Takara Bio USA

Matchmaker® Gold Yeast Two-Hybrid System User Manual

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I. Introduction & Protocol Overview

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). In the **Matchmaker Gold Yeast Two-Hybrid System** (Cat. No. 630489), when bait and library (prey) fusion proteins interact, the DNA-BD and AD are brought into proximity to activate transcription of four independent reporter genes (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*) (Figure 1).

This technology can be used to:

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

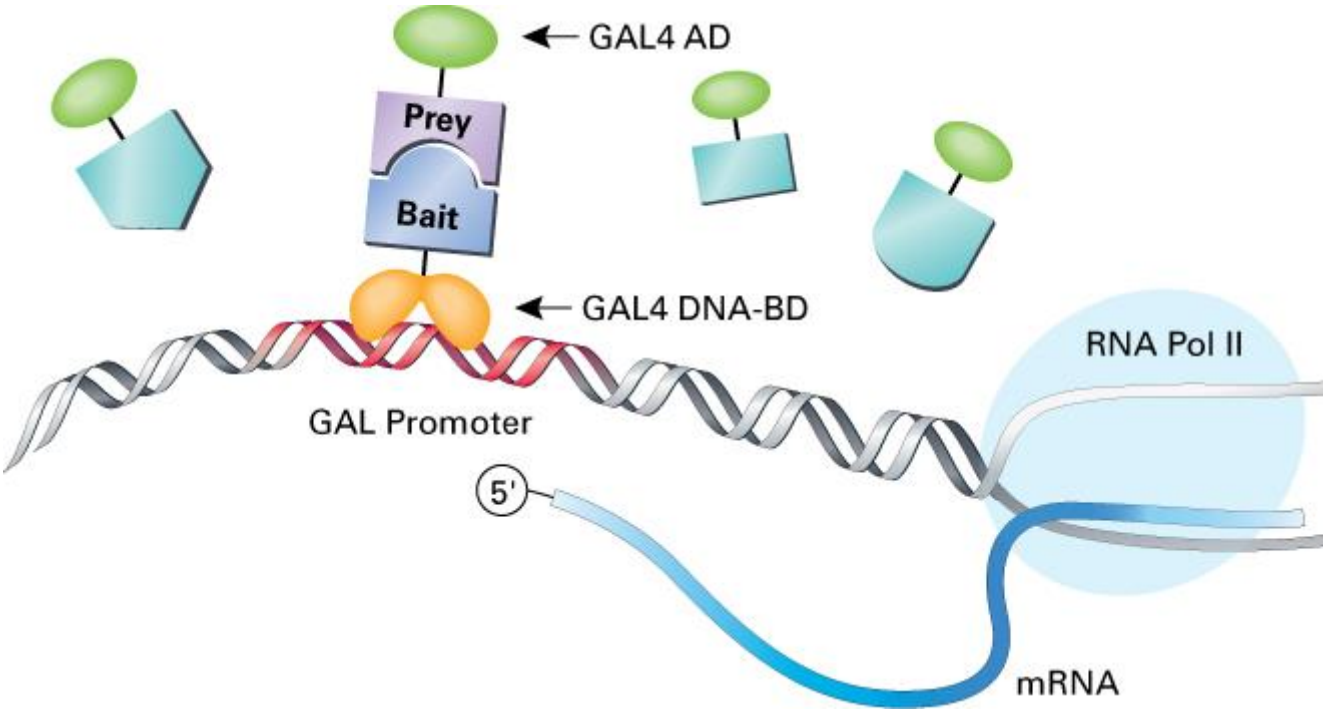

Figure 1. The two-hybrid principle. Two proteins are expressed separately, one (a bait protein) fused to the Gal4 DNA-binding domain (BD) and the other (a prey protein) fused to the Gal4 transcriptional activation domain (AD). In yeast strain Y2HGold, activation of the reporters (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*) only occurs in a cell that contains proteins which interact and bind to the Gal4-responsive promoter.

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 Matchmaker Gold Two-Hybrid System (Cat. No. 630489). 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

There are four integrated reporter genes under the control of three distinct Gal4-responsive promoters (Figure 2) in Takara Bio's Y2HGold Yeast Strain, which are used to detect two-hybrid interactions.

AUR1-C. A dominant mutant version of the *AUR1* gene that encodes the enzyme inositol phosphoryl ceramide synthase. *AUR1-C* is expressed in Y2HGold Yeast Strain in response to protein-protein interactions that bring the *GAL4* transcriptional activation and DNA binding domains into close proximity. In *Saccharomyces cerevisiae*, its expression confers strong resistance (*AbA*^{r}) to the otherwise highly toxic drug Aureobasidin A (Cat Nos. 630466 & 630499). **This drug reporter is preferable to nutritional reporters alone, due to lower background activity.** For example, the use of this reporter alone results in considerably less background than a histidine reporter alone.

HIS3. Y2HGold 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 –His minimal medium.

ADE2. Y2HGold 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 a-galactosidase, an enzyme occurring naturally in many yeast strains. As a result of two-hybrid interactions, a-galactosidase (*MEL1*) is expressed and secreted by the yeast cells. Yeast colonies that express Mel1 turn blue in the presence of the chromogenic substrate X-alpha-Gal (Cat. Nos. 630462 & 630462).

NOTE: X-alpha-Gal is not X-Gal and is not a substrate for β-galactosidase.

Three Different Binding Sites

Three promoters controlling the four reporter genes *AUR1-C*, *HIS3*, *ADE2*, and *MEL1* in Y2HGold 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.

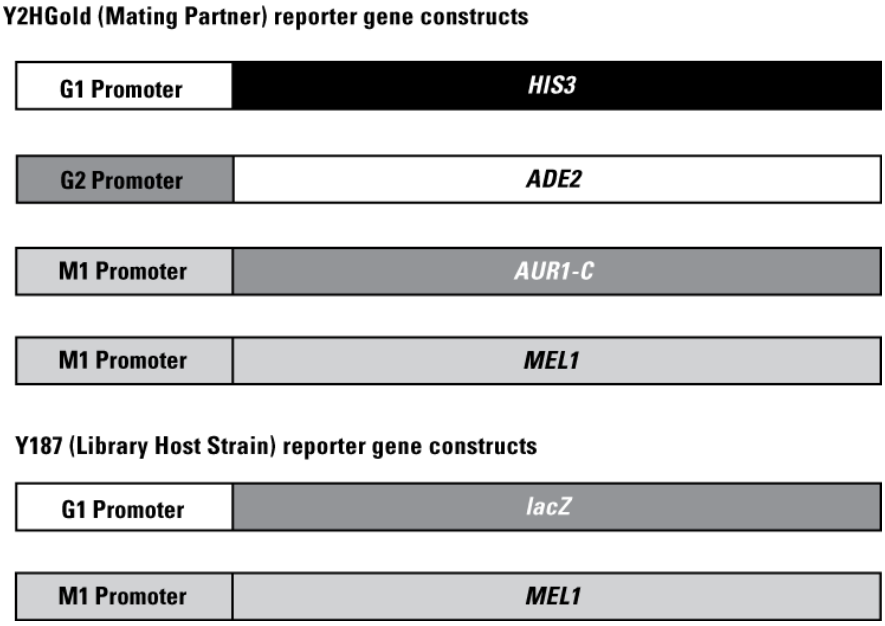

Figure 2. Reporter gene constructs in Matchmaker yeast strains. In Y2HGold, the *HIS3*, *ADE2*, and *MEL1*/*AUR1-C* reporter genes are under the control of three completely heterologous Gal4-responsive promoter elements—G1, G2, and M1, respectively. The protein-binding sites within the promoters are different, although each is related to the 17-mer consensus sequence recognized by Gal4 (Giniger et al. 1985; Giniger & Ptashne, 1988).

