Matchmaker™ One-Hybrid Library Construction & Screening Kit

PT3529-1 (PR732190)
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I. Introduction & Protocol Overview

The **Matchmaker One-Hybrid Library Construction & Screening Kit** provides a simple and highly efficient method for constructing and screening cDNA libraries for yeast one-hybrid screening. Your library is constructed and screened directly in yeast, by *in vivo* recombination.

There is no need for labor-intensive library cloning, amplification and harvesting in *E.coli*. Matchmaker Library Construction & Screening Systems use SMART™ cDNA Synthesis technology, which allows you to construct cDNA libraries from any tissue source starting with as little as 100 ng of total RNA.

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

One-hybrid assays enable you to identify and characterize proteins that bind to a target, cis-acting DNA sequence. In a Matchmaker one-hybrid assay, potential DNA-binding proteins, **the Prey**, are expressed as fusions to the GAL4 activation domain in pGADT7-Rec2. The target DNA sequence, or **Bait Sequence**, is cloned into pHIS2.1 as one copy or tandem repeats. Interaction between a DNA-binding protein and the target sequence stimulates transcription of *HIS3* (Figure 1), enabling the yeast host strain, Y187, to grow on minimal media lacking histidine. Both Bait and Prey plasmids in this system are low-copy-number vectors, yielding fewer false positives than the high-copy-number vectors that are generally used for two-hybrid studies.

This technology can be used to:

- identify novel DNA-protein interactions
- confirm suspected interactions
- define interacting domain sequences

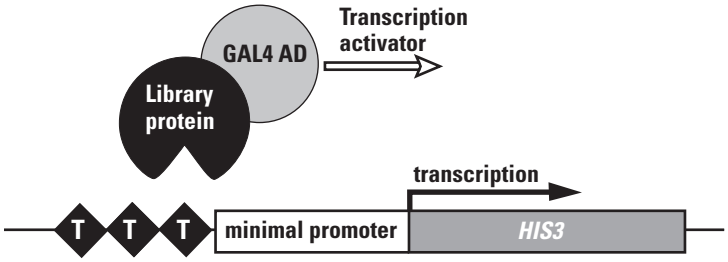

Figure 1. Screening for protein-DNA interactions with the Matchmaker One-Hybrid System. In this construct, three copies of the DNA target (T) have been inserted into the pHIS2.1 reporter vector.

Nutritrion Reporter to Detect One-Hybrid Interactions

HIS3. Yeast strainY187 is unable to synthesize histidine and is therefore unable to grow on media that lack this essential amino acid. When the prey and your bait sequence interact, His3p is expressed from the pHIS2.1 reporter vector and permits the cell to biosynthesize histidine and grow on a his- minimal medium. 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3p, is used to inhibit low levels of His3p expressed in the absence of an activating prey protein (Fields, 1993; Durfee et al., 1993).

Matchmaker Screening Protocol Overview

The entire Matchmaker screening process consists of the following steps:

- Step 1. Perform control experiments
- Step 2. Clone your target sequence (bait) and optimize 3-AT
- Step 3. Construct and screen library by cotransformation and *in vivo* recombination
- Step 4. Confirm and interpret results

