-Leu/-Trp DO Supplement	0.60 g
L-Leucine	100 mg
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

Appendix D: Yeast Media Recipes continued

F. Quadruple Dropout (QDO) Media

SD/-Ade/-His/-Leu/-Trp Liquid (1 L)	
Reagent	**Amount**
Minimal SD Base	26.7 g
-Ade/-His/-Leu/-Trp DO Supplement	0.60 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

SD/-Ade/-His/-Leu/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-Ade/-His/-Leu/-Trp DO Supplement	0.60 g
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light.	

Notes

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