User Manual

Matchmaker™ Gold Yeast One-Hybrid Library Screening System User Manual

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I. Introduction & Protocol Overview

A. Introduction

The **Matchmaker Gold Yeast One-Hybrid Library Screening System** provides a simple and highly efficient method for constructing cDNA libraries for yeast one-hybrid screening. Matchmaker Gold Systems employ the strong selective power of **Aureobasidin A** resistance to produce screens with very low backgrounds. The one-hybrid assay derives from the original yeast two-hybrid system, but enables you to screen a library to identify and characterize proteins that bind to a target, *cis*-acting DNA sequence (Figure 1), instead of screening for protein-protein interactions (Fields & Song, 1989).

Your one-hybrid cDNA library is simultaneously constructed and screened directly in yeast as a result of *in vivo* plasmid recombination. There is no need for labor-intensive library cloning, amplification and harvesting in *E. coli*. Library construction in the Matchmaker Gold System uses SMART™ cDNA synthesis technology, which allows you to create cDNA libraries from any tissue source, starting with as little as 100 ng of total RNA.

B. Principles of the Yeast One-Hybrid System—A Protein-DNA Interaction Assay

The Bait - The target DNA sequence, or bait sequence, is cloned into the pAbAi Vector as a single copy or tandem repeats. Your pBait-AbAi construct is then efficiently integrated into the genome of the Y1HGold yeast strain by homologous recombination to generate a bait-specific reporter strain (Figure 2).

The Prey - In a Matchmaker one-hybrid assay, potential DNA-binding proteins, or prey proteins, are expressed as fusion proteins containing the yeast GAL4 transcription activation domain (GAL4 AD). A prey library is constructed directly in yeast by cotransforming PCR-amplified SMART cDNA and the linear pGADT7-Rec Vector into the your Y1HGold bait reporter yeast strain (Figure 3).

Detecting Protein-DNA Interactions - Aureobasidin A (AbA) is a cyclic depsipeptide antibiotic which is toxic to yeast at low concentrations. Resistance to AbA is conferred by the AbA^r gene (*AUR-1C*) which is the reporter on the bait vector pAbAi. When a prey protein binds to your bait sequence, the GAL4 AD activates expression of AbA^r which allows the cells to grow on media containing the AbA antibiotic (Figure 1).

Matchmaker Gold One-hybrid technology can be used to:

- Identify novel protein-DNA interactions
- Confirm and characterize known and suspected DNA-protein interactions
- Define interacting protein domains and cognate DNA sequences

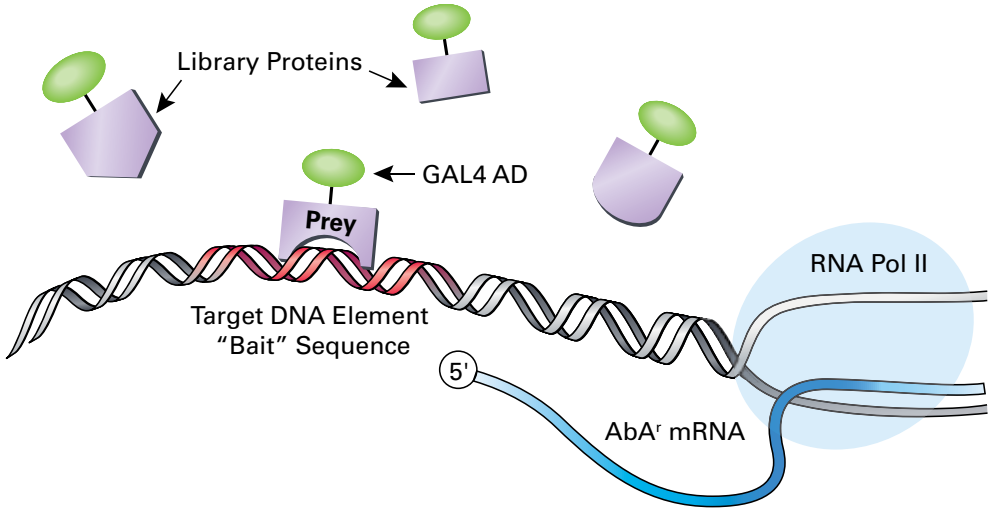


Figure 1. Screening for protein-DNA interactions with the Matchmaker Gold One-Hybrid System. One to three copies of the DNA target sequence are cloned into the pAbAi reporter vector which is then integrated into the Y1HGold genome to create a bait-specific reporter strain. Activation of the AbA resistance gene (AbA^r) occurs if a prey protein from the library binds to the bait sequence.

I. Introduction & Protocol Overview continued

Protocol Overview: The Matchmaker Gold One-Hybrid Library Screening process consists of the following steps:

- Step 1. Clone your target sequence (bait) into the pAbAi Vector
- Step 2. Create a bait/reporter strain by integrating the pBait-AbAi plasmid into the Y1HGold yeast genome (Figure 2)
- Step 3. Test the Y1HGold bait strain for background AbA^r expression.
- Step 4. Construct and screen a cDNA library by cotransformation and *in vivo* homologous recombination (Figure 3).
- Step 5. Confirm and interpret screening results

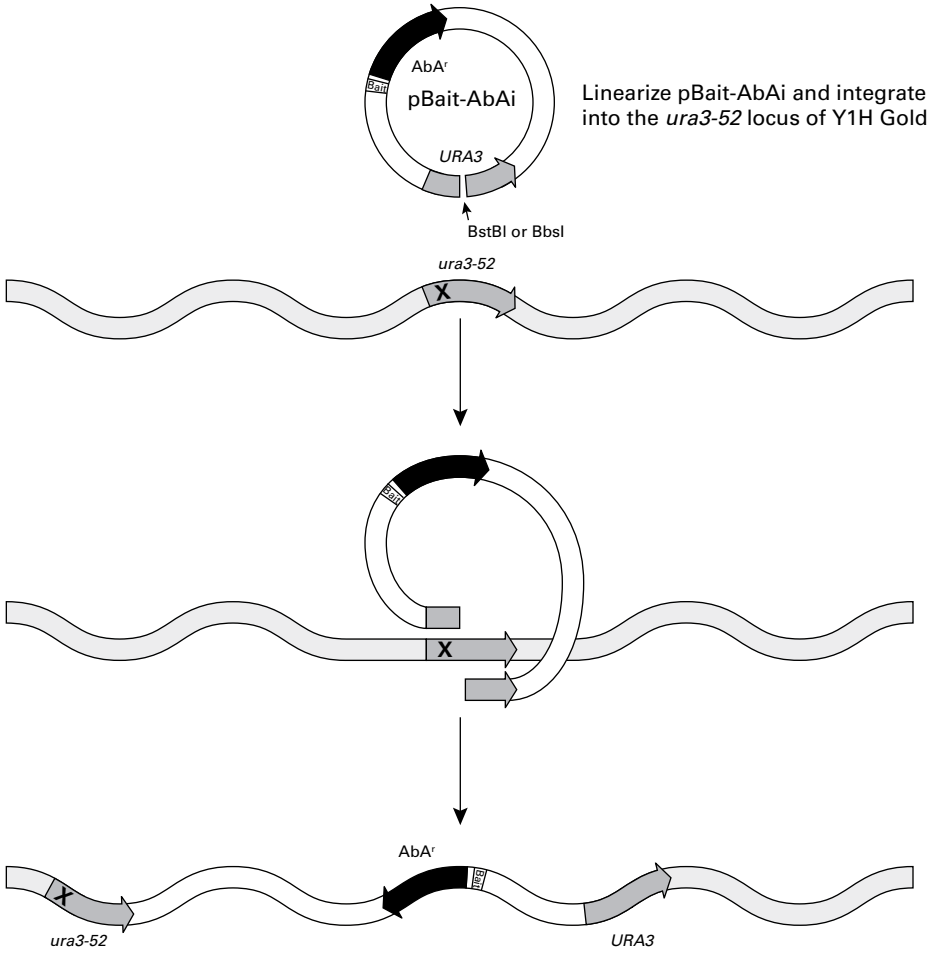


Figure 2. Create your bait reporter strain by homologous recombination into the genome of Y1HGold. The inactive *ura3-52* locus of Y1HGold consists of an irreversible transposon disruption that can only be repaired by homologous recombination with the wild type *URA3* gene, which is provided by the pBait-AbAi vector. Transformation of Y1HGold with a pBait-AbAi vector linearized with BstBI or BbsI, results in colonies that can grow in the absence of uracil on SD/-Ura agar plates. These colonies also contain a stable Bait-AbAi reporter that can be used to screen for protein-DNA interactions.



ATTENTION: Successful use of any yeast one-hybrid system depends upon no/low recognition of your target sequence by endogenous yeast transcription factors, which can cause high background expression of the reporter gene. For this reason it is critical to test your Y1HGold[Bait-AbAi] strain for AbA^r expression (AbA resistance) before screening a library (see Section VI).