I. Introduction & Protocol Overview continued

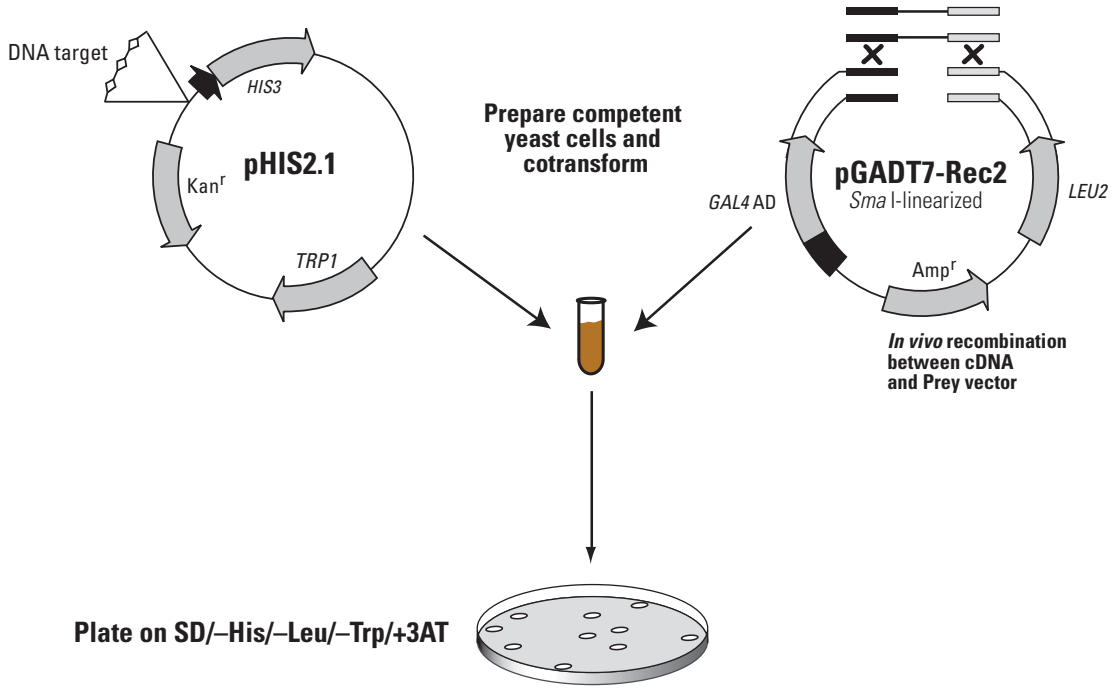

Figure 2. One-hybrid library construction and screening. Your target bait sequence is cloned upstream of the His3 reporter in pHIS2.1. The high-complexity pretransformed cDNA library, which expresses fusions with the Gal4 AD, is generated by cotransformation of the library cDNA with pGADT7-Rec2. Expression from the HIS3 reporter is detected in colonies that are able to grow on minimal medium that lacks histidine and contains 3-AT.

II. List of Components

This kit contains sufficient reagents to make five one-hybrid libraries.

Store deionized H_2O, CHROMA SPIN Columns, NaCl Solution, Dropout (DO) Supplements, NaOAc, LiAc, PEG, TE Buffer, and YPD Plus Medium at room temperature. Store yeast strains, Control Poly A+ RNA, and the SMART III Oligo at –70°C. Store all other reagents at –20°C.

First-strand cDNA synthesis

- 10 µl SMART III* Oligo (10 µM; 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3')

  *The SMART III Oligo is a modified oligo.
- 10 µl CDS III Primer (10 µM; 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$VN-3')*
- 10 µl CDS III/6 Primer (10 µM; 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-NNNNNN-3')*

  *N = A, G, C, or T; V = A, G, or C
- 20 µl MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase
- 7 µl RNase H
- 100 µl 5X First-Strand Buffer (250 mM Tris (pH 8.3); 30 mM $MgCl_2$; 375 mM KCl)
- 100 µl DTT (dithiothreitol; 20 mM)
- 5 µl Control Poly A^+ RNA (Human Placenta; 1 µg/µl)
- 50 µl dNTP Mix (dATP, dCTP, dGTP, dTTP, 10 mM each)

cDNA amplification

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

cDNA purification

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

One-Hybrid Library Construction

- 20 µg pHIS2.1 Reporter Vector (500 ng/µl)
- 20 µg pGADT7-Rec2 AD Cloning Vector (Sma I-linearized; 500 ng/µl)
- 20 µg pGAD-Rec2-53 Control Vector (500 ng/µl)
- 20 µg p53HIS2 Control Vector (500 ng/µl)
- 0.5 ml *S. cerevisiae* strain Y187
- 50 ml NaCl Solution (0.9%)
- 10 g –Leu DO Supplement
- 10 g –Trp DO Supplement
- 10 g –Leu/–Trp DO Supplement
- 10 g –His/–Leu/–Trp DO Supplement

II. List of Components continued

Yeastmaker Yeast Transformation System 2 (provided with the One-Hybrid Kit)

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

Other

- Matchmaker One-Hybrid Library Construction & Screening Kit User Manual (PT3529-1)
- Yeast Protocols Handbook (PT3024-1)
- pGADT7-Rec2 Vector Information Packet (PT3704-5)
- pHIS2.1 Vector Information Packet (PT3951-5)

III. List of Abbreviations

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
Bait	pHIS2.1 containing repeats of your target sequence of interest
Prey	pGADT7-Rec2 vector containing a library gene

Yeast Phenotypes

His–, Leu–, or Trp–	Requires histidine (His), leucine (Leu), or tryptophan (Trp) in the medium to grow; i.e., is 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
TDO	Triple dropout medium: SD/–His/–Leu/–Trp or SD/–Ade/–Leu/–Trp
YPD	A blend of yeast extract, peptone, and dextrose in optimal proportions for growth of most strains of *S. cerevisiae*
YPDA	YPD medium supplemented with adenine (0.003% final concentration)

IV. Host Strain Information

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

Table I: Yeast Host Strain Genotypes

Strain	Genotype	Reporters	Transformation Markers	Reference
Y187[1]	*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

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

Table II: Phenotype Testing on Various SD Media

Strain	SD/–His	SD/–Leu	SD/–Trp	SD/–Ura
Y187	–	–	–	+
Y187[p53HIS2]	–	–	+	+
Y187[pGAD-Rec2-53]	–	+	–	+
Y187[pGAD-Rec2-53/p53HIS2]	+	+	+	+