Matchmaker Screening Protocol Overview

The entire Matchmaker screening process (Figure 3) consists of the following steps:

- Step 1. Perform control experiments
- Step 2. Clone and test bait for autoactivation and toxicity
- Step 3. Screen Mate & Plate library
- Step 4. Confirm and interpret results

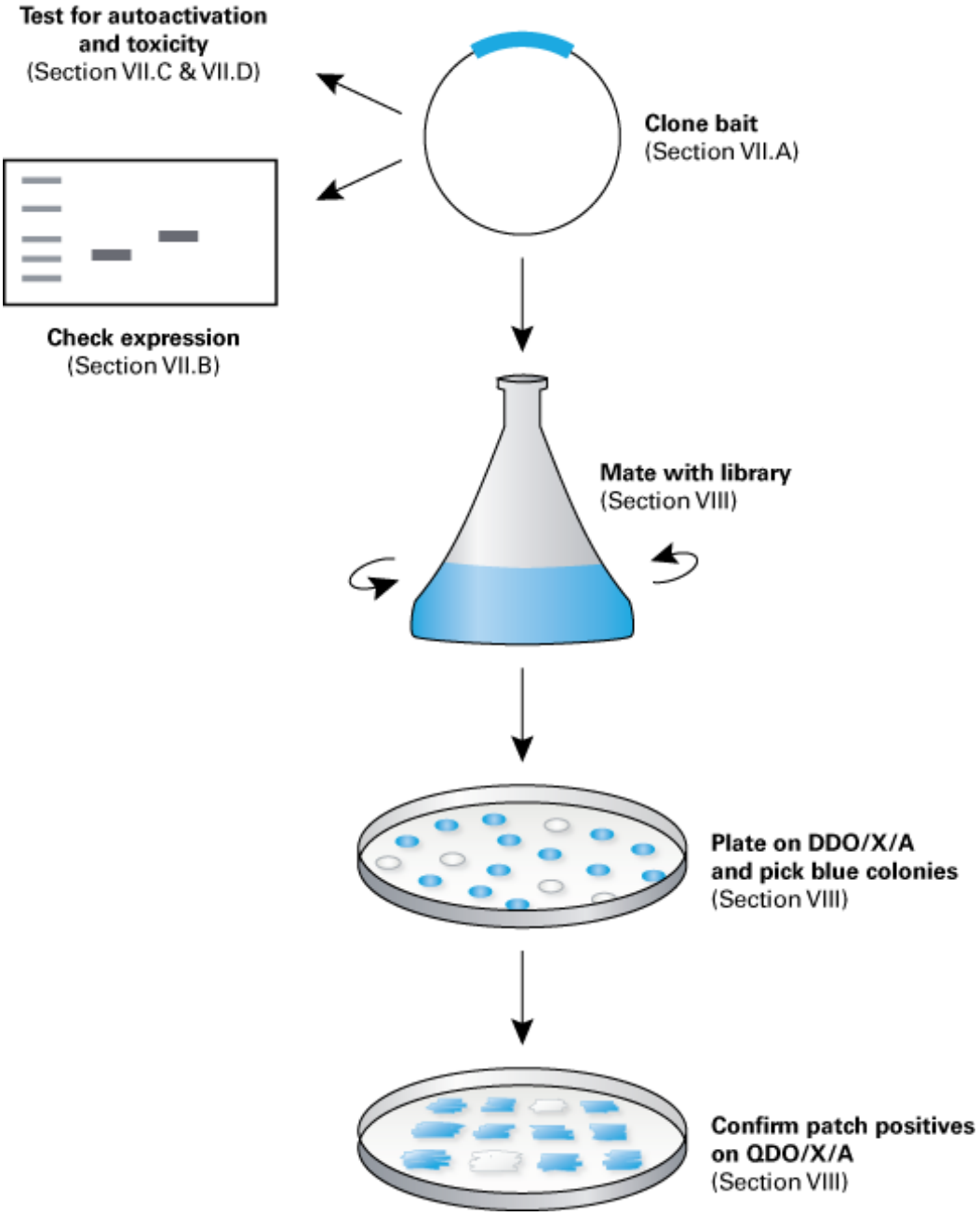

Figure 3. Two-hybrid screening using Mate & Plate Libraries. Your bait protein is expressed as a fusion with the Gal4 DNA-BD in yeast strain Y2HGold. The high-complexity library, which expresses fusions with the Gal4 AD, is provided in yeast strain Y187. When cultures of the two transformed strains are mixed together overnight, they mate to create diploids. Diploid cells contain four reporter genes: *HIS3*, *ADE2*, *MEL1*, and *AUR1-C*, that are activated in response to two-hybrid interactions.

II. List of Components

Matchmaker Gold Yeast Two-Hybrid System (Cat. No. 630489)

Store all Matchmaker vectors at –20°C, all yeast (*S. cerevisiae*) strains at –70°C, and all Yeast Media Pouches at room temperature.

For the Yeastmaker™ Yeast Transformation System 2, store carrier DNA and control plasmid at –20°C, and all other components at room temperature.

Matchmaker Vectors

- 50 µl pGBKT7 DNA-BD Cloning Vector (0.1 µg/µl)
- 50 µl pGADT7 AD Cloning Vector (0.1 µg/µl)
- 50 µl pGBKT7-53 Control Vector (0.1 µg/µl)
- 50 µl pGADT7-T Control Vector (0.1 µg/µl)
- 50 µl pGBKT7-Lam Control Vector (0.1 µg/µl)

Matchmaker Yeast Strains

- 0.5 ml Y2HGold Yeast Strain
- 0.5 ml Y187 Yeast Strain

Yeastmaker Yeast Transformation System 2 (also available separately as Cat No. 630439)

- 2 x 1 ml Yeastmaker Carrier DNA, denatured (10 mg/ml)
- 20 µl pGBT9 (0.1 µg/µl; positive control plasmid)

- 2 x 50 ml 50% PEG
- 50 ml 1 M LiAc (10X)
- 50 ml 10X TE Buffer
- 50 ml YPD Plus Liquid Medium

Complimentary Yeast Media Pouches

- 1 x 0.5 L YPDA
- 1 x 0.5 L YPDA with Agar
- 1 x 0.5 L SD–Leu with Agar
- 1 x 0.5 L SD–Trp with Agar

III. Additional Materials Required

The following reagents are required but not supplied.

A. Mate & Plate Libraries

(several catalog items; see **www.takarabio.com** for current list)

Store all Mate and Plate Libraries and Control vials at –70°C.

5 x 1.0 ml Library Aliquots (Universal Human and Mouse are also sold as 2 x 1 ml vials)

1 x 1.0 ml Mate & Plate Control

Alternatively, you can make your own Mate & Plate library using our Make Your Own "Mate & Plate" Library System (Cat. No. 630490).

B. Accessory Kits

- **Yeastmaker Yeast Transformation System 2** (supplied with your system—see Section II; also sold separately as Cat. No. 630439)
- **Easy Yeast Plasmid Isolation Kit** (50 preps; Cat. No. 630467)
- **Matchmaker Insert Check PCR Mix 2** (Cat. No. 630497; for characterizing the cDNA inserts of positive clones from your library screening.)

C. Tools for Plating Yeast

Tools for plating yeast include a sterile glass rod—and a bent Pasteur pipette or 5-mm glass beads for spreading cells on plates. (Use 5–7 beads per 100-mm plate, or 15–20 beads for a 150-cm plate).

D. Yeast Media

Table IV (in Appendix D) lists the components of the **Yeast Media Set 2** (Cat. No. 630494) and the **Yeast Media Set 2 Plus** (Cat. No. 630495). These media sets contain a complete assortment of mixes for preparing eight specialized broth and agar media, designed for use with the Matchmaker Gold Yeast Two-Hybrid System, in convenient, "ready-mixed" foil pouches. The Yeast Media Set 2 Plus also contains the additional media supplements **Aureobasidin A** and **X-α-Gal**, which are required for the protocols described in this user manual. Table V (in Appendix D) contains information for purchasing each of the media mixes separately, in packs of 10 pouches, and Table VI (in Appendix D) contains preparation instructions for all additional required media supplements and information for purchasing Aureobasidin A and X-α-Gal separately.

Additionally, the following should be considered when culturing yeast for a two-hybrid screen:

- See Appendix D for working and stock concentrations of Aureobasidin A and X-α-Gal.
- **SD medium** (synthetically defined medium) is minimal media that is routinely used for culturing *S. cerevisiae*. **SD base** supplies everything that a yeast cell needs to survive (including carbon and nitrogen sources). Essential amino acids, which are added to SD base to create minimal medium, are already included premixed in Takara Bio's **Yeast Media Pouches**. The particular minimal medium that is chosen will determine which plasmids and/or activated reporters are selected for.