I. Introduction & Protocol Overview continued

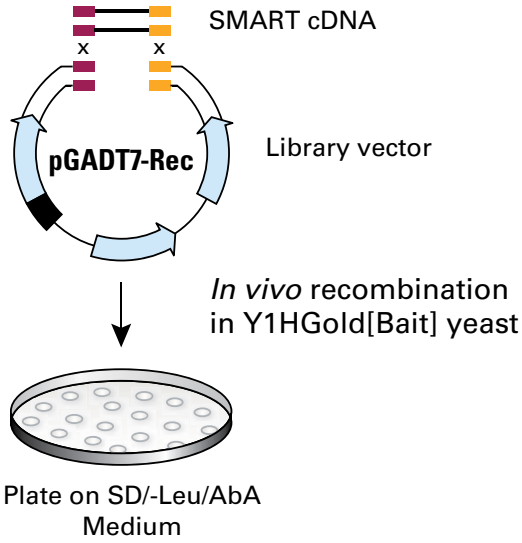


Figure 3. Use SMART Technology and yeast biology to construct and screen your library. Your cDNA library is simultaneously constructed and screened directly in yeast. First, SMART cDNA synthesis technology creates a cDNA pool with flanking end sequences that are homologous to the prey vector, pGADT7-Rec. When the cDNA and the linear pGADT7-Rec Vector are cotransformed into your newly created Y1HGold[Bait] reporter strain, the yeast recombine the cDNA and the vector with high efficiency. Transformed cells are plated on SD/-Leu/+AbA to select for colonies whose prey proteins have activated the AbA^r reporter.

II. List of Components

The **Matchmaker™ Gold Yeast One-Hybrid Library Screening System** (Cat. No. 630491) contains the materials needed to make five one-hybrid libraries. We recommend using the **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207) to amplify the SMART cDNA to produce ds cDNA (not included).

Box 1 (Store at –20°C):

- 10 µl SMART™ MMLV RT (200 units/µl; also available as Cat. No. 639522)
- 300 µl 5X First-Strand Buffer
- 165 µl DTT (100 mM)
- 10 µl CDS III Primer (12 µM)
- 10 µl CDS III/6 Primer (10 µM)
- 50 µl 5' PCR Primer (10 µM)
- 50 µl 3' PCR Primer (10 µM)
- 7 µl RNaseH (2 units/µl)
- 500 µl Melting Solution
- 50 µl dNTP Mix (10 mM each dNTP)
- 25 µg pGADT7-Rec AD Cloning Vector (SmaI-linearized; 500 ng/µl)
- 20 µg pAbAi Vector (500 ng/µl)
- 10 µg p53-AbAi Control Vector (500 ng/µl)
- 625 ng p53 Control Insert (25 ng/µl)
- 50 µl pGADT7 AD Vector (100 ng/µl)

Box 2 (Store at –20°C):

Yeastmaker™ Yeast Transformation System 2*
(Box 1 of 2)

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

Box 3 (Store at RT):

Yeastmaker™ Yeast Transformation System 2*
(Box 2 of 2)

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

* also available separately as Cat. No. 630439

Box 4 (Store at –70°C):

- 10 µl SMART™ III Oligonucleotide (12 µM)
- 5 µl Control Poly A$^+$ RNA (Human Placenta; 1 µg/µl)
- 0.5 ml *S. cerevisiae* Y1HGold

Box 5 (Store at RT):

- 1 pouch YPDA Broth (0.5 L)
- 1 pouch YPDA with Agar (0.5 L)
- 1 pouch SD/-Ura with Agar (0.5 L)
- 50 ml NaCl Solution (0.9%)
- 300 µl Sodium Acetate (3 M)
- 500 µl Deionized H_2O
- 10 ea CHROMA SPIN™+TE-400 Columns

Other

- Matchmaker™ Gold One-Hybrid Library Screening System User Manual (PT4087-1)
- Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1)
- pGADT7-Rec Vector Information (PT3530-5)
- pAbAi Vector Information (PT4091-5)
- pGADT7 AD Vector Information (PT3249-5)

III. List of Abbreviations

AD/library plasmid	Plasmid encoding a fusion of the GAL4 activation domain and a library cDNA; also "prey plasmid".
AD/library protein	A fusion protein comprised of the GAL4 activation domain and a polypeptide encoded by a library cDNA; also "prey protein".
AD vector	Plasmid encoding the yeast GAL4 activation domain, e.g. pGADT7-Rec.
Bait	pAbAi containing one or more repeats of your target DNA sequence of interest
Prey	A GAL4 AD fusion protein expressed from the pGADT7-Rec vector which contains a library cDNA

Yeast Phenotypes

Ura-, Leu-, or Trp-	Strains that require uracil (Ura), leucine (Leu), or tryptophan (Trp), respectively, in the medium to grow; i.e., they are auxotrophic for one (or more) of these specific nutrients.

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
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 (120 mg/L final concentration) to prevent yeast cultures from becoming pink in color.

IV. Y1HGold Host Strain Information

The genotype and several phenotypes of the Y1HGold yeast strain are shown in Tables I and II. For additional information on the growth and maintenance of yeast, see *Guide to Yeast Genetics and Molecular Biology* (Guthrie & Fink, 1991).

Table I: Y1HGold Strain Genotype

Strain	Genotype
Y1HGold	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, MEL1*

Table II: Y1HGold Phenotype Testing on Various SD Media

Strain	SD/–Leu	SD/–Ura	SD/AbA^{200}
Y1HGold	–	–	–
Y1HGold[p53-AbAi]	–	+	–
Y1HGold[pGADT7-Rec-p53]	+	–	–
Y1HGold[pGADT7-Rec-p53/p53-AbAi]	+	+	+

V. Additional Materials & Yeast Media Required

A. Additional Materials Required for cDNA Amplification, Library Construction, and Screening

- **Thermostable DNA polymerase for PCR.** We recommend using the **Advantage® 2 PCR Kit** (Cat Nos. 639206 & 639207) to amplify ds cDNA from the first-strand SMART cDNA that is made with the Matchmaker Gold kit. Advantage 2 Polymerase Mix allows you to amplify cDNA (as large as 20 kb) with much higher fidelity than conventional PCR. *The Advantage 2 PCR Kit is NOT included in the Matchmaker Gold Yeast One-Hybrid Library Screening System.*
- **Restriction enzymes BstBI** or **BbsI**, for linearizing your pBait-AbAi plasmid prior to transforming it into Y1H-Gold for integration.
- **Matchmaker Insert Check PCR Mix 1** (Cat. No. 630496). This complete 2X mix contains PCR enzyme, specific primers, dNTPs, and buffer for performing rapid yeast colony PCR for confirming that your pBait-AbA plasmid containing your target sequence has integrated into the Y1HGold genome. ’
- **Matchmaker Insert Check PCR Mix 2** (Cat. No. 630497), or **Matchmaker AD LD-Insert Screening Amplimer Set** (Cat. No. 630433) for amplifying and characterizing the cDNA inserts from the library that are contained in the positive clones that emerge from your screening.
- **Easy Yeast Plasmid Isolation Kit** (Cat. No. 630467), for simple and efficient rescue of library plasmids from yeast.
- **Thermal cycler,** for oligonucleotide annealing and cDNA amplification, preferably equipped with a heated lid.
- Standard molecular biology reagents, enzymes, and supplies needed for plasmid and oligonucleotide cloning, ligation, and analysis. See individual protocols for requirements.

B. Yeast Media and Supplements Required

- **Yeast Media Set 1** (Cat. No. 630492) and/or **Yeast Media Set 1 Plus** (Cat. No. 630493). These media sets each contain a complete assortment of ready-mixed foil pouches for preparing the five specialized broth and agar media needed for the Matchmaker Gold Yeast One-Hybrid Screening System. The Yeast Media Set 1 **Plus** also contains Aureobasidin A (See Appendix B).
- **Aureobasidin A** (Cat. Nos. 630466 & 630499) See Appendix B for stock solution preparation instructions, and for its use in media preparation.
- **Additional yeast media.** Appendix B lists information for purchase and preparation for each of the required media mixes which are available as **Yeast Media Pouches** in packs of 10.
  - **YPDA** is a rich medium used for routine culturing of all *S. cerevisiae* yeast strains. The additional adenine prevents yeast cultures from becoming pink in color.
  - **SD medium** (synthetically defined medium) is minimal media that used for culturing *S. cerevisiae* and selecting for plasmid transformation. SD base supplies everything that a yeast cell needs to survive (including carbon and nitrogen sources). Essential amino acids, which are added separately to the SD base to create minimal medium, are already included premixed in Clontech’s **Yeast Media Pouches**. Selecting for the growth of yeast transformed by specific plasmids requires plating the yeast on the minimal medium specific for the marker or reporter present on the plasmid.
  - **SD/-Ura DO supplement** is used to select for integration of the bait-reporter construct into the genome of YIHGold. SD/-Ura dropout supplement (SD/-Ura DO), is so called because the medium includes all essential nutrients except uracil, which is omitted from the formulation (or “dropped out”). pAbAi integrants are able to grow because the plasmid encodes the wild-type *URA3* biosynthesis gene, that is otherwise inactive in the parent strain, and allows the yeast to grow in the absence of uracil.
  - **SD/-Ura/AbA* media** is used to select for Aureobasidin A resistance which is activated by prey proteins that specifically interact with your bait sequence. The concentration of AbA used for selection (*) must be titrated for each individual bait strain.