V. Yeast Media & Additional Materials Required

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

- Minimal media that is routinely used for culturing *S. cerevisiae* is called "synthetically defined" medium or SD. **SD base** supplies everything that a yeast cell needs to survive (including carbon and nitrogen sources) with the exception of essential amino acids, which are added separately as a **dropout (DO) supplement**. The particular DO supplement that is chosen will determine which plasmids and/or activated reporters are selected for.
- For example, SD base mixed with -Leu/-Trp dropout supplement (SD/-Leu/-Trp) is used to select for the bait and prey plasmids. Cells harboring these plasmids are able to grow because the vectors encode tryptophan and leucine biosynthesis genes, respectively, that are otherwise absent from the cell. **We often refer to SD/-Leu/-Trp as Double Dropout (DDO) in this user manual.**
- Similarly, SD/-His/-Leu/-Trp selects for the presence of bait and prey plasmids, but also selects for the activation of the *HIS3* reporter as part of the one-hybrid assay. Colonies that grow on this **Triple Dropout (TDO)** contain both bait and prey plasmids and also express proteins that interact with the target sequence cloned into pHIS2.1.

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

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

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

V. Yeast Media & Additional Materials Required continued

Table IV: Additional Media Supplements

Supplement Name	Clontech Cat. No. [1]	Stock Solution Concentration
L-Adenine Hemisulfate	Sigma A9129	0.2% stock solution
L-Leucine	Sigma L8000	–
Kanamycin Sulfate	–	50 mg/ml stock solution
Dimethyl Formamide	–	–
3-AT (3-Amino-1,2,4-Triazole)	Sigma A8056	1 M (84 mg/ml) stock solution

[1] Unless otherwise specified

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

Rich Media

YPDA liquid

YPDA agar

Single DO Media

SD/-Trp liquid

SD/-Trp agar

SD/-Leu liquid

SD/-Leu agar

Double DO Media

SD/-Leu/-Trp liquid

SD/-Leu/-Trp agar

Triple DO Media

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

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

VI. Control Experiments

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

A. General Considerations

To familiarize yourself with the procedures and expected results of a one-hybrid assay, perform these control transformations before you begin screening the library.

- **3-amino-1,2,4-triazole (3-AT)** is a competitive inhibitor of the yeast *HIS3* protein (His3p). 3-AT is used throughout the protocols in this user manual to inhibit low levels of His3p expressed in the absence of an activating prey protein (Fields, 1993; Durfee et al., 1993).
- **p53HIS2** is a positive control reporter vector that contains three tandem copies of the *cis*-acting DNA consensus sequence recognized by p53. p53HIS2 was constructed by inserting the DNA targets into the multiple cloning site of pHIS2. As a result, the DNA targets are positioned just upstream of the minimal promoter of the *HIS3* locus ($P_{\text{min}HIS3}$) and the *HIS3* reporter gene.
- **pGAD-Rec2-53** is a positive control vector that encodes murine p53 as a fusion with the GAL4 AD. Yeast cells that contain both p53HIS2 and pGAD-Rec2-53 should grow on minimal SD media lacking histidine and containing 50 mM 3-AT—i.e., on SD/–His/–Leu/–Trp/3-AT.
- A **negative control** can also be performed using pHIS2.1 and pGAD-Rec2-53. Yeast cotransformed with these two plasmids will grow on SD/-Leu, SD/-Trp, and SD/-Leu/-Trp minimal media; colonies should not grow on TDO (-His/-Leu/-Trp) + 50mM 3-AT.
- Table V indicates the selection media required for transformants containing the bait vector, the prey vector, or both, as well as the selection for protein-DNA interactions.

Table V: Cotransforming Y187 with Control Plasmids

Cotransformation	Plate on SD Minimal Agar Medium	Selects for
p53HIS2.1 + pGAD-Rec2-53	–Leu	pGAD-Rec2-53
	–Trp	pHIS2.1
	–Leu/–Trp (DDO)	Cotransformed pHIS2.1/pGAD-Rec2-53
	His/-Leu/-Trp/50 mM 3-AT	One-hybrid Interactions

Protocol 7–10 days

B. Protocol: Cotransformation

1. Materials:
   - Y187 Yeast Strain
   - Yeastmaker Yeast Transformation System 2
     [provided with the One-Hybrid Kit or available separately (Cat. No. 630439)]
   - SD-/Trp agar plates (Appendix D)
   - SD-/Leu agar plates (Appendix D)
   - SD/-His/-Leu/-Trp/50 mM 3-AT agar plates (Appendix D)
   - YPD liquid medium + 25% glycerol (Freezing Medium)
   - YPDA agar (Appendix D)