- **SD/–Leu/–Trp** dropout supplement is used to select for the bait and prey plasmids. SD/–Leu/–Trp dropout is so called because the medium includes every essential amino acid except for leucine and tryptophan, which are omitted from the formulation (or "Dropped Out"). Cells harboring Matchmaker bait and prey plasmids are able to grow because the plasmids 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.**
- **SD/–Ade/–His/–Leu/–Trp** dropout supplement is used to select for the bait and prey plasmids, and in addition, for the activation of the Gal-responsive *HIS3* and *ADE2* genes as part of the confirmation step 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*. This medium is used at the end of the two-hybrid screen to confirm interactions.

IV. General Considerations Regarding Yeast Two-Hybrid Libraries

Use of Mate & Plate Libraries

Please note that the protocols described in this manual assume that you are using a **Mate & Plate Library**. These libraries utilize the natural ability of haploid yeast strains such as Y187 and Y2HGold to mate with each other to form a diploid cell, providing a very easy way to introduce an entire library (prey) to your bait.

- Takara Bio **strongly** recommends using **Mate & Plate Libraries** with the **Matchmaker Gold System**. These libraries provide by far the simplest method for yeast two-hybrid screening because no library-scale transformations or labor-intensive amplifications are needed. Thus, very little optimization and hands-on time are required.
- Several Mate & Plate libraries are available for purchase from Takara Bio, supplied as 5 x 1 ml vials (and also sold as 2 x 1 ml vials for Universal Human and Universal Mouse libraries). **Alternatively, you can easily "Make Your Own Mate & Plate Library"** using Cat No. 630490, and store enough 1 ml vials for more than 100 library screens.
- A single 1-ml Mate & Plate Library aliquot is sufficient for each complete library screening (>1 x 10^6 independent clones).
- See Appendix A for more details on prey vectors used to construct Mate & Plate Libraries. These libraries are supplied in *Saccharomyces cerevisiae* strain Y187, in Freezing Medium. Depending on which library you purchase, your library may be cloned into pGADT7-Rec2, pGADT7-RecAB, or pACT2. (See Certificate of Analysis for details.). All are compatible with Matchmaker Gold.
- A Mate & Plate Control is supplied with all Mate & Plate libraries. This control is Y187 Yeast Strain pretransformed with our pGADT7-T positive control plasmid, which expresses the Gal4 AD-SV40 large T-antigen fusion protein. See Section VI for control experiments.
- Once a library aliquot has been thawed, do not refreeze it. With every freeze/thaw cycle, there is a ~10% loss in viability, which can affect the quality of the library.
- The recommended Freezing Medium consists of YPDA broth + 25% glycerol (see Appendix D).

V. List of Abbreviations

AD fusion library [or AD library]	A cDNA library (such as a Mate & Plate 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

AbA	The antibiotic Aureobasidin A, which is toxic to yeast at low concentrations (0.1–0.5 µg/ml). It acts by inhibiting a yeast enzyme, inositol phosphoryl ceramide synthase.
AbA^r	Resistance to the antibiotic Aureobasidin A, conveyed by expression of the *AUR1-C* gene product
AUR1-C	A dominant mutant version of the *AUR1* gene, which encodes the enzyme inositol phosphoryl ceramide synthase. This gene is expressed in yeast strain Y2HGold, in response to protein-protein interactions that bring the *GAL4* activation and binding domains in proximity, thus conferring resistance to the antibiotic Aureobasidin A.
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 (beta-gal) activity
Mel1$^+$	Expresses the *MEL1* reporter gene; i.e., is positive for α-galactosidase (alpha-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
DDO	Double dropout medium: SD/–Leu/–Trp
DDO/X/A	Double dropout medium: SD/–Leu/–Trp supplemented with X-alpha-Gal and Aureobasidin A
TDO	Triple dropout medium: SD/–His/–Leu/–Trp or SD/–Ade/–Leu/–Trp
QDO	Quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp
QDO/X/A	Quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp supplemented with X-alpha-Gal and Aureobasidin A
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 hemisulfate (1X concentration = 120 µg/ml)

VI. 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

To familiarize yourself with the procedures and expected results of a two-hybrid assay, use these procedures to perform a control mating before you begin screening the library. Select for diploids and for two-hybrid protein-protein interactors as described below.

- pGBKT7-53 encodes the Gal4 DNA-BD fused with murine p53; pGADT7-T encodes 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 Y2HGold [pGBKT7-53] with Y187 [pGADT7-T] will result in diploid cells containing both plasmids that can activate all four reporters (Table I).
- A negative control should also be performed using pGBKT7-Lam (which encodes the Gal4 BD fused with lamin) and pGADT7-T. Diploid yeast containing pGBKT7-Lam and pGADT7-T will grow on SD/–Leu, SD/–Trp and SD/–Leu/–Trp (DDO) minimal media, but no colonies should grow on DDO + AbA.
- Table I 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 I. Mating the Pretransformed Control Strains

Mating Strain [plasmid]	Plate on SD Minimal Agar Medium	Selects for	*MEL1* Phenotype
Y2HGold [pGBKT7-53] x Y187[pGADT7-T]	–Leu	pGADT7-T	White
	–Trp	pGBKT7-53	White
	–Leu/–Trp[1] (DDO)	Diploids containing pGBKT7-53 and pGADT7-T	White
	–Leu/–Trp/X-alpha-Gal/AbA[2] (DDO/X/A)	Diploids that have also activated Aureobasidin A resistance and a-galactosidase through protein-protein interactions	Blue

[1]Controls for mating efficiency.

[2]Selects for diploids expressing interacting proteins.

B. Protocol: Control Mating Protocol

1. Materials:

    - SD/–Trp with Agar (see Appendix D)
    - SD/–Leu with Agar (see Appendix D)
    - SD/–Trp/X-alpha-Gal agar plates (see Appendix D)
    - SD/–Leu/–Trp agar plates (see Appendix D)
    - SD/–Leu/–Trp/X-alpha-Gal /AbA agar plates (see Appendix D)
    - 2 x YPDA Broth (see Appendix D)
    - YPDA Broth + 25% glycerol (Freezing Medium; see Appendix D)
    - Y2HGold Yeast Strain (Bait Strain)
    - Y187 Yeast Strain (Prey Strain)
    - pGBKT7-53 Positive Control Bait Plasmid
    - pGBKT7-Lam Negative Control Bait Plasmid
    - pGADT7-T Positive Control Prey Plasmid

    NOTES:
    - Use the Yeastmaker Yeast Transformation System 2 (supplied with this system) for all transformations.
    - X-alpha-Gal is not the same as X-Gal.

2. Use the Yeastmaker Yeast Transformation System 2 according to the small-scale protocol in the accompanying user manual to perform the following three transformations.

Strain	Transformation Plasmid	Plating Medium
Y2HGold	pGBKT7-53	SD/–Trp with Agar
Y2HGold	pGBKT7-Lam	SD/–Trp with Agar
Y187	pGADT7-T	SD/–Leu with 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 4ºC in subdued lighting for up to one month.

4. Pick one 2–3 mm colony of each type for use with the following small-scale mating procedure (Steps 5–7).

    - **Positive Control Mating:** Y2HGold [pGBKT7-53] and Y187 [pGADT7-T]
    - **Negative Control Mating:** Y2HGold [pGBKT7-Lam] and Y187 [pGADT7-T]

5. Place both colonies in a single 1.5-ml centrifuge tube containing 500 µl of 2X YPDA and vortex to mix.

6. Incubate with shaking at 200 rpm at 30°C overnight [20–24 hr].

7. From the mated culture (0.5 ml), spread 100 µl of 1/10, 1/100, and 1/1,000 dilutions on each of the following agar plates. Incubate plates (colony side facing downward) at 30°C for 3–5 days.

    - SD/–Trp
    - SD/–Leu
    - SD/–Leu/–Trp (=DDO)
    - SD/–Leu/–Trp/X-alpha-Gal/AbA (=DDO/X/A)

8. Expected results after 3–5 days:

    Positive control:

    - Similar number of colonies on DDO and DDO/X/A agar plates
    - Colonies on DDO/X/A are blue

    Negative control:

    - Colonies on DDO, but no colonies on DDO/X/A agar plates

    NOTES:

    - For positive interactions, theoretically, the number of colonies should be the same on both media. DDO selects for the presence of both plasmids (i.e., proper mated diploids) and DDO/X/A selects for the plasmids as well as for the interactions of the hybrid proteins encoded by them to activate the *AbA*^{r} and *MEL1* reporters. However, a difference (approximately 10–20% lower on DDO/X/A) 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.