VI. Constructing the Bait Plasmid and Bait Yeast Strain

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Detailed instructions are provided for the synthesis and cloning of your target element (Protocol A) and for generating your Y1HGold target reporter bait strain (Protocol B).

1–2 days

A. Protocol: Synthesizing and Cloning Your pBait-AbAi Plasmid

Your target-reporter construct (pBait-AbAi) should contain at least one copy of the DNA target element inserted upstream of the *AbA*^{r} reporter gene in pAbAi (see Target Sequence Notes below). Numerous studies have indicated that the most effective constructs contain at least three tandem copies of the DNA target. Although three copies may be preferred, published reports have demonstrated that a single copy may also suffice in certain cases. Tandem copies may be generated by different methods, but we have found oligonucleotide synthesis to be the most convenient and reliable, especially since well-defined regulatory elements are usually <20 bp.

1. For each target sequence, design and synthesize two antiparallel oligonucleotides with overhanging sticky ends compatible with the sticky ends of the appropriately digested pAbAi Vector. We strongly recommend that the 3'-end of your annealed oligonucleotide (or insert) contain either a SalI or XhoI overhang, whereas the 5'-end of your annealed oligonucleotide (or insert) can be compatible with any one of the unique restriction sites indicated in the MCS that is upstream of the SmaI site. We recommend that you also generate a *mutant bait* construct containing a target sequence that you would expect to disrupt the binding of the proteins you seek in the library.
2. Anneal the oligonucleotides as follows (use a thermal cycler). Once annealed, the ds oligonucleotides are ready for ligation into the pAbAi vector. Alternatively, annealed oligonucleotides can be stored at –20°C until ready to use.
   a. Resuspend each ss oligonucleotide in TE buffer to a final concentration of 100 µM.
   b. Mix the top strand and bottom strand oligos at a ratio of 1:1. This mixture will ultimately yield ds oligo at a concentration of 50 µM (assuming 100% theoretical annealing).
   c. Heat the mixture to 95°C for 30 sec to remove all secondary structure.
   d. Heat at 72°C for 2 min.
   e. Heat at 37°C for 2 min.
   f. Heat at 25°C for 2 min.
   g. Store on ice.
3. Linearize 1 µg of pAbAi, using restriction enzymes that generate overhanging sequences or ends compatible with your target sequence oligonucleotides (see suggestions in Section VI A.1). Purify the linear DNA on an agarose gel or by using a spin column method such as that provided by NucleoSpin® Extract II (Cat. No. 740609.50).

TARGET SEQUENCE NOTES:

- The annealed oligos consist of one or more copies of the target element and should be designed with a different restriction site overhang on each end. When the two strands are annealed, the resulting double-stranded DNA will have a different overhang at each end for directional cloning into the appropriately digested pAbAi Vector.
- We recommend that you also create a pAbAi construct containing a mutant sequence having point mutations expected to disrupt protein binding, for use as a negative control in confirming the specificity of your positive clones in Section X.
- If a cloned protein is known to interact with your sequence, you may wish to clone it into the supplied pGADT7 vector for use as a positive control for your bait sequence.

VI. Constructing the Bait Plasmid and Bait Yeast Strain continued

A. Protocol: Synthesizing and Cloning Your pBait-AbAi Plasmid (cont'd)

4. Ligate the ds oligonucleotide into the linearized pAbAi Vector.

   a. Dilute the annealed oligos (from Step 2g) 1/100 with TE buffer to obtain a concentration of 0.5 µM.

   Note: To ensure good ligation efficiency it is necessary to dilute the oligo so that it is only in moderate excess. Using a large excess of the oligo will inhibit ligation.

   b. Assemble a ligation reaction for each annealed oligonucleotide by combining the following reagents in an microfuge tube:

1.0 µl	**pAbAi vector (50 ng/µl),** linearized and with oligo-compatible overhangs
1.0 µl	**Annealed oligonucleotide (0.5 µM)** (target or mutant)
1.5 µl	**10X T4 DNA ligase buffer**
0.5 µl	**BSA (10 mg/ml)**
10.5 µl	**Nuclease-free H_2O**
0.5 µl	**T4 DNA ligase (400 U/µl)**
15 µl	Total volume

   Note: If desired, a control ligation can be assembled using 1 µl of nuclease-free H_2O instead of annealed oligonucleotide.

   c. Incubate the reaction mixture for 3 hr at room temperature, transform *E. coli*, and identify the correct constructs using standard techniques.

B. Protocol: Generating the Bait-Reporter Yeast Strains

Protocol 4–5 days

The bait-reporter strain is generated by homologous integration into Y1HGold (see Figure 2) and includes the following steps:

- Step 1: Digest the pBait-AbAi, pMutant-AbAi, and p53-AbAi vectors with BstBI or BbsI.*
- Step 2: Transform the linearized plasmids into Y1HGold. Select for transformants on SD/-Ura media.
- Step 3: Confirm that the plasmid integrated correctly by using a colony PCR analysis and the **Matchmaker Insert Check PCR Mix 1** (Cat. No. 630496).
- Step 4: Determine minimal inhibitory concentration of Aureobasidin A for your bait strain.

*We strongly recommend that you also create a positive control strain with linearized p53-AbAi and a negative control strain with your pMutant-AbAi using the following protocol. These will help with troubleshooting and with the confirmation of positive clones.

Materials Required:

- Y1HGold yeast strain
- Your pBait-AbAi and pMutant-AbAi plasmids from Protocol A, and p53-AbAi (included)
- BstBI or BbsI restriction enzyme
- DNA clean-up kit (e.g. NucleoSpin® Extract II, Cat. No. 740609.50)
- Yeastmaker Yeast Transformation System 2 (included)
- **Matchmaker Insert Check PCR Mix 1** (Cat. No. 630496)
- SD/-Ura with Agar
- YPDA broth

VI. Constructing the Bait Plasmid and Bait Yeast Strain continued

B. Protocol: Generating the Bait-Reporter Yeast Strains (cont'd)

1. Linearize 2 µg of pBait-AbAi, pMutant-AbAi, and p53-AbAi at either of the unique BstBI or BbsI sites within the *URA3* gene of the vectors. Purify the linear DNA using a spin column method such as that provided by NucleoSpin® Extract II (Cat. No. 740609.50).

   Note: Clontech does not usually gel purify the linearized vectors, we simply perform a clean-up step after confirming on an agarose gel that the plasmid has been linearized.

2. Using the protocol for the Yeastmaker Yeast Transformation System 2, transform Y1HGold with 1 µg of each of the linear plasmids.

3. Dilute each transformation reaction 1/10, 1/100, and 1/1000. Plate 100 µl from each dilution on SD/-Ura agar medium.

4. After 3 days pick 5 colonies and analyze by colony PCR using the Matchmaker Insert Check PCR Mix 1, which will identify correctly integrated clones according to the strategy shown in Figure 4. Use untransformed Y1HGold colonies as negative controls.

   Expected Colony PCR Analysis Results:

   – Positive Control: 1.4 kb

   – Negative Control: No Band

   – Bait Strain: 1.35 kb + Insert size

5. Pick one colony for each confirmed bait, and one from the p53-AbAi control, and streak them onto SD/-Ura agar medium. After 3 days at 30ºC, store at 4ºC for up to 1 month. These are your newly constructed Y1HGold[Bait/AbAi] strains and [p53/AbAi] control strain.

6. Also for long term storage, grow an overnight culture in YPDA broth (3 ml), collect the cells by centrifugation, and resuspend them in 1 ml of freezing medium (Appendix B). Quick freeze, and store at –70ºC.

   Note: The integrated plasmids are very stable, overnight broth media culturing without *URA3* selection will not result in loss of the integrant.

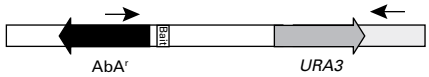


Figure 4. Confirming pBait-AbAi integration by colony PCR. The primers in the Matchmaker Insert Check PCR Mix 1 are located in the AbA^r gene and in the Y1Hgold genome, downstream of the *URA3* locus. They will amplify a region of ~1.4 kb that encompasses your bait sequence and confirms the presence of the integrated plasmid. See Figure 2 for context

VII. Testing Your Bait Strain for AbA^r Expression

A. Protocol: Determining the Minimal Inhibitory Concentration of Aureobasidin A for Your Bait

Depending on the bait sequence cloned into pAbAi, the basal expression of the bait reporter stain in the absence of prey can vary. For example, the p53-AbAi control has a minimal inhibitory concentration of 100 ng/ml Aureobasidin A.