2. Streak the provided Y187 strain from the glycerol stock on YPDA.

VI. Control Experiments continued

3. Grow at 30°C for 3 days.

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

4. Pick one 2–3 mm colony and cotransform the following using the Small Scale Transformation Protocol (Section XII.B).
   - Positive Control: p53HIS2 and pGAD-Rec2-53
   - Negative control: pHIS2.1 and pGAD-Rec2-53
5. Plate 100 µl of 1/10, 1/100, and 1/1,000 dilutions on each of the following agar plates. Incubate plates (colony side down) at 30°C for 3–5 days.
   - SD/-Trp
   - SD/-Leu
   - SD/-Leu/-Trp (DDO)
   - SD/-His/-Leu/-Trp//50 mM 3-AT (TDO + 3-AT)

NOTE: We generally do not recommend plating undiluted transformed cells for the reasons described in Section XIII.A.

6. Expected results after 3–5 days:

   Positive control: healthy colonies on DDO, similar number of healthy colonies on TDO/3-AT agar plates.

   Negative control: healthy colonies on DDO, no colonies on TDO/3-AT agar plates.

Notes:

- For positive interactions, theoretically, the number of colonies should be the same on both DDO and TDO/3-AT. DDO selects for both plasmids and TDO/3-AT selects for the plasmids as well as for the interactions of the p53 protein with its target sequence. However, a difference (approximately 10% lower on TDO) 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, respectively.

7. Pick healthy 2 mm colonies from DDO plates, restreak onto fresh DDO plates, and incubate at 30°C for 3–4 days.
   - Short-term storage (< 4 weeks): Seal with Parafilm and store at 4°C.
   - Long-term storage: Scoop a large healthy colony and fully resuspend in 500 µl of YPD + 25% glycerol. Store at –80°C.

Notes:

- These cotransformants 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. Constructing and Testing your Bait

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Detailed instructions are provided to synthesize and clone your target element (Section A) and to test your target reporter (bait) for histidine expression (Section B).

2 days

A. Synthesize and Clone Your Target Element

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

1. Design and synthesize two antiparallel oligonucleotides with overhanging sticky ends compatible with the sticky ends of digested pHIS2.1.

NOTES:

- The annealed oligos consist of one or more copies of the target element with a different restriction site on each end. When the two strands are annealed, the resulting double-stranded DNA will have a different overhang at each end for directional cloning into pHIS2.1.
- We recommend that you also create a pHIS2.1 construct containing a mutant sequence with point mutations to use as a negative control in Section IV.
- If there is a protein that is already known to interact with your sequence, you may wish to clone this into pGADT7-Rec2 for use as a positive control for your bait sequence. (See Appendix A for a simple cloning procedure via cotransformation.)

2. Anneal the oligonucleotides (use a thermal cycler)
   a. Resuspend each oligonucleotide in TE buffer to a final concentration of 100 µM.
   b. Mix the oligos for the top strand and the bottom strand at a 1:1 ratio. This mixture will ultimately yield 50 µM of ds oligo (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.

   The annealed oligonucleotide is now ready for ligation into the pHIS2.1 vector. Alternatively, the annealed oligonucleotide can be stored at –20°C until ready to use.

3. Ligating the ds oligonucleotide into pHIS2.1
   a. Dilute the annealed oligo (from Step 2.g) 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 an excess of the oligo will inhibit ligation.

VII. Constructing and Testing your Bait continued

b. Assemble a ligation reaction for each experimental annealed oligonucleotide,. For each ligation, combine the following reagents in an Eppendorf tube:

- 1 µl digested pHIS2.1 Vector (50 ng/µl)
- 1 µl diluted, annealed oligonucleotide (0.5 µM)
- 1.5µl 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 and transform *E. coli*.

Protocol 5–7 days

B. Protocol: Testing your Target-Reporter (Bait)

3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the yeast *HIS3* protein (His3p). 3-AT is used throughout the protocols in this user manual to inhibit low levels of His3p expressed in the absence of an activating prey protein (Fields, 1993; Durfee et al., 1993).

ATTENTION: Successful use of any yeast one-hybrid system is dependent upon no/low recognition of your target sequence by endogenous yeast transcription factors. For this reason it is critical to test your construct for histidine expression before screening the library. The following experiment will determine how much 3-AT you will require in your library screen to suppress any basal expression from your specific bait construct.

1. Materials
   - Y187 Yeast Strain
   - Yeastmaker Yeast Transformation System 2
     [provided with the One-Hybrid Kit or available separately (Cat. No. 630439)]
   - SD-/Trp Agar plates (Appendix D)
   - SD/-His/-Trp/ agar plates (Appendix D)
   - SD/-His/-Trp/ 50mM 3-AT agar plates (Appendix D)
   - SD/-His/-Trp/ 100mM 3-AT agar plates (Appendix D)
   - YPD liquid medium + 25% glycerol (Freezing Medium)
2. Perform Control Experiments (Section VI)
3. Transform your bait construct using the Small Scale Transformation Protocol (Section XII).