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 YPDA Broth + 25% glycerol (Appendix D). 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.

VII. 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 the bait plasmid pGBKT7 (see Appendix C and **www.takarabio.com** for map).

For an extremely simple cloning procedure, we recommend using one of Takara Bio's In-Fusion® HD Cloning Plus Kits (see **www.takarabio.com** for details).

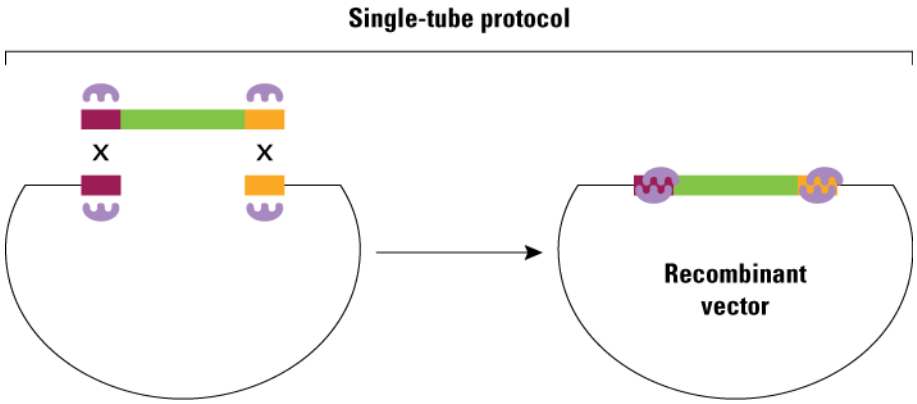

Figure 4. Simple, one-step PCR cloning with In-Fusion HD Cloning Plus Kits.

PCR Cloning of Your Bait into pGBKT7

The following method describes a simple and highly efficient method to clone your gene in-frame with the *GAL4* BD in pGBKT7. The magic of In-Fusion HD Cloning Plus (Cat. Nos. 638909, 638910, 638911 & 638920) means that:

- Your bait is automatically cloned in-frame with the bait.
- Virtually every clone contains your insert.
- It does not matter what sites are present on your bait sequence since for In-Fusion Cloning you do not digest it.

1. Digest pGBKT7 to completion with BamHI and EcoRI, then spin column-purify.
2. Amplify your bait insert by PCR using oligos that contain a 24-bp homology to your bait, and a 15-bp homology to the linear ends of pGBKT7, which are designed as follows:

   Forward Primer (111 = first codon of your bait)
   5'-C ATG GAG GCC GAATTC 111 222 333 444 555 666 777 888

   Reverse Primer (LLL = reverse complement of last codon of your bait)
   5'-GC AGGTCGACGGATCC LLL NNN NNN NNN NNN NNN NNN

   NOTE: These primers actually contain 16 bp of homology in order to keep the BamHI and EcoRI sites intact.
3. Mix the bait and linear pGBKT7 together and "fuse"' using the In-Fusion enzyme.

   See the In-Fusion HD Cloning Plus Kit User Manual at **www.takarabio.com** for additional details regarding PCR cloning procedures.

- 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 a standard 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 (yeast cannot simply be boiled or sonicated to extract protein), we strongly recommend that you use the supporting protocols provided at **www.takarabio.com**

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

NOTE: Use Y2HGold [pGBKT7-53] as a positive control that expresses a 57 kD protein.

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 Y2HGold, 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 Y2HGold cells [see Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1), supplied with this system]
   - SD/–Trp/X-alpha-Gal agar plates (Appendix D)
   - SD/–Trp/X-alpha-Gal/AbA agar plates (Appendix D)

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

2. Transform 100 ng of your pGBKT7+Bait construct using the Yeastmaker Yeast Transformation System 2 (supplied with this system).

   NOTE: (POSITIVE and NEGATIVE CONTROLS) For comparison, we recommend that you also plate the diploid controls that you created in Section VI; but plate them on **DDO/X/A** since they contain both bait and prey plasmids).

3. Spread 100 µl of a 1/10 dilution and a 1/100 dilution of your transformation mixture onto separate plates, as follows:
   - SD/–Trp plates = SDO
   - SD/–Trp/X-alpha-Gal = SDO/X plates
   - SD/–Trp/X-alpha-Gal/AbA = SDO/X/A plates

4. Expected results after 3–5 days:

Sample	Selective Agar Plate	Distinct 2-mm Colonies	Color
Bait autoactivation test	SDO	Yes	White
Bait autoactivation test	SDO/X	Yes	White or very pale blue
Bait autoactivation test	SDO/X/A	No	N/A
Positive control (Section VI)	DDO/X/A	Yes	Blue

NOTE: If your bait autoactivates the *AbA*^{r} reporter (i.e., blue colonies appear on SDO/X/A), check to see if it also activates the His3/Ade2 reporters by plating on SD–Trp/–His/–Ade. See Section XI (Troubleshooting) if your bait activates all the reporters.

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 transformation control in Takara Bio's Yeastmaker Transformation System 2 (supplied with this system).

1. Materials:
    - Y2HGold competent cells [see Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1), supplied with this system]
    - SD/–Trp agar plates (Appendix D)
    - SD/–Trp broth (Appendix D)
2. Transform 100 ng of the following vectors:
    - 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.

VIII. 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.

- Simply mix a concentrated bait culture with 1 ml of your Mate & Plate library and incubate overnight before plating on DDO/X/A selective media (see Figure 3).
- In addition to plating on DDO/X/A media, it is imperative that you determine the number of clones that you screened on DDO (following the protocol below ensures this). Screening fewer than 1 million clones may result in an inability to detect positive interactions.

With a "normalized" Mate & Plate library, up to threefold fewer clones need to be screened, because gene representation has been equalized, significantly reducing the abundance of housekeeping genes in the library. Several normalized libraries are available from **www.takarabio.com**.

1. Materials:
    - Mate & Plate Library—make your own, using our **Make Your Own "Mate & Plate" Library System** (Cat. No. 630490) or purchase separately.
    - Bait construct transformed into Y2HGold on SD/–Trp (Section VII)
    - SD/–Trp Broth (Appendix D)
    - 2X YPDA Broth (Appendix D)
    - 0.5X YPDA broth (Appendix D)
    - kanamycin sulfate (50 mg/ml)
    - YPDA + 25% glycerol [Freezing Medium] (Appendix D)
    - The following selective SD agar plates (also see Appendix D):

Agar Media	Acronym	Number of Plates
SD/–Trp	—	5–10 (100 mm plates)
SD/–Leu	—	5–10 (100 mm plates)
SD/–Leu/–Trp	DDO	5–10 (100 mm plates)
SD/–Leu/–Trp/X-alpha-Gal/AbA	DDO/X/A	50–55 (150 mm plates)
SD/–Ade/–His/–Leu/–Trp/X-alpha-Gal/AbA	QDO/X/A	5–10 (100 mm plates)

NOTE: Takara Bio's Mate & Plate libraries are supplied in yeast strain Y187, so your bait must be in yeast strain Y2HGold.

If you wish to "Make your Own Mate & Plate Library", use our kit (Cat. No. 630490)

(see **www.takarabio.com** for details)

2. Construct your bait, test for autoactivation and toxicity (Section VII).
3. Perform the control experiments (Section VI).

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 (Y2HGold [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 >1 x 10^8 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 library aliquot should contain >2 x 10^7 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 2 x 10^7 cells/ml, you will obtain 200 colonies on the 1/10,000 dilution plate.

    b. Combine 1 ml of your Mate & Plate Library with 4–5 ml Bait Strain (from Step 4) 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).

IMPORTANT: Use the lowest shaking speed possible that prevents the cells from settling at the base of the flask. Vigorous shaking can reduce the mating efficiency, but shaking too slowly will cause the cells to sediment, also lowering 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 (see Figure 5). The lobes represent the two haploid parental cells and the budding diploid cell. Some zygotes may resemble a clover leaf, while others may take on a shape similar to a “Mickey Mouse” face.