ATTENTION: Successful use of any yeast one-hybrid system depends upon no/low recognition of your target sequence by endogenous yeast transcription factors. For this reason, it is critical to test your construct for AbA^r expression before screening the library. The following experiment will determine the concentration of AbA you need to use in your library screen to suppress basal expression of your bait construct.

Materials required:

- Y1HGold[Bait/AbAi] strain, and the Y1H[Mutant/AbAi] and Y1HGold[p53/AbAi] control strains generated in Section V.B
- SD/-Ura with Agar
- Aureobasidin A (AbA)
- SD/-Ura/AbA agar plates containing 100–200 ng/ml AbA

Protocol:

1. Pick a large healthy colony from the bait and control strains. Resuspend each colony in 0.9% NaCl and adjust the OD_{600} to ~0.002 (for approximately 2000 cells per 100 µl).
2. Plate 100 µl on each of the following media. Allow colonies to grow for 2–3 days at 30°C.
   - SD/-Ura
   - SD/-Ura with AbA (100 ng/ml)
   - SD/-Ura with AbA (150 ng/ml)
   - SD/-Ura with AbA (200 ng/ml)
3. Expected results for the plating of ~2000 colonies are shown in Table III.

Table III: Expected Results for AbA^r Basal Expression

[AbA] ng/ml	Y1HGold[p53-AbAi] colonies	Y1HGold[pBait-AbAi] colonies
0	~2000	~2000
100	0	Bait dependent
150	0	Bait dependent
200	0	Bait dependent



Note: If 200 ng/ml does not suppress basal expression, you can try increasing the concentration of AbA to 500–1000 ng/ml. However, if 1000 ng/ml does not suppress AbA^r in the absence of prey, your bait DNA sequence is likely being recognized by endogenous yeast transcription factors and therefore your sequence cannot be used for a yeast one hybrid screen.

4. For library screening, use the minimal concentration of AbA, or a concentration that is slightly higher (by 50–100 ng/ml) that completely suppress the growth of your bait strain.

VIII. Generating cDNA for Library Construction

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for first-strand cDNA synthesis (Protocol A), cDNA amplification using long distance PCR (LD-PCR) (Protocol B), and column purification of ds cDNA using a CHROMA SPIN™+TE-400 column (Protocol C).

Use the following protocol for generating cDNA using Clontech's simple and high efficiency SMART™ technology (Figure 5). For a detailed description of SMART technology, refer to Appendix C. We recommend using the **Advantage® 2 PCR Kit** (Cat No. 639206) to amplify your SMART cDNA as described in Protocol B. This kit is not included in the Matchmaker Gold Yeast One-Hybrid Library Screening System.

Preparing cDNA for library construction consists of three protocols:

A. First-strand SMART cDNA synthesis

B. Amplifying SMART cDNA by long distance PCR (LD-PCR)

C. Purifying the ds cDNA with a CHROMA SPIN+TE-400 column

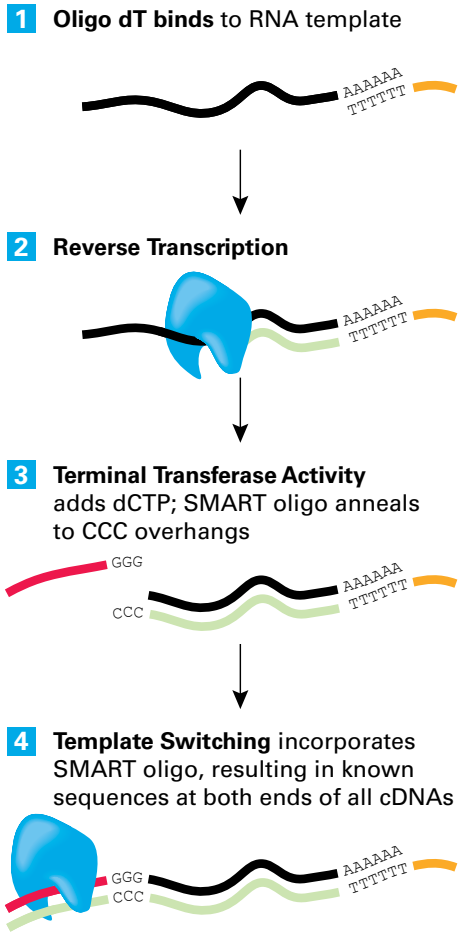


Figure 5. SMART cDNA synthesis generates cDNA ends that are homologous to the cloning site in pGADT7-Rec.

VIII. Generating cDNA for Library Construction continued

Protocol
1 day

A. Protocol: First Strand SMART cDNA Synthesis

It is strongly recommended that you perform a positive control cDNA synthesis with the Human Placenta Poly A+ RNA provided with the system. This control verifies that all components are working properly and provides a standard for comparing the yield and size range of the ds cDNA synthesized from your experimental RNA sample.

In the protocol that follows, you have the option of priming first-strand cDNA synthesis with an oligo-dT (CDS III) or random primer (CDS III/6). The reaction conditions vary slightly depending on the primer used.

Attention

IMPORTANT: Do not increase the size or volume of any of the reactions. All components have been optimized for the volumes specified.

1. Prepare or obtain high quality Poly A+ or total RNA. We recommend using **NucleoSpin® RNA II** (e.g. Cat. No. 740955.20) for purifying total RNA form a variety of sources, and using **NucleoTrap® mRNA Kit** (e.g. Cat. No. 740655) to enrich for poly A+ mRNA from total RNA.
2. Combine and mix the following reagents in a sterile microcentrifuge tube. Use a second tube for the control cDNA reaction:

   1–2 µl RNA sample (0.025–1.0 µg poly A⁺ or 0.10–2.0 µg total RNA)*
   1.0 µl CDS III (oligo-dT) or **CDS III/6 (random) Primer**
   1–2 µl Deionized H_2O (for a total volume of 4.0 µl).
   4.0 µl Total volume

   *For the control reaction, use 1 µl (1 µg) of the Control Poly A⁺ RNA.
3. Incubate at 72°C for 2 min.
4. Cool on ice for *exactly 2 min*, spin briefly, and immediately add the reagents in Step 5.
5. To each reaction, add the reagents listed below* and mix by tapping or by gentle pipetting. Spin briefly.

   2.0 µl 5X First-Strand Buffer
   1.0 µl DTT (100 mM)
   1.0 µl dNTP Mix (10 mM)
   1.0 µl SMART MMLV RT
   9.0 µl Total volume

NOTE

***NOTE:** A master mix of these reagents can be prepared prior to Step 2 and kept on ice. This initial step of cDNA synthesis is critical and the denatured RNA/primer mix should not be kept on ice longer than 2 min.

6. **If using the CDS III/6 random primer**, incubate at 25-30°C for 10 min at room temperature.
   If using the CDS III primer, omit this step and continue to Step 7.
7. **For either primer,** incubate at 42° for 10 min.

   NOTE: If possible, perform the incubation in a thermal cycler with a heated lid. If using a water bath or non-hot-lid cycler, add a drop of mineral oil to prevent sample evaporation.
8. Add 1 µl of the SMART III oligo, mix and incubate at 42°C for 1 hr.
9. Place the tube at 75°C for 10 min to terminate first strand synthesis.
10. Cool to room temperature, add 1 µl RNaseH (2 units).
11. Incubate at 37° for 20 min.
12. Proceed to LD-PCR amplification (Section VII.B). Any first strand SMART cDNA synthesis reaction that is not used immediately should be stored at –20°C. It can be used for up to 3 months.

VIII. Generating cDNA for Library Construction continued

Protocol
5 hr

B. Protocol: Amplifying SMART cDNA by Long Distance PCR (LD-PCR)

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

Table IV. Relationship Between Amount of RNA and Optimal Number of Thermal Cycles

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

Materials Required:

- First-strand cDNA (from Protocol A)
- Thermal cycler, preheated
- Equipment and reagents for agarose gel electrophoresis

Protocol:

1. Set up **TWO** 100 µl PCR reactions for each experimental sample and one reaction for the control sample:

2	**µl**	**First-Strand SMART cDNA** (from Protocol A)
70	**µl**	**Deionized H_2O**
10	**µl**	**10X Advantage® 2 PCR Buffer***
2	**µl**	**50X dNTP Mix**
2	**µl**	**5' PCR Primer**
2	**µl**	**3' PCR Primer**
10	**µl**	**Melting Solution**
2	**µl**	**50X Advantage 2 Polymerase Mix***
100	µl	Total volume

*We highly recommend using the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) for generating and amplifying ds cDNA.

2. Begin thermal cycling using the following parameters:
   - **1 cycle: 95°C x 30 sec**
   - **X[a] cycles: 95°C x 10 sec**
     68°C x 6 min[b]
   - **1 cycle: 68°C x 5 min**

   a Refer to Table IV to estimate the number of cycles.
   b Program the cycler to increase the extension time by 5 sec with each successive cycle. For example, in the second cycle, the extension should last 6 min and 5 sec; in the third, 6 min and 10 sec, and so on.
3. Analyze 7 µl from each PCR reaction alongside a 1 kb DNA ladder on a 1.2% agarose/EtBr gel. Typical results for the control RNA after spin column purification are shown in Figure 6. If your amplified cDNA does not appear as expected, refer to the Troubleshooting Guide (Section XI).
4. Proceed to cDNA purification (Protocol C) or store the ds cDNA at –20°C until use.