VII. Constructing and Testing your Bait continued

4. Plate 100 µl of 1/10 and 1/100, dilutions on on each of the following agar plates. Incubate plates (colony side down) at 30°C for 3–5 days.

   - SD/-Trp
   - SD/-His/-Trp
   - SD/-His/-Trp/50 mM 3-AT
   - SD/-His/-Trp/100 mM 3-AT

NOTE: We generally do not recommend plating undiluted transformed cells for the reasons described in Section XIII.A.

5. Expected results after 3–5 days:

- Many healthy colonies on SD/-Trp
- Siginificantly fewer colonies on SD/-His/-Trp
- No colonies on either SD/-His/-Trp/50 mM 3-AT or SD/-His/-Trp/100 mM 3-AT

NOTES:

- If you still have many colonies on media containing 50 mM 3-AT but no colonies on 100 mM 3-AT, use 100 mM 3-AT for your library screen.
- If you have no colonies with either dose of 3-AT, you have a choice of using either concentration; 100 mM for highest stringency.
- If you have a similar number of colonies on SD/-His/-Trp/100mM 3-AT, but they grow very slowly compared to the positive controls on SD/-His/-Leu/-Trp/100 mM 3-AT, you still may be able to perform the one-hybrid library screening. Dilute your co-transformed bait/library (Section IX, Step 6) onto more plates and avoid picking very small colonies after 4–5 days on SD/-His/-Leu/-Trp/100 mM 3-AT. Large healthy colonies may harbor prey proteins that interact strongly with your bait sequence.

VIII. Generating the cDNA for Your Library

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Detailed instructions are provided for first-strand cDNA synthesis (Section A), cDNA amplification using long distance PCR (LD-PCR) (Section B), and column purification of ds cDNA using a CHROMA SPIN TE-400 column (Section C).

Use the following protocol for generating cDNA using Clontech's simple and highly efficiency SMART™ technology (Figure 3). For detailed description of SMART technology, refer to Appendix C.

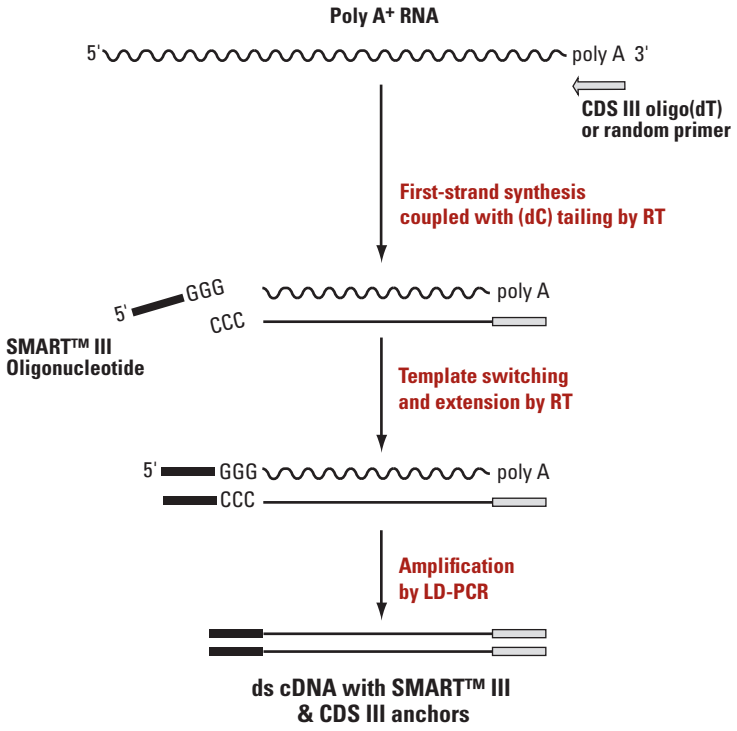

Figure 3. Synthesis of high-quality ds cDNA using SMART technology.

Protocol
1 day

A. Protocol: First Strand cDNA Synthesis

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

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

The procedure consists of three steps:

- First-strand cDNA synthesis
- Ampification of cDNA by long distance PCR (LD-PCR)
- Column purification of ds cDNA with a CHROMA SPIN TE-400 column

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

VIII. Generating the cDNA for Your Library continued

1. **Prepare:** high quality Poly A or total RNA

   NOTE: We recommend NucleoSpin® RNAII kits (Cat. Nos. 635990, 635991 & 635992) for purification of total RNA form a variety of sources, and NucleoTrap mRNA Kits for polyA+ mRNA enrichment from total RNA.