8. Centrifuge to pellet the cells (1,000 g for 10 min).

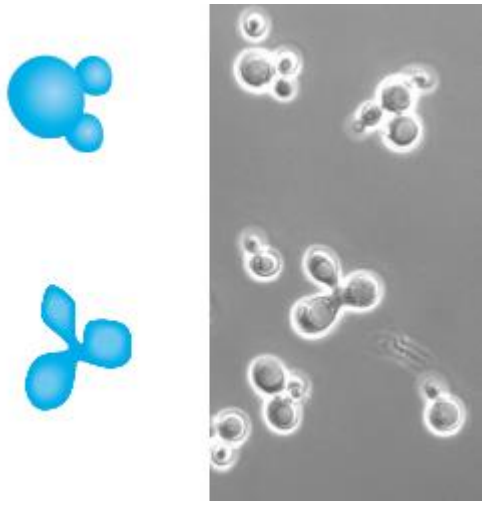

Figure 5. An example of a typical yeast zygote.

9. Meanwhile, rinse the 2-L flask twice with 50 ml 0.5X YPDA (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 pelleted cells in 10 ml of 0.5X YPDA/Kan liquid medium. Measure the total volume of cells + medium.

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

12. From the mated culture, spread 100 µl of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on each of the following 100-mm 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 DDO/X/A (50–55 plates). Incubate at 30°C for 3–5 days.
14. **Calculate the number of screened clones** (diploids) by counting the colonies from the 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, since using less than this will result in less chance of detecting genuine interactions on Aureobasidin A plates (DDO/X/A).

    ### 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

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 XI) 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. Patch out all the blue colonies that grew on DDO/X/A onto higher stringency QDO/X/A agar plates using a flat sterile toothpick or yellow pipette tip (Figure 6).

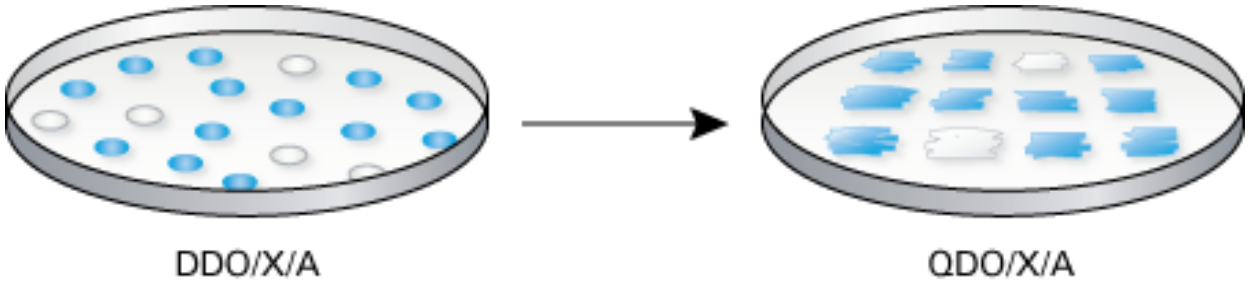

Figure 6. High stringency screening of potential interactors.

NOTE: Although it is possible to screen directly on high stringency QDO/X/A, Takara Bio recommends screening first on lower stringency DDO/X/A to detect as many positives interactors as possible before confirming those by patching on highest stringency plates.

17. All QDO/X/A positive interactions must be further analyzed (Section X) to identify duplicates and to verify that the interactions are genuine.

IX. Analysis of Results

After high-stringency patching 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 >1 million independent clones? Refer to Section VIII, Step 14 to determine if you screened 1 million independent clones? Optimize the mating/transformation procedure (see Section XI. Troubleshooting Guide) and repeat the screening procedure.

- Check that your DDO/X/A and QDO/X/A growth media performs as expected with the positive and negative controls. Very little AbA (125 ng/ml) is required, so make certain not to use too much.
- If you screened >1 million independent clones and detected no positive colonies on DDO/X/A, repeat the screen with a reduced concentration of Aureobasidin A (150 ng/ml instead of 200 ng/ml).

B. Too Many Positives

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

- Check that your DDO/X/A and QDO/X/A 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 X.A). After the clones have been sorted into groups, a representative of each unique type can then be analyzed for false positive interactions (Section X.C).
- Alternatively, you may wish to try a Normalized Mate & Plate Library; see **www.takarabio.com** for details.

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

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

The following represents the recommended order of events to confirm that the positive interactions are genuine. The strategy is summarized in Figure 7. 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 X.A)** 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 and proceed directly to the **Easy Yeast Plasmid Isolation Kit** (Cat. No. 630467).

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

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

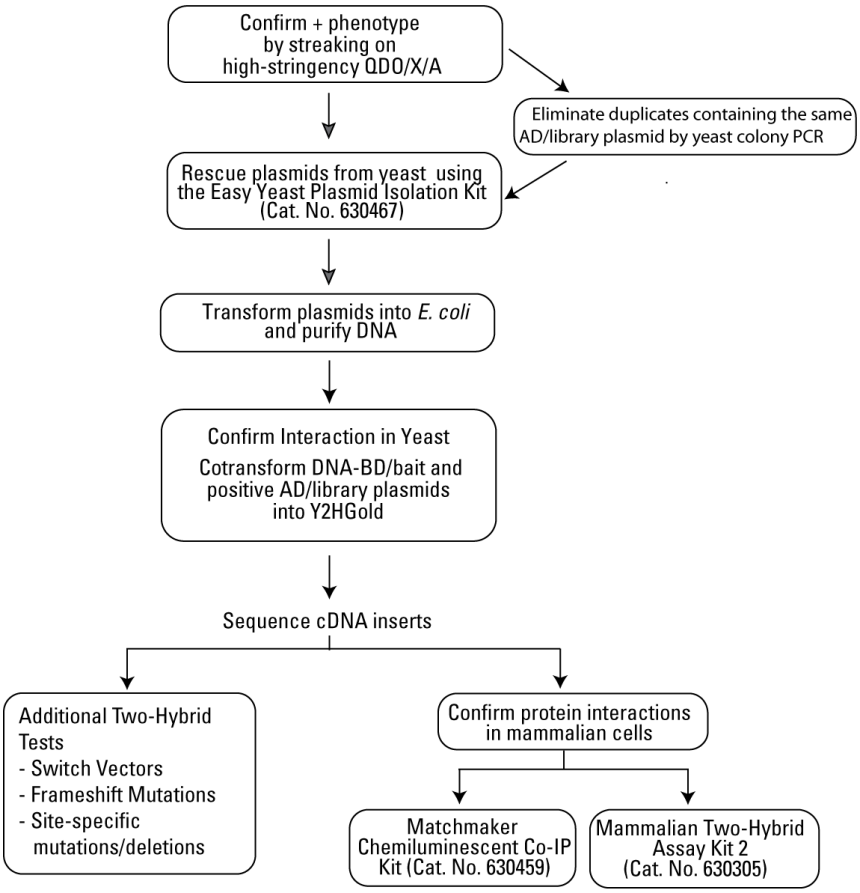

Figure 7. Strategies for analyzing and verifying putative positive interactions.

A. Protocol: Yeast Colony PCR Analysis to Eliminate Duplicate Clones

1. Use the **Matchmaker Insert Check PCR Mix 2** (Cat. No. 630497) to amplify your prey library inserts. The kit includes a premix of enzyme, reagents, and primers to amplify cDNA inserts from pGADT7 vectors. You can then characterize the inserts in Steps 2–4 using restriction enzyme analysis to identify potential duplicates clones. We strongly recommend this complete premix because we find that it performs very well in yeast colony samples.
2. Analyze PCR products by electrophoresis on a 0.8% TAE Agarose/EtBr gel. The presence of more than a single band is common, indicating the presence of more than one prey plasmid in a cell.

   NOTE: To confirm that similar sized bands contain the same insert, digest the PCR product with AluI or HaeIII or another frequently cutting enzyme, and analyze the products on a 2% agarose/EtBr gel.
3. 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.
4. 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.

B. 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, a yeast cell 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 (no Aureobasidin A), 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/X using the **Easy Yeast Plasmid Isolation Kit** (Cat. No. 630467)
- **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 Stellar™ Competent Cells (Cat. No. 636763).

C. Protocol: Distinguishing Genuine Positive from False Positive Interactions

Y2HGold Yeast Strain 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 Normalized Mate & Plate Libraries 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 8):

- **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.