VIII. Generating cDNA for Library Construction continued

Protocol
2 hr

C. Protocol: Purifying the ds cDNA with CHROMA SPIN™+TE-400 Columns

In the following protocol, a CHROMA SPIN+TE-400 Column is used to select for DNA molecules >400–600 bp.

CHROMA SPIN Columns are gel filtration columns that fractionate molecules based on size. DNA molecules larger than the pore size are excluded from the resin. Large molecules move quickly move through the gel bed when the column is centrifuged, while molecules smaller than the pore size are held back. For more information about these columns, please refer to the CHROMA SPIN Columns User Manual (PT1300-1) available on our web site at **www.clontech.com**.

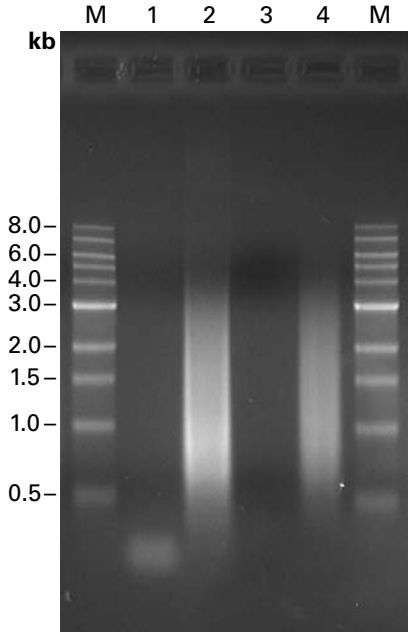


Figure 6. High-quality cDNA generated using SMART cDNA synthesis. Oligo dT-primed cDNA synthesis was carried out with or without 1 µg of human placenta poly A^+ RNA (positive and negative controls, respectively). LD PCR was performed using the Advantage® 2 Polymerase Mix (with duplicate samples) and one set of products was purified (size-selected) using CHROMA SPIN+TE-400 columns. Analysis of each respective sample on a 1% agarose gel revealed that the resulting cDNAs ranged from 300 bp to 6 kb. Lanes M: 1 kb ladder molecular weight standard. Lane 1: unpurified negative control. Lane 2: unpurified positive control. Lane 3: purified negative control. Lane 4: purified positive control. Lane 4 shows reduced abundance of cDNA below 400 bp compared to Lane 2, after size selection with CHROMA SPIN+TE-400 columns.

Materials Required:

- PCR-amplified ds cDNA samples, prepared as in Protocol B (2 x 93 µl)
- Sodium acetate (3 M; pH 5.3)
- Ice cold ethanol (95–100%)
- CHROMA SPIN+TE-400 columns (1 each/cDNA sample)

Protocol:

1. Prepare one CHROMA SPIN+TE-400 column for each cDNA sample to be purified (see Figure 7).
   - Invert each column several times to resuspend the gel matrix completely
   - Snap off the break away end from the bottom of each column
   - Place the columns in 2 ml collection tubes (supplied)
   - Remove the top caps

VIII. Generating cDNA for Library Construction continued

C. Protocol: Purifying ds cDNA with CHROMA SPIN+TE-400 Columns (cont'd)

2. Centrifuge the columns at 700 g for 5 min to purge the equilibration buffer, then discard collection tube and buffer. The matrix will appear semi-dry.

NOTE: We recommend using swinging bucket or horizontal rotors. Fixed angel rotors can be used but there is a risk that the sample will pass down the inner side of the columns instead of through the gel matrix, and may result in inconsistent purification.

3. Place each spin column in a second collection tube (or 1.5 ml microfuge tube, if preferred) and apply a cDNA sample to each column at the CENTER of the flat surface of the gel matrix. Do not allow sample to flow along inner wall of the column.
4. Centrifuge the columns at 700 g for 5 min. The purified cDNA is now in the collection tubes.
5. Combine the two purified cDNA samples into a single microcentrifuge tube, measure the combined volume with a micropipet, and precipitate the cDNA with ethanol:
   a. Add 1/10th vol 3 M sodium acetate (pH 5.3), and mix.
   b. Add 2.5 vol of ice-cold ethanol (95–100%)
   c. Place in –20°C freezer for 1 hr
   d. Centrifuge at 14,000 rpm for 20 min at room temperature
   e. Carefully remove and discard the supernatant; do not disturb the pellet
   f. Centrifuge briefly at 14,000 rpm and remove remaining supernatant
   g. Air dry the pellet for 10 min
7. Resuspend the cDNA in 20 µl deionized water. The cDNA is now ready for library construction using homologous recombination in yeast (Section VIII). At this point, you should have 2–5 µg of ds cDNA

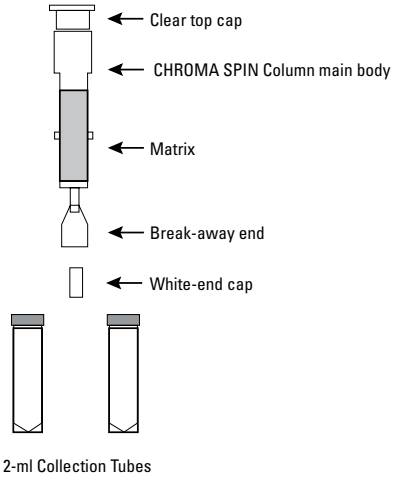


Figure 7. CHROMA SPIN+TE-400 columns and collection tubes. Note that a conventional, tapered 1.5 ml microcentrifuge tube can be substituted for the 2 ml collection tube for the final cDNA collection step. This will produce a more compact pellet of cDNA upon precipitation.

IX. Creating and Screening a One-Hybrid Library

PLEASE READ THIS ENTIRE SCREENING PROTOCOL BEFORE STARTING
Detailed instructions are provided for constructing and screening a one-hybrid library.

The protocol below describes how to make and screen your one-hybrid library. If you wish to test one-on-one bait/prey interactions, simply clone your prey into pGADT7 and follow the protocol for the p53 controls but you must first optimize the Aureobasidin A concentration for your bait as described in Section VI.A.

Materials required:

- Yeast strains Y1HGold[Bait/AbAi] and Y1HGold[p53/AbAi] from Section V.B.
- pGADT7-Rec AD Cloning Vector (SmaI-linearized)
- p53 Control Insert
- Purified SMART ds cDNA, 2–5 µg in 20 µl deionized water (Section VII.C)
- Yeastmaker Yeast Transformation System 2
- The following SD agar plates (Appendix B)
  - SD/-Leu (5–10 x 100 mm plates)
  - SD/-Leu/AbA^{200} (Aureobasidin A, 100 ng/ml) (5 x 100 mm plates)
  - SD/-Leu/AbA*; **You will need 100 x 150 mm plates.**
    (*The AbA concentration should be determined empirically for your bait strain, as described in Section VI.A. It is usually 100–200 ng/ml.)



Protocol 3–5 days

A. Protocol: Creating and Screening a One-Hybrid cDNA Library

1. Create and test your Y1HGold[Bait/AbAi] strain on SD/-Leu/AbA* (Sections V & VI)
2. Synthesize ds cDNA using SMART technology to obtain 2–5 µg of cDNA in a volume of 20 µl (Section VII).
3. Using the Yeastmaker Transformation System 2 and the protocol included with that system(PT1172-1), set up the following transformation reactions:
   a. **Library scale** transformation of Y1HGold[Bait/AbAi]
      - 20 µl SMART-amplified ds cDNA (2–5 µg)
      - 6 µl pGADT7-Rec, (SmaI-linearized) (3 µg)
   b. **Small scale** transformation of the Y1HGold[53/AbAi] control strain
      - 5 µl p53 fragment (125 ng)
      - 2 µl pGADT7-Rec, (SmaI-linearized) (1 µg)
3. From each of the transformation reactions, spread 100 µl of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on one of each of the following 100 mm agar plates.
   - SD/-Leu, and
   - SD/-Leu/AbA*, for the library reaction or
   - SD/-Leu/AbA^{200} for the p53 control reaction

IX. Creating and Screening a One-Hybrid Library continued

A. Protocol: Creating and Screening a One-Hybrid cDNA Library (cont'd)

4. Plate the remainder of the library transformation reaction (~15 ml), on the 150 mm SD/-Leu/AbA* plates using 150 µl per plate.
5. Incubate the plates (colony side down) for 3–5 days.
6. Calculate the number of screened clones by counting the number of colonies on the SD/-Leu 100 mm plates after 3–5 days.
   - Number of screened clones = [cfu/ml on SD/-Leu] x [dilution factor] x [resuspension volume (15 ml)]



NOTE: It is imperative that at least 1 million clones are screened. Using fewer clones will diminish the chances of detecting genuine interactions.