2. Combine and mix the following reagents in a sterile microcentrifuge tube:

   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 or CDSIII/6 Primer

   1–2 µl Deionized H_2O (to bring volume up to 4.0 µl).

   4.0 µl total volume

   NOTE: For the control reaction, use 1 µl [1 µg] of the control RNA.

   CDSIII = Oligo-dT

   CDSIII/6 = Random Primer

3. Incubate at 72°C for 2 min
4. Cool On Ice for 2 min, spin briefly
5. To the reaction add the following and mix by tapping:

   2.0 µl 5X First-Strand Buffer

   1.0 µl DTT (20 mM)

   1.0 µl dNTP Mix (10 mM)

   1.0 µl MMLV Reverse Transcriptase

   9.0 µl total volume

6. **ONLY if using Random Primer (CDSIII/6)** [Omit this step if using Oligo-dT (CDSIII), and continue to Step 7]

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

7. Incubate at 42° for 10 min.

   NOTE: Carry out the incubation in a hot lid thermal cycler. If you are using a water bath or non-hot-lid cycler, add a drop of mineral oil to prevent loss in volume due to evaporation.

8. Add 1 µl SMART III oligo, mix and incubate at 42°C for 1 hr.

9. Place the tube at 75°C to terminate first strand synthesis.
10. Cool to room temperature, add 1µl RNase H (2 units)
11. Incubate at 37° for 20 min
12. Proceed to LD-PCR amplification (Section VIII.B).

    NOTE: Any first strand synthesis reaction that is not used immediately should be stored at –20°C for up to 3 months.

VIII. Generating the cDNA for Your Library continued

Protocol
5 hr

B. Protocol: Amplify cDNA Using Long Distance PCR (LD-PCR)

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

Table VI. Relationship between Amount of RNA and Optimal Number of Thermal Cycles

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

1. **Prepare:**
   - First-strand cDNA (Section A)
   - Preheat thermal cycler
2. Set up **TWO** 100 µl PCR reactions for each experimental sample and one reaction for the control sample:

   2 µl First-Strand cDNA (Section 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 10X GC-Melt Solution

   2 µl 50X Advantage 2 Polymerase Mix

   100 µl total volume
3. Begin thermal cycling using the following parameters:
   - 95°C 30 sec
   - x Cycles[a]

     95°C 10 sec

     68°C 6 min[b]
   - 68°C 5min

a Refer to table VI 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.

VIII. Generating the cDNA for Your Library continued

4. Analyze a 7 µl aliquot of the PCR product from each sample alongside 0.25 µg of a 1 kb DNA size marker on a 1.2% agarose/EtBr gel.

   Typical results obtained with Human Placenta Poly A+ RNA after column chromatography are shown in Figure 4.

   NOTE: If your PCR product does not appear as expected, refer to the Troubleshooting Guide (Section XII).

5. Proceed with Column Chromatography (Section C) or store ds cDNA at –20°C until use.

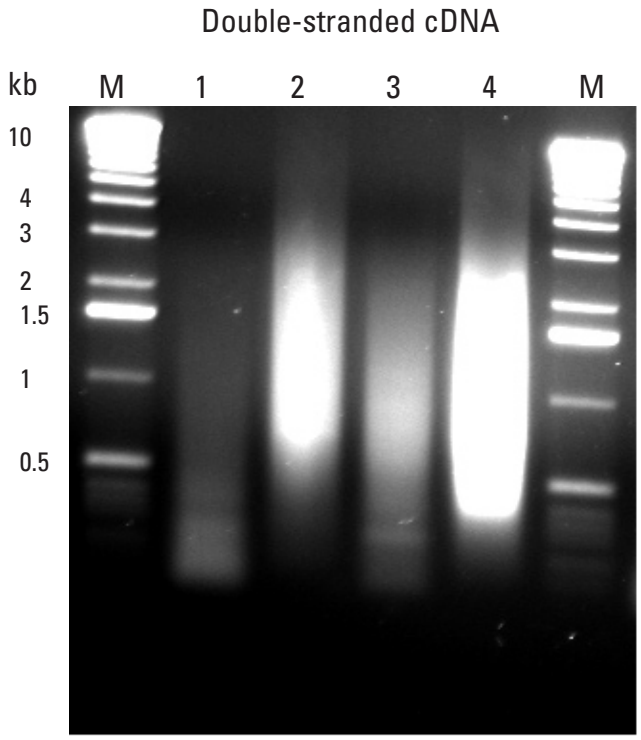

Figure 4. Double-stranded cDNA synthesized from Control Human Placenta Poly A+ RNA. 1 µl (1.0 µg) of Control Human Placenta Poly A+ RNA was used as the template for first-strand cDNA synthesis. Two first-strand samples were prepared: One with a random primer (our CDS III/6 Primer; Lanes 1 & 3), and the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

C. Protocol: Purify ds cDNA with CHROMA SPIN TE-400 Columns

Protocol 2 hr

In the following protocol, a CHROMA SPIN TE-400 Column is used to select for DNA molecules >200 bp.