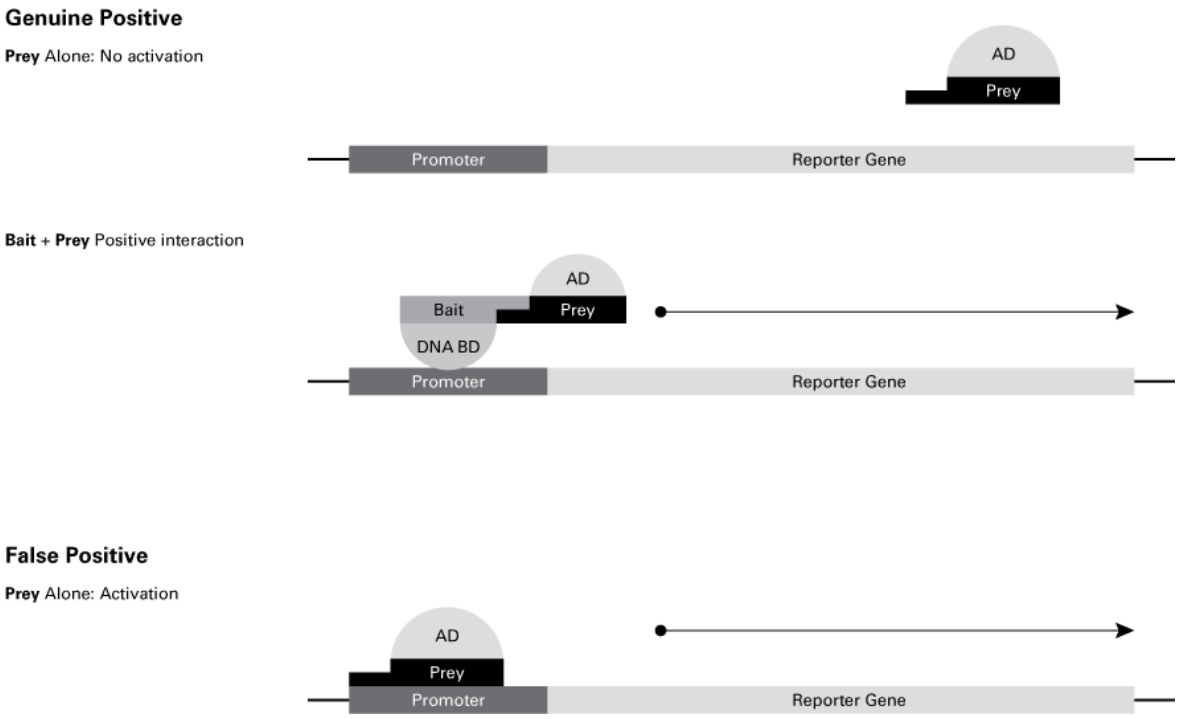

Figure 8. Illustration of the activation of reporter gene expression in genuine and false positives.

You can confirm protein interactions in yeast on selective media (see Appendix D for recipes) using the following cotransformation procedure (Figure 9). This can also be done by yeast mating (see Section VIII).

1. Materials:
    - Competent Y2HGold cells [see Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1), supplied with this system]
    - SD/-Leu/-Trp/X-alpha-Gal Agar (Appendix D) = DDO/X
    - SD/-Ade/-His/-Leu/-Trp/X-alpha-Gal/AbA (Appendix D) = QDO/X/A
2. Using the small-scale transformation procedure, cotransform100 ng of each of the following pairs of vectors into Y2HGold Competent Cells:
    - pGBKT7/Bait + Prey (in pGADT7, pGADT7-Rec, or pGADT7-RecAB)
    - Empty pGBKT7 + Prey (in pGADT7, pGADT7-Rec, or pGADT7-RecAB)

NOTE: We recommend that you perform the experiment side by side with the positive and negative controls (Section VI).

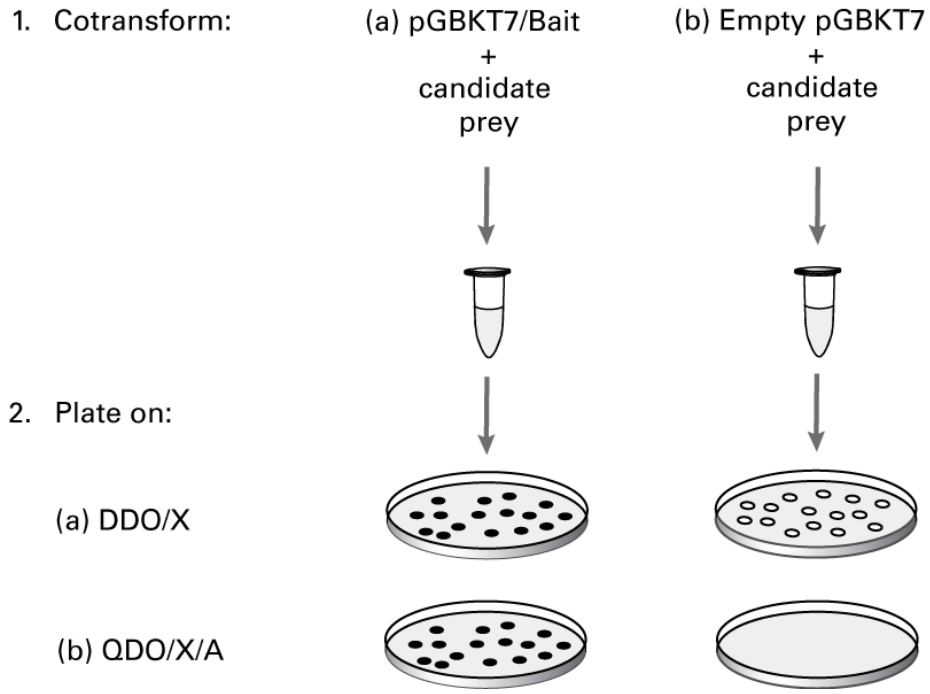

Figure 9. Using cotransformation on selective media to verify protein interactions. Expected results from genuine interactions.

3. Spread 100 µl of 1/10 and 1/100 dilutions of the transformation mix on the following plates:
   - DDO/X
   - QDO/X/A

4. Expected results after 3–5 days at 30°C (Figure 9):

   a. Genuine Positive:

Sample	Selective Agar Plate	Distinct 2-mm Colonies	Color
Bait + candidate prey	DDO/X	Yes	Blue
	QDO/X/A	Yes	Blue
Empty pGBKT7 + candidate prey	DDO/X	Yes	White
	QDO/X/A	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	Yes	Blue
	QDO/X/A	Yes	Blue
Empty pGBKT7 + candidate prey	DDO/X	Yes	Blue
	QDO/X/A	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/X/A selects for the plasmids as well as for the interaction of the hybrid proteins encoded by them. However, a difference is usually observed (10–60% lower on QDO/X/A, depending on the strength of the interaction).

D. 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

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 X.C).

- 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-alpha-Gal. This may be due to inaccessibility of a particular prey fusion protein to a specific UAS. Confirm the interaction by additional means such as coimmunoprecipitation.
- 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.
- 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 Takara Bio's In-Fusion PCR Cloning Systems (see **www.takarabio.com**) and determine if the *AbA*^{r}, *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 using **Gal4 AD Antibody** (Cat. No. 630402) 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 on the bait plasmid (Chien et al. 1991).

E. 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).
- 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.

F. 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 (beta-galactosidase) integrated in Y187 is not secreted (in contrast to alpha-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 yeast strain Y187 for such quantitative studies because the *LacZ* promoter in this strain expresses strongly (Y2HGold/Y187 diploids can also be used). Quantitative *LacZ* assays are described in supporting Matchmaker protocols at **www.takarabio.com**.

XI. Troubleshooting Guide

Problem	Possible Explanation	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/–Leu/–Trp/X-alpha-Gal/AbA medium.
	• 0.5X YPDA medium is too rich for the resuspension of transformed cells	Use water, TE, or 0.9% NaCl.
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 hemocytometer. 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] and with that of Y187[pGADT7-T] mated to Y2HGold[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.