Calculating the number of screened clones: example calculation

- Resuspension volume = 15 ml
- Plating Volume = 100 µl
- 250 colonies grew on the 1/100 dilution on SD/-Leu plates.

Therefore, the number of clones screened = 250 cfu/0.1 ml x 100 x 15 ml = 3.75 million

7. Expected results

- Positive Control Experiment:
  - Similar number of colonies on SD/Leu and SD/-Leu/AbA^{200}
- Library Screening:
  - Calculated >1 million colonies on SD/-Leu plates
  - Far fewer colonies on SD/-Leu/AbA* plates. The number of positives will be bait sequence dependent.

Library Screening Notes:

For positive control interactions, the number of colonies should theoretically be the same on both types of selective media. SD/-Leu selects for the presence of the prey plasmid (i.e, properly transformed cells) and SD/-Leu/ +AbA^{200} selects for the prey plasmid as well as for the positive interaction of p53 with its recognition site. However, a difference of approximately 10–20% lower on SD/-Leu/ AbA^{200} is usually observed.

X. Analysis of Results

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

A. Too Few Positives

- Have you screened >1million independent clones? Refer to Section IX.A, step 6 to calculate whether you have screened at least 1 million independent clones. Optimize the transformation procedure and repeat the screening procedure.
- Check that your growth media performs as expected with the positive and negative controls.
- Retest the minimal inhibitory concentration of AbA for your bait strain
- Try increasing the number of repeats of your target sequence. Generally we find that three repeats work well.

B. Too Many Positives

Have you determined the optimal AbA concentration for your bait as described in Section VII.A?

- Check that your media performs as expected with the positive and negative controls.
- If you used 100 ng/ml AbA, repeat the screen with 200 ng/ml.
- Pick only large healthy colonies after 3–5 days to analyze further in Section X.
- Your bait may interact with a partner that is abundant in the library. Sort duplicates by yeast colony PCR (Section X.B). After the clones have been sorted into groups, a representative of each unique type can then be analyzed for false positive interactions (Section X.D).

XI. Confirming Positive Interactions & Rescuing the Prey Plasmid

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for confirmation of phenotype (Protocol A); yeast colony PCR to eliminate duplicates (Protocol B); rescue and isolation of library plasmids responsible for activation of the* AUR1-C *reporter (Protocol C); and distinguishing genuine positive from false positive interactions (Protocol D).

The following represents the recommended order of events to confirm that the positive interactions are genuine. 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 sequence 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.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 reporter phenotype by restreaking onto fresh selective media
- Yeast Colony PCR
- Rescue and isolation of the library plasmid responsible for activation of reporters
- Distinguishing genuine positive from false positive interactions



Protocol 2–4 days

A. Protocol: Confirmation of Reporter Phenotype by Restreaking

Materials required:

- Single colonies selected from the library screen and growing on SD/-Leu/AbA* media
- SD/-Leu/AbA* agar plates (Appendix B)

Protocol:

1. Restreak positive clones to generate **single colonies** on SD/-Leu/AbA* plates (Appendix B).
2. Genuine positive clones should grow as healthy single colonies in 2–4 days. These colonies should be used to generate cultures for subsequent analysis.

XI. Confirming Positive Interactions & Rescuing the Prey Plasmid continued



Protocol 4 hr–1 day

B. 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.



Protocol 2–7 days

C. Rescue and Isolation of Library Plasmids Responsible for Reporter Activation

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 *AbA*^{r} reporter, 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 SD/-Leu/AbA*, each time picking a single colony for restreaking. The plasmid should be rescued from one of these clones (see Step 2).

2. Rescuing the Library Plasmid from Yeast

To identify the gene responsible for the positive interaction, we recommend using the **Easy Yeast Plasmid Isolation Kit** (Cat. No. 630467) to rescue the plasmid from yeast cells grown on SD/-Leu/AbA*.

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

Since pGADT7-Rec contains a ampicillin resistance gene, prey plasmids can be selected for on LB plus 100 µg/ml ampicillin using any commonly used cloning strain of *E. coli* (e.g. DH5α, or Fusion-Blue™ from Clontech).

XI. Confirming Positive Interactions & Rescuing the Prey Plasmid continued

Protocol
5 days

D. Protocol: Distinguishing Genuine Positive from False Positive Interactions

With all one-hybrid screens, there is a possibility of detecting false positives and it is important to these from authentic interactions between your bait sequence and a prey protein using the following criteria (Figure 8).

- **Genuine Positive**: Authentic Bait sequence and Prey are both required to activate the *AbA*^{r} reporter
- **False Positive**: Prey activates the *AbA*^{r} reporter in the presence of a mutated bait sequence.

One-hybrid interactions can be confirmed on selective media using the following cotransformation procedure described in Figure 9.

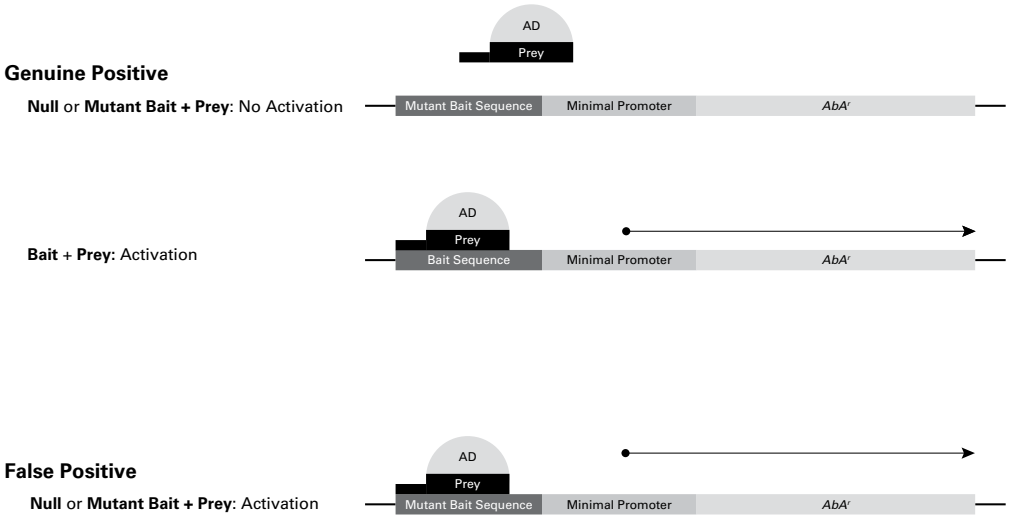


Figure 8. Illustration of the activation of reporter gene expression in genuine and false positives.

Materials required:

- Yeastmaker Yeast Transformation System 2 reagents
- Y1HGold[Bait/AbAi] yeast
- Y1HGold[Mutant/AbAi] yeast
- SD/-Leu plates
- SD/-Leu/AbA* plates

Protocol:

1. Using the Yeastmaker Transformation System 2 reagents and the small-scale transformation procedure, transform 100 ng of your rescued prey vector into the following yeast strains:
   - Y1HGold[Bait/AbAi]
   - Y1HGold[Mutant/AbAi]

   NOTE: We recommend that you perform the experiment side by side with the positive and negative controls (Section V).
2. Spread 100 µl of 1/10 and 1/100 dilutions of the transformation mix on the following plates:
   - SD/-Leu
   - SD/-Leu/AbA*

XI. Confirming Positive Interactions & Rescuing the Prey Plasmid continued

D. Protocol: Distinguishing Genuine Positive from False Positive Interactions (cont'd)

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

a. **Genuine Positive:**

Sample	Selective Agar Plate	Distinct 2 mm Colonies
Y1HGold[Bait/AbAi] + Prey	SD/-Leu	Yes
	SD/-Leu/AbA*	Yes
Y1HGold[Mutant/AbAi] + Prey	SD/-Leu	Yes
	SD/-Leu/AbA*	No (or very small)

b. **False Positive:**

Sample	Selective Agar Plate	Distinct 2 mm Colonies
Y1HGold[Bait/AbAi] + Prey	SD/-Leu	Yes
	SD/-Leu/AbA*	Yes
Y1HGold[Mutant/AbAi] + Prey	SD/-Leu	Yes
	SD/-Leu/AbA*	Yes

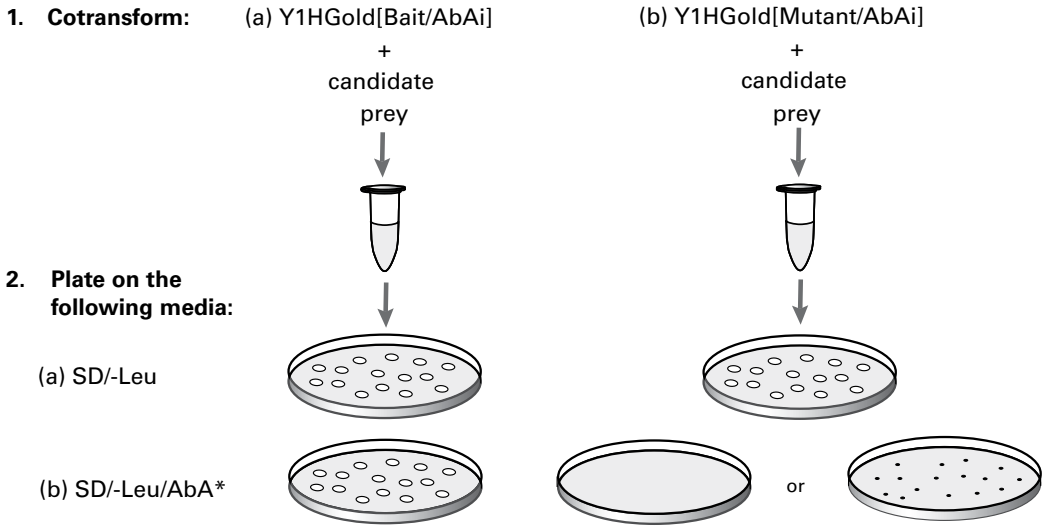


Figure 9. Using cotransformation on selective media to verify interactions. Expected results from genuine interactions.