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

1. **Prepare:**
   - dsCDNA by LD-PCR (Section B)
   - sodium acetate (3 M; pH 5.3)
   - ice cold ethanol (95–100%)

VIII. Generating the cDNA for Your Library continued

2. Prepare one CHROMA SPIN TE-400 column for each 93µl cDNA sample (see Figure 5)
   - Invert each column several times to resuspend the gel matrix completely
   - Snap off the break away from the bottom of the column
   - Place the column in a 2ml collection tube (supplied)
   - Remove the top cap

   NOTE: You will use 2 columns for each library to be constructed.

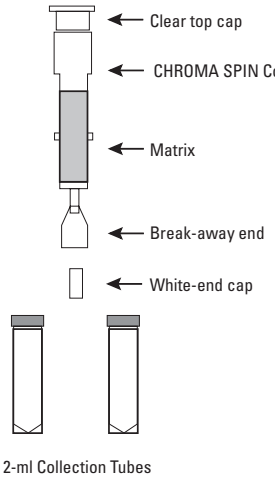

Figure 5. CHROMA SPIN column and collection tubes. Note that a conventional, tapered 1.5-ml microcentrifuge tube can be substituted for the 2-ml collection tube. This will allow the sample to be confined to a narrower area for easier handling.

3. Centrifuge at 700 g for 5 min to purge the equillibration buffer, then discard collection tube and buffer.

   The matrix will appear semi-dry.

   NOTE: We recommend swing 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, resulting in inconsistent purification.

4. Replace spin column in second collection tube and apply your 93 µl sample to the CENTER of the flat surface of the gel matrix.

   NOTE: Do not allow sample to flow along inner wall of the column.

5. Centrifuge at 700 g for 5 min, your purified sample is now in the collection tube.
6. Combine your two purified samples into a single microcentrifuge tube and ethanol-precipitate the cDNA:
   - Add 1/10th vol 3M Sodium Acetate (pH 5.3)
   - Add 2.5 vol of ice-cold ethanol (95–100%)
   - Place in –20°C freezer for 1hr
   - Centrifuge at 14,000 rpm for 20 min at room temperature
   - Discard the supernatant; do not disturb the pellet
   - Centrifuge briefly at 14,000 rpm and remove remaining supernatant
   - Air dry the pellet for 10 min
7. Resuspend the cDNA in 20 µl deionized water

   The cDNA is now ready for library construction by *in vivo* recombination in yeast (Section IX).

   NOTE: At this point you should have 2-5 µg of ds cDNA

IX. One-Hybrid Library Screening

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

IMPORTANT: See Appendix A if you plan to test specific prey proteins and not an entire library.

Protocol 5–7 days

1. Materials:
   - Competent Y187 yeast cells (Section XII.A)
   - pHIS2.1/Bait (Section VII)
   - pGADT7-Rec2 (Linear)
   - The following SD agar plates (Appendix D)
     - SD/-Trp (5–10x 100 mm plates)
     - SD/-Leu (5–10x 100 mm plates)
     - SD/-Leu/-Trp (5–10x 100 mm plates)
     - SD/-His/-Leu/-Trp/ 50–100 mM 3-AT (Section VII)(100x 150 mm plates)
   - 2xYPDA liquid medium (Appendix D)
   - 0.5xYPDA liquid medium (Appendix D)
   - kanamycin sulfate (50 mg/ml)
   - YPD + 25% glycerol liquid (freezing) medium
2. Perform Control Experiments and test your pHIS2.1/Bait on SD/-His/Leu/Trp/ 50 mM–100 mM 3-AT (Sections VI and VII)
3. Synthesize ds cDNA using SMART technology (Section VIII) to obtain 2–5 µg of cDNA in a volume of 20 µl.
4. Perform cotransformation of the following using the library scale transformation procedure (Section XII.B):
   - 20 µl SMART-amplified ds cDNA (2–5 µg)
   - 6 µl Linear pGADT7-Rec2 (3 µg)
   - pHIS2.1/Bait vector (5 µg)
5. From the transformation mix spread 100 µl of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on each of the following agar plates.
   - SD/-Trp
   - SD/-Leu
   - SD/-Leu/-Trp
6. Plate the remainder of the transformation mix (~15ml), 150 µl per 150mm plate on the following plates
   - SD/-His/-Leu/-Trp/ 50–100mM 3-AT (~100 plates)
7. Incubate the plates (colony side down) for 3-5 days.