Problem	Possible Explanation	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 four reporters independent of the DNA-BD/bait	False positives. The AD/library hybrid activates transcription and binds because it recognizes the promoter regions of the reporters.	Refer to Section X for methods to verify protein interactions; see Serebriiskii et al. (2000) and Bartel et al. (1993a) for further discussion of false positives.

XII. 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.takarabio.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: Mate & Plate Library Construction

A. Host Strain Information

The phenotypes and complete genotypes of Y2HGold and Y187 (the library strain) are shown in Tables II and III. For additional information on the growth and maintenance of yeast, see the supporting Matchmaker protocols at **www.takarabio.com**. We also recommend the Guide to Yeast Genetics and Molecular Biology (Guthrie & Fink, 1991).

Table II. Yeast Host Strain Genotypes

Strain	Genotype[a]	Reporters	Transformation Markers	Reference
Y2HGold[b, d]	*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$_{TATA}$ AUR1-C MEL1*	*AbA^r, HIS3, ADE2, MEL1*	*trp1, leu2*	Nguyen, 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] Y2HGold 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*, Y2HGold 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.

[d] The *AUR1-C* reporter construct was integrated into the yeast genome by homologous recombination at the ura3-52 mutation (Y. Nguyen, unpublished). Recombinants were selected on SD/–Ura. The met– phenotype in this strain is unstable.

Table III. Phenotype Testing on Various SD Media

Strain	SD/–Ade	SD/–His	SD/–Leu		SD/–Trp	SD/–Ura
Y2HGold	–	–	–		–	+
Y187	–	–	–		–	+
Y2HGold [pGBKT7-53]	–	–	–		+	+
Y187 [pGADT7-T]	–	–	+		–	+
Control Diploid[1]	+	+	+		+	+

[1] Diploid strain derived from mating Y2HGold [pGBKT7-53] with Y187 [pGADT7-T].

B. General Considerations for Mate & Plate Library Construction

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.takarabio.com**.

- **Libraries constructed in pGADT7-Rec**

  Mate & Plate Libraries cloned in pGADT7-Rec are constructed using our Make Your Own "Mate & Plate" Library System (Cat. No. 630490). pGADT7-Rec offers a simple and efficient method for constructing two-hybrid libraries via recombination-mediated cloning in vivo directly in *S. cerevisiae*. See the Make Your Own "Mate & Plate" Library System User Manual (PT4085-1) at **www.takarabio.com** for a description of the procedure.

- **Libraries constructed in pGADT7-RecAB**
  Normalized Mate & Plate 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**
  Mate & Plate Libraries that are cloned in pACT2 were 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.

C. 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)25d(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 (Borson et al. 1992). 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.

D. 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 Takara Bio’s SMART technology, as outlined in the user manual of the ”Make Your Own Mate & Plate Library System” (Cat. No. 630490)

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 EcoRI 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 EcoRI/NotI/SalI 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.

E. 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.

F. Normalized Mate & Plate Libraries

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

G. 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.

H. 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 since they are made directly in yeast.

I. 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.

J. Quality Control for the Mate & Plate 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.

B. Library Titering

1. Materials:
   - YPDA Broth (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 VIII) to 1 ml of YPDA Broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution A (dilution factor = 10^{-2}).

   NOTE: Matchmaker Gold Libraries are extremely viscous, so pay special attention during aliquoting.
3. Remove 10 µl from Dilution A and add it to 1 ml of YPDA Broth 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 Broth 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}

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

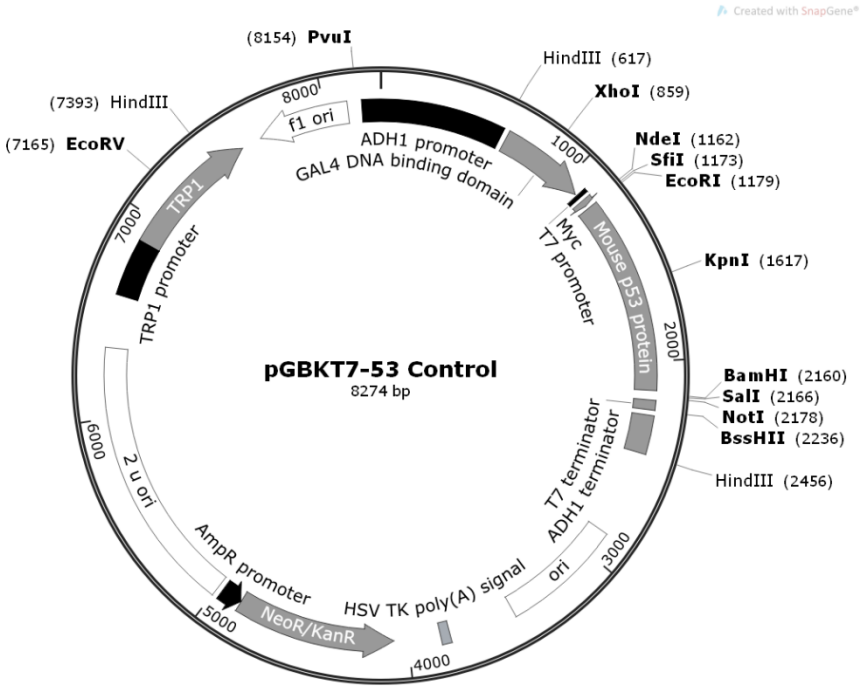

Figure 10. Map of pGBKT7-53 DNA-BD control plasmid. pGBKT7-53 is a positive control plasmid that encodes a fusion of the murine p53 protein (a.a. 72–390) and the *GAL4* DNA-BD (a.a. 1–147). The murine p53 cDNA (GenBank Accession No. K01700) was cloned into pGBKT7 at the EcoRI and BamHI sites. The p53 insert was derived from the plasmid described in Iwabuchi et al. (1993); plasmid modification was performed at Takara Bio. pGBKT7-53 has not been sequenced.

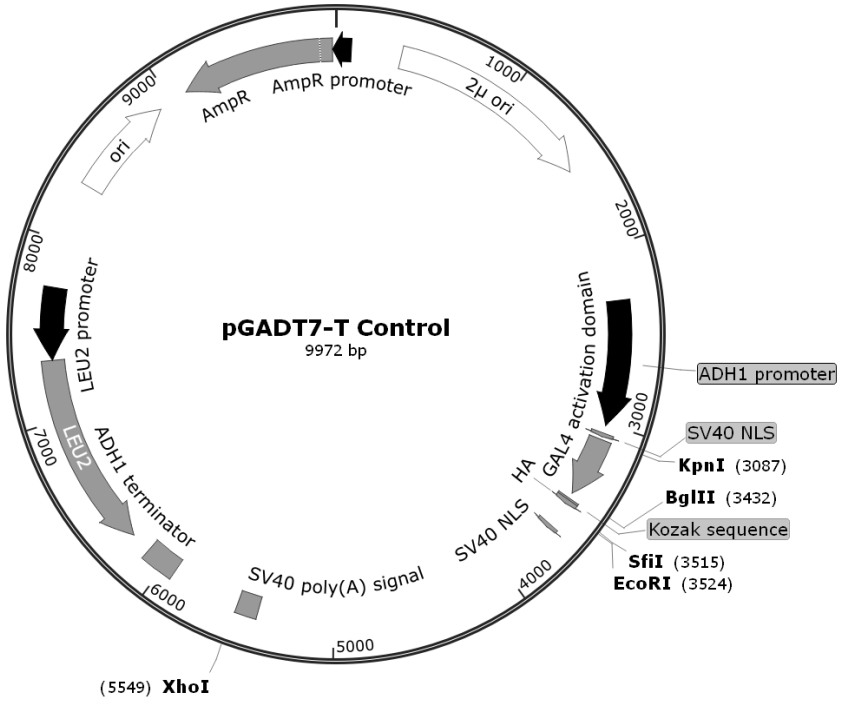

Figure 11. Map of pGADT7-T AD control plasmid. pGADT7-T is a positive control plasmid that encodes a fusion of the SV40 large T antigen (a.a. 87–708) and the GAL4 AD (a.a. 768–881). The SV40 large T antigen cDNA (GenBank Locus SV4CG), which was derived from the plasmid referenced in Li & Fields (1993), was cloned into pGADT7; plasmid modification was performed at Takara Bio. pGADT7-T has not been sequenced and it is not known whether any of the sites are unique.