XI. Confirming Positive Interactions & Rescuing 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

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 confirming that your clone is not a false positive (Section X.D).

- Most library clones will contain some of the 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 Clontech's In-Fusion PCR Cloning System (see www.clontech.com) and determine if the prey retains its bait-dependent activity.
- If 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 HA-Tag Polyclonal Antibody (Cat. No. 631207) 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), although this is a very rare occurrence.

XII. Troubleshooting Guide

Table V: Matchmaker Gold One-Hybrid Troubleshooting Guide

PROBLEM	CAUSE	SOLUTION
Inability to suppress basal *AbA^r* expression with 200 ng/ml AbA.	Improper media preparation (see Appendix B)	Repeat experiment with the control vectors, to confirm, and remake media, if necessary.
	Your target sequence may be strongly recognized by endogenous yeast transcription factors (see Section X)	Test AbA concentrations up to 1000 ng/ml, if that does not eliminate background colony growth, the one-hybrid system may not be suitable for your particular target sequence.
Too few or too many positives	See Section IX	See Section IX
Low transformation efficiency	Problems with starter culture or plasmid DNA quality	• Make sure that you set up your starter culture from a fresh healthy colony and use high quality plasmid DNA. Set up 3–4 starter cultures from separate colonies and proceed with the fastest growing culture. • Perform the control transformation with the pGBT9 vector supplied in the Yeastmaker Yeast Transformation System 2.
	Problems with Yeastmaker Carrier DNA quality	Re-denature (boil) and cool your Yeastmaker Carrier DNA prior to the transformation. If your carrier DNA aliquot is old, purchase a fresh aliquot from Clontech (Cat. No. 630440).
	Problems with cDNA quality, quantity	Ensure that the quality of your cDNA is good (see Section VII), and that you have >2 µg.
	Problems with DMSO quality	Use a fresh bottle of DMSO. We find that some batches of DMSO result in low transformation efficiencies.
	pH of growth medium is not optimal	Ensure that the pH of your growth medium is correct. All SD media should be adjusted to pH 5.8 prior to autoclaving.
Yeast growth media issues	SD Agar media did not set properly	Ensure that the media is pH 5.8 prior to autoclaving. If you did not adjust the pH, the media may be too acidic and the agar will be hydrolyzed in the autoclave, and thus will not set. The agar also breaks down if the media is over-autoclaved, preventing it from setting properly.
	Colonies appear pink on YPD or YPDA media	The red pigment exhibited by *ade2* mutants is an oxidized, polymerized derivative of 5'aminoimidazole ribotide which accumulates in *ade2* or *ade1* strains grown in medium low in adenine. YPD contains low levels of adenine, however Clontech YPDA contains 120 µg/ml adenine, which much higher than standard recipes and why we recommend using it exclusively (Appendix B).

XII. Troubleshooting Guide continued

Table V: Matchmaker Gold One-Hybrid Troubleshooting Guide, cont'd

PROBLEM	CAUSE	SOLUTION
Failure to detect known protein-DNA interactions	Insufficient number of target sequence repeats (only one or two repeats used).	Try increasing the number of repeats of your target sequence. At Clontech, we find that three repeats often results in stronger interactions than one or two repeats. Presumably, the central repeat is acting as a spacer between two binding sites.
	The AD hybrid protein (prey) may be toxic to the cell. In this case, transformants will not grow or may grow very slowly on selective medium.	Truncation of the prey protein may alleviate the toxicity and still allow the interaction to occur.
	If one of the following situations is occurring, it may interfere with the ability of the prey proteins to interact with the target element: • The hybrid protein is not stably expressed in the host cell. • The GAL4 AD occludes the site of interaction. • The hybrid protein folds improperly. • The hybrid protein cannot be localized to the yeast nucleus. (See van Aelst et al. [1993] for one example).	In these cases, it may help to construct hybrids containing different domains of the DNA-binding protein. Matchmaker antibodies are available for characterizing the expression of hybrid prey protein. See www.clontech.com

XIII. References

An extensive list of Matchmaker System citations are available at **www.clontech.com**

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Appendix A: Plasmid Information

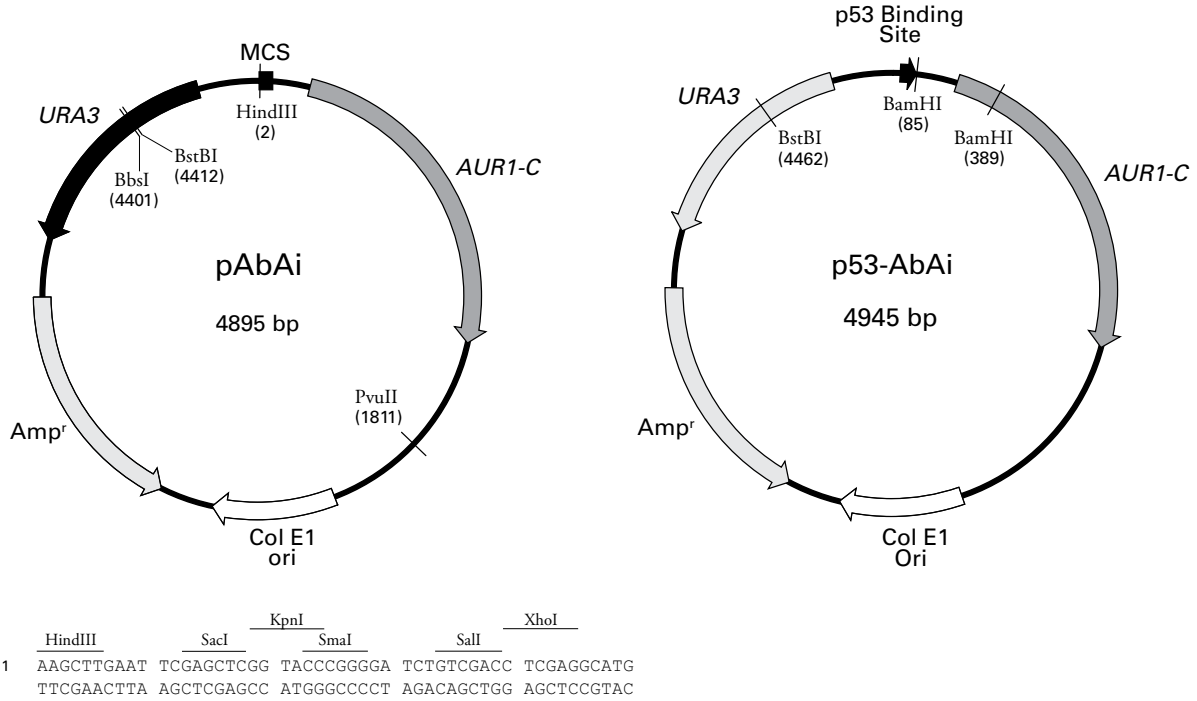


Figure 10. Maps of the pAbAi Vector and the p53-AbAi Control Vector. pAbAi is a yeast reporter vector that can be used in one-hybrid assays to identify and characterize DNA-binding proteins. The vector, which was specifically designed for use with the Matchmaker Gold Yeast One-Hybrid Library Screening System, contains a multiple cloning site (MCS; lower panel) upstream of the yeast iso-1-cytochrome C minimal promoter (not shown) and the *AUR1-C* gene, an antibiotic resistance gene that confers resistance to Aureobasidin A (AbA). A *cis*-acting element (i.e., a target sequence or "bait") can be cloned in the MCS and used as a bait to screen GAL4 AD/cDNA fusion libraries for proteins that interact with the target sequence. Positive protein-DNA (i.e., one-hybrid) interactions drive the expression of *AUR1-C*. As a result, one-hybrid interactions can be detected by selecting for yeast that are resistant to AbA.

p53-AbAi is a yeast reporter vector that serves as a positive control in the Matchmaker Gold Yeast One-Hybrid Library Screening System. The vector contains a p53 binding site (consisting of 3 tandem copies of the p53 consensus binding sequence) cloned in the MCS of pAbAi. Expression of *AUR1-C*, and thus AbA resistance, is induced by the binding of a GAL4 activation domain-p53 fusion protein (GAL4 AD-p53) to the p53 binding site.