Appendix D: Yeast Growth Media & Supplements

A. Ready-to-go Media Pouches Available from Takara Bio

Takara Bio offers media sets with a complete assortment of mixes in convenient, "ready-mixed" foil pouches, for use with the Matchmaker Gold Yeast Two-Hybrid System. We also provide the media supplements Aureobasidin A and X-alpha-Gal in an optional combination with the media set. In addition, each of the media mixes as well as these two supplements may be purchased separately.

- See Table IV for a list of the components of the **Yeast Media Set 2** (Cat. No. 630494) and the **Yeast Media Set 2 Plus** (Cat. No. 630495). The Yeast Media Set 2 Plus also contains the additional media supplements **Aureobasidin A** and **X-alpha-Gal**, which are required for the protocols described in this user manual.
- See Table V for information for purchasing each of the media mixes separately, in packs of 10 pouches.
- See Table VI for information on preparing all additional required media supplements and purchasing Aureobasidin A and X-alpha-Gal separately.

Table IV. Components of Yeast Media Set 2 & Yeast Media Set 2 Plus

Media Pouch	Quantity of Pouches Supplied	Volume of Media Each Pouch Makes
YPDA Broth	2	0.5 L
YPDA with Agar	1	0.5 L
SD/–Leu Broth	1	0.5 L
SD/–Leu with Agar	1	0.5 L
SD/–Trp Broth	1	0.5 L
SD/–Trp with Agar	1	0.5 L
SD/–Leu/–Trp with Agar	10	0.5 L
SD/–Ade/–His/–Leu/–Trp with Agar	1	0.5 L
Additional Components in Yeast Media Set 2 Plus		
X-alpha-Gal	250 mg	—
Aureobasidin A	1 mg	—

Table V. Individual Yeast Media Pouches for Matchmaker Gold Protocols

Yeast Media Pouches	Takara Bio Cat. No.	Quantity of Pouches Supplied	Volume of Media Each Pouch Makes
Rich Media (for Routine Culturing of Untransformed Yeast)			
YPDA Broth	630306	10	0.5 L
YPDA with Agar	630307	10	0.5 L
Minimal Media Single Dropouts (SDO)			
SD–Trp Broth	630308	10	0.5 L
SD–Trp with Agar	630309	10	0.5 L
SD–Leu Broth	630310	10	0.5 L
SD–Leu with Agar	630311	10	0.5 L
Minimal Media Double Dropouts (DDO)			
SD–Leu/–Trp Broth	630316	10	0.5 L
SD–Leu/–Trp with Agar	630317	10	0.5 L
Minimal Media Triple Dropouts (TDO)			
SD–His/–Leu/–Trp Broth	630318	10	0.5 L
SD–His/–Leu/–Trp with Agar	630319	10	0.5 L
Minimal Media Quadruple Dropouts (QDO)			
SD–Ade/–His/–Leu/–Trp Broth	630322	10	0.5 L
SD–Ade/–His/–Leu/–Trp with Agar	630323	10	0.5 L

Table VI. Additional Media & Media Supplements Required for a Two-Hybrid Screen

Freezing Medium	Preparation Instructions	
YPDA Medium & 25% glycerol	See Section E of Appendix D	
Supplement Name	**Takara Bio Cat. No. [1]**	**Stock Solution Concentration**
Aureobasidin A	630466 630499	500 µg/ml (see Section F of Appendix D)
X-alpha-Gal (250 mg)	630462 630463	20 mg/ml in dimethyl formamide
Kanamycin sulfate	—	50 mg/ml stock solution
Dimethyl formamide	—	—

[1]Unless otherwise specified.

B. General Media Preparation Instructions

- Prepare media by dissolving pouch contents in 500 ml ddH_2O, autoclave for 15 min at 121˚C, and allow to cool before use (or filter-sterilize broth media). Do not over-autoclave.
- This media does not usually require pH adjustment, but if your source water is particularly acidic, you may need to adjust the pH of the media to 5.8.
- For additional information on preparing media from the pouches, please see the Takara Bio Yeast Media Protocol-at-a-Glance (PT4057-2) at **www.takarabio.com**

C. 2X YPDA Broth

Reconstitute one YPDA Broth pouch in 250 ml ddH_2O and sterilize.

D. 0.5X YPDA Broth

Reconstitute one YPDA Broth pouch in 1 L ddH_2O and sterilize.

E. Freezing Medium

Mix 100 ml YPDA (sterile) and 50 ml 75% glycerol (sterile).

F. Aureobasidin A Stock Solution Recipe:

Dissolve 1 mg Aureobasidin A (Cat. No. 630466) in 2 ml of absolute ethanol for a stock concentration of 500 µg/ml. Store at 4°C.

G. Aureobasidin A Working Concentration

Add just 200 µl of the Aureobasidin A stock solution to 500 ml of dropout agar media for yeast two-hybrid screening, yielding a final concentration of 200 ng/ml.

H. X-alpha-Gal Stock Solution Recipe

Dissolve X-alpha-Gal (Cat. No. 630463) at 20 mg/ml in dimethylformamide (DMF). Store X-alpha-Gal solutions at –20°C in the dark.

I. X-alpha-Gal Working Concentration

Add 1 ml of the X-alpha-Gal stock solution to 500 ml dropout media for yeast two-hybrid screening.

J. SDO Agar Plates

Single dropout (–Trp) or (–Leu) media is prepared as follows:

- Prepare 500 ml agar media using SD/–Trp (or SD/–Leu) with Agar, autoclave, and cool to 55–60°C in a water bath.
- Mix, pour immediately, and allow to dry.

K. SDO/X Agar Plates

Single dropout (–Trp) media containing 40 µg/ml X-alpha-Gal is prepared as follows:

- Prepare 500 ml agar media using SD/–Trp with Agar, autoclave, and cool to 55–60°C in a water bath.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Mix, pour immediately, and allow to dry.

L. SDO/X/A Agar Plates

Single dropout (–Trp) media containing 40 µg/ml X-alpha-Gal and 200 ng/ml Aureobasidin A is prepared as follows:

- Prepare 500 ml agar media using SD/–Trp with Agar, autoclave, and cool to 55–60°C in a water bath.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Add 200 µl of Aureobasidin A stock solution.
- Mix, pour immediately, and allow to dry.

M. DDO Agar Plates

Double dropout media is prepared as follows:

- Prepare 500 ml agar media using SD–Leu/–Trp with Agar, autoclave, and cool to 55–60°C in a water bath.
- Mix, pour immediately, and allow to dry.

N. DDO/X Agar Plates

Double dropout media containing 40 µg/ml X-alpha-Gal is prepared as follows:

- Prepare 500 ml agar media using SD–Leu/–Trp with Agar, autoclave, and cool to 55–60°C in a water bath.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Mix, pour immediately, and allow to dry.

O. DDO/X/A Agar Plates

Double dropout media containing 40 µg/ml X-alpha-Gal and 200 ng/ml Aureobasidin A is prepared as follows:

- Prepare 500 ml agar media using SD–Leu/–Trp with Agar, autoclave, and cool to 55–60°C in a water bath.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Add 200 µl of Aureobasidin A stock solution.
- Mix, pour immediately, and allow to dry.

P. QDO/X Agar Plates

Quadruple dropout media containing 40 µg/ml X-alpha-Gal is prepared as follows:

- Prepare 500 ml agar media using SD/–Ade/–His/–Leu/–Trp with Agar, autoclave, and cool to 55–60°C in a waterbath.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Mix, pour immediately, and allow to dry.

Q. QDO/X/A Agar Plates

Quadruple dropout media containing 40 µg/ml X-alpha-Gal and 200 ng/ml Aureobasidin A is prepared as follows:

- Prepare 500 ml agar media using SD/–Ade/–His/–Leu/–Trp with Agar, autoclave, and cool to 55–60°C in a waterbath.
- Add 200 µl of Aureobasidin A stock solution.
- Add 1 ml of X-alpha-Gal Stock Solution.
- Mix, pour immediately, and allow to dry.

R. Kanamycin Supplement to Yeast Media

Kanamycin sulfate can be added to all yeast media at a final concentration of 50 µg/ml to stop bacterial contamination. Please note that kanamycin does not stop contaminating fungal growth, so proper sterile technique must still be used. Also note that this antibiotic does not select for any plasmids in yeast.

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