The pAbAi vectors cannot be propagated episomally in yeast; but can only be stably maintained through integration into the host genome. Integration is accomplished via homologous recombination between the vector's *URA3* gene and the *ura3-52* locus of the Y1HGold yeast strain provided in the Matchmaker Gold Yeast One-Hybrid System. *URA3* is a nutritional marker that can also be used for selection of recombinant yeast. The vectors also contain a Col E1 origin of replication and an ampicillin resistance gene (Amp^r) for propagation and selection in *E. coli*. For more detailed information, see the pAbAi and the p53-AbAi Vector Information packets (PT4091-5 and PT4092-5, respectively).

Appendix A: Plasmid Information continued

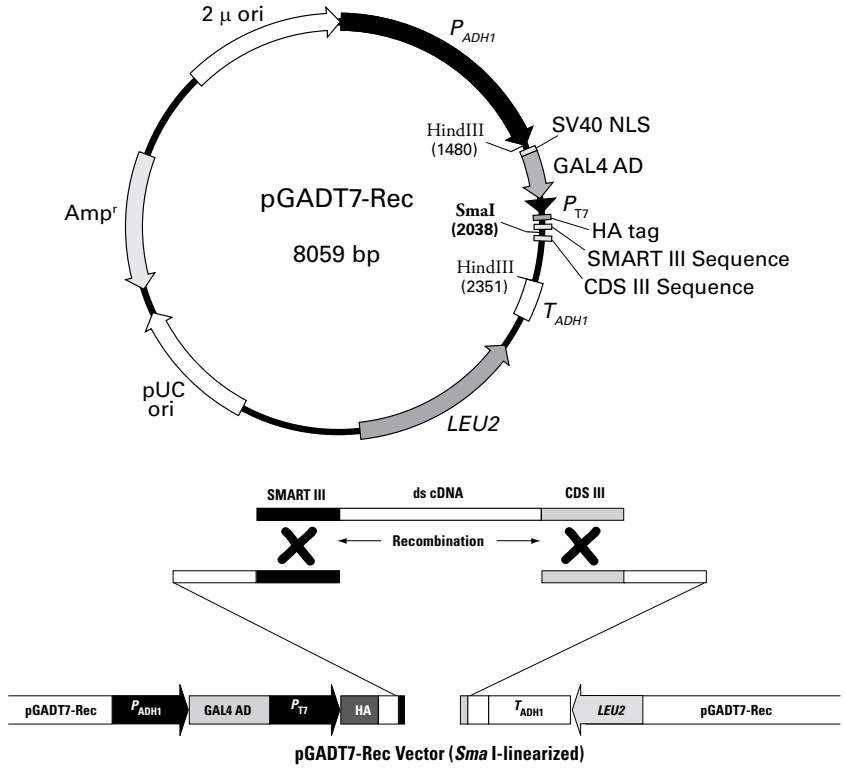


Figure 11. Map of pGADT7-Rec Vector. pGADT7-Rec is engineered for constructing GAL4 AD/cDNA libraries by homologous recombination in yeast. To construct AD fusions in pGADT7-Rec, first generate double-stranded (ds) cDNA using SMART DNA Synthesis. Then transform yeast with the cDNA products and SmaI-linearized pGADT7-Rec. Cellular recombinases will use the ds cDNA to repair the gap in pGADT7-Rec (Lower Panel). Successful recombination results in a fully functional, circular expression vector, which confers the Leu+ phenotype to Leu auxotrophs such as yeast strain Y1HGold. For more detailed information, see the pGADT7-Rec Vector Information packet (PT3530-5).

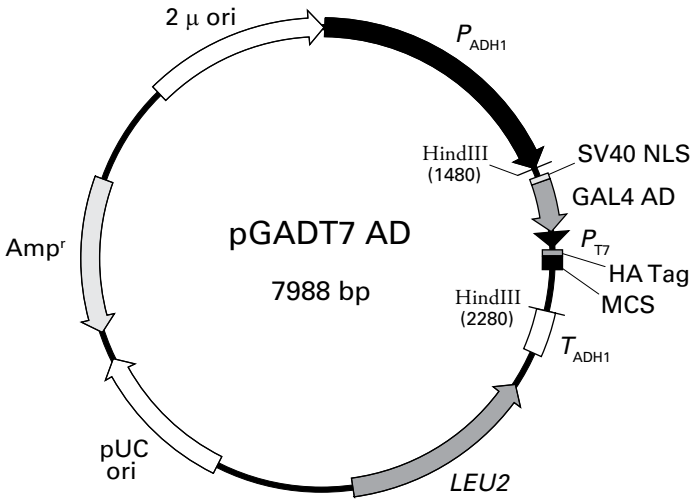


Figure 12. Map of pGADT7 Vector. pGADT7 AD is a yeast expression vector designed to express a protein of interest fused to a GAL4 activation domain (AD; amino acids 768–881). Transcription of the GAL4 AD fusion is driven by the constitutively active ADH1 promoter (P_{ADH1}), and is terminated at the ADH1 transcription termination signal (T_{ADH1}). For more detailed information, see the pGADT7 AD Vector Information packet (PT3249-5).

Appendix B: Yeast Growth Media & Supplements

A. Ready-to-go Media Pouches Available from Clontech

Clontech offers media sets with a complete assortment of mixes in convenient, "ready-mixed" foil pouches, for use with the Matchmaker Gold Yeast One-Hybrid Library Screening System. See Table VI for a list of the components of the **Yeast Media Set 1** (Cat. No. 630493), and the **Yeast Media Set 1 Plus** (Cat. No. 630494), which also contains **Aureobasidin A**, which is required for yeast one-hybrid library screening using the Matchmaker Gold Yeast One-Hybrid Library Screening System.

- See Table VII for information for purchasing each of the media mixes separately, in packs of 10 pouches,

Table VI: Components of Yeast Media Set 1 & Yeast Media Set 1 Plus

Media Pouch	Quantity of Pouches Supplied	Volume of Media/Pouch
YPDA Broth	2	0.5 L
YPDA with Agar	1	0.5 L
SD/–Leu Broth	1	0.5 L
SD/–Leu with Agar	10	0.5 L
SD/–Ura with Agar	2	0.5 L
Also included in Yeast Media Set 1 Plus		
Aureobasidin A	1 mg	—

Table VII: Individual Yeast Media Pouches for Matchmaker Gold Protocols

Yeast Media Pouches	Cat. No.	Quantity of Pouches	Volume of Media/ Pouch
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/–Ura Broth	630314	10	0.5 L
SD/–Ura with Agar	630315	10	0.5 L
SD/–Leu Broth	630310	10	0.5 L
SD/–Leu with Agar	630311	10	0.5 L

Appendix B: Yeast Growth Media & Supplements continued

B. General Media Preparation Instructions

- Prepare media by dissolving pouch contents in 500 ml dd$H_2$0, autoclave for 15 min at 121˚C, and allow to cool before use (or filter-sterilize broth media). Do not over-autoclave.
- The premixed media pouches supplied by Clontech do 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 Clontech Yeast Media Protocol-at-a-Glance (PT4057-2) available at **www.clontech.com**

C. Freezing Medium

Mix 100 ml YPDA (sterile) and 50 ml 75% glycerol (sterile). Sterile filter if necessary.

D. Aureobasidin A: Stock Solution Recipe:

To prepare a stock solution of 500 µg/ml, dissolve 1 mg Aureobasidin A (Cat. No. 630466) in 2 ml of absolute ethanol. Store at 4°C.

E. Aureobasidin A: Working Concentrations of 100–200 ng/ml in agar media

To autoclaved agar media that has been allowed to cool sufficiently for pouring plates (~50°C), add the appropriate amount of Aureobasidin A stock solution (500 µg/ml) per 500 ml of media to achieve the desired final concentration. Mix well and pour plates immediately.

Final Concentration	Volume of AbA Stock Solution/500 ml media
100 ng/ml	100 µl
200 ng/ml	200 µl

Appendix C: SMART™ Technology Overview

A. SMART Technology

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

B. Mechanism of cDNA Synthesis

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

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

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

In the second step, ss cDNA is amplified by LD PCR to produce ds cDNA. We recommend using the **Advantage 2 PCR Polymerase Mix** (Cat. No. 639201) for cDNA amplification. The Advantage® 2 Polymerase Mix consists of TITANIUM™ Taq DNA Polymerase (a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase), TaqStart Antibody to provide automatic hot-start PCR, and a minor amount of a proofreading polymerase. This polymerase system lets you amplify cDNA (as large as 20 kb) with a fidelity rate significantly higher than that of conventional PCR.

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Aureobasidin Resistance Gene
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