IX. One-Hybrid Library Screening continued

8. **Calculate the number of screened clones** by counting the colonies from the SD/-Leu/-Trp (DDO) plates after 3–5 days.
   - Number of Screened Clones = cfu/ml on DDO x resuspension volume (ml)
   - It is imperative that at least 1 million clones are screened, using less than this will result in less chance of detecting genuine interactions.

Example Calculation

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

Therefore Number of Clones screened = 250 x 15 x 10 x 100 = 3.75 million

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, Step 8 to determine if you screened 1 million independent clones. Optimize the transformation procedure (see Section XIII:Troubleshooting Guide) and repeat the screening procedure.
- Check that your TDO/3-AT growth media performs as expected with the positive and negative controls.
- If you screened >1 million independent clones and detected no positive colonies on high stringency TDO/ 100mM 3-AT , repeat the screen with a reduced 3-AT concentration.
- 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 3-AT concentration for your bait (Section VII.B)?

- Check that your TDO/3-AT media performs as expected with the positive and negative controls.
- If you used 50 mM 3-AT, repeat the screen with 100 mM 3-AT.
- Pick only large healthy colonies after 3-5 days to analyze further in Section XI.
- Your bait may interact with a partner that is abundant in the library. Sort duplicates by Yeast Colony PCR (Section XI.B). After the clones have been sorted into groups, a representative of each unique type can then be analyzed for false positive interactions (Section XI.D).

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

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Detailed instructions are provided for confirmation of phenotype (Section A), yeast colony PCR to eliminate duplicates (Section B), rescue and isolation of library plasmids responsible for activation of the* HIS3 *reporter (Section C), and distinguishing genuine positive from false positive interactions (Section D).

The following represents the recommended order of events to confirm that the positive interactions are genuine. 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 XI.B) to sort the duplicate clones before segregating and rescuing the plasmid. If you have a low number of positive clones, you may choose to omit the colony PCR screening step altogether.

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

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

Protocol 3–5 days

A. Confirmation of Phenotype by Restreaking

1. Materials:
   - Single colonies of yeast obtained from the library screen growing on TDO/3-AT
   - SD/-His/-Leu/-Trp/3-AT agar plates (Appendix D)
2. Restreak positive clones to **single colonies** on TDO/3-AT plates (Appendix D).
3. Expected results:

   Positive colonies will grow as healthy single colonies in 2-4 days.

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

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

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

Protocol
4 hr–1 day

B. Protocol: Yeast Colony PCR to Eliminate Duplicates

This procedure uses the Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No 630433) and Advantage 2 PCR Polymerase Mix (Cat. No. 639201). **We strongly recommend using the Advantage 2 Polymerase Mix**, rather than any other DNA polymerase formulation, because we find that it performs well in yeast cell samples.

1. Materials
   - Single colonies of yeast from one-hybrid screen, growing on TDO/3-AT (Appendix D)
   - 0.8% TAE Agarose/EtBr gel
   - Master mix using Advantage 2 Polymerase, as described below.
   - LD insert, screening amplimer (Cat. No. 630433), Advantage 2 (Cat. No. 639201)
2. Prepare a PCR master mix by combining the components specified in Table VI.

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

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

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

Attention

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

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

NOTE

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

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

6. If a high percentage of the colonies appear to contain the same AD/library insert, expand your PCR analysis to another batch of 50 colonies.

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

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

Protocol 2–7 days

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

1. Segregation of Library Plasmid in Yeast

Transformed yeast cells (unlike transformed *E.coli* cells) can harbor more than one version of a related plasmid. This means that in addition to containing a prey vector that expresses a protein responsible for activating the *HIS3* 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 TDO/3-AT, 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

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 TDO/3-AT using the Yeastmaker Yeast Plasmid Isolation Kit (Cat. No. 630441) or other suitable method. An alternative procedure is described in the Yeast Protocols Handbook.
- The nucleic acid rescued directly from yeast will be a mixture of Bait Plasmid, Prey Plasmid, and Yeast Genomic DNA, so you will need to isolate the prey plasmid by transformation and selection in *E.coli,* followed by any standard plasmid preparation procedure (see Step 3).

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

If your bait is cloned in pHIS2.1:

pHIS2.1 contains a kanamycin resistance gene, therefore you can select for the prey plasmid simply by selection on LB plus 100 µg/ml ampicillin using any commonly used cloning strain of *E.coli* (e.g. DH5α, or Fusion-Blue™ from Clontech).

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

D. Protocol: Distinguishing Genuine Positive from False Positive Interactions

With every one-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 6)

- **Genuine Positive**: Both Bait and Prey are required to activate the *HIS3* reporter
- **False Positive**: Prey can activate the *HIS3* reporter even in the presence of a mutated bait sequence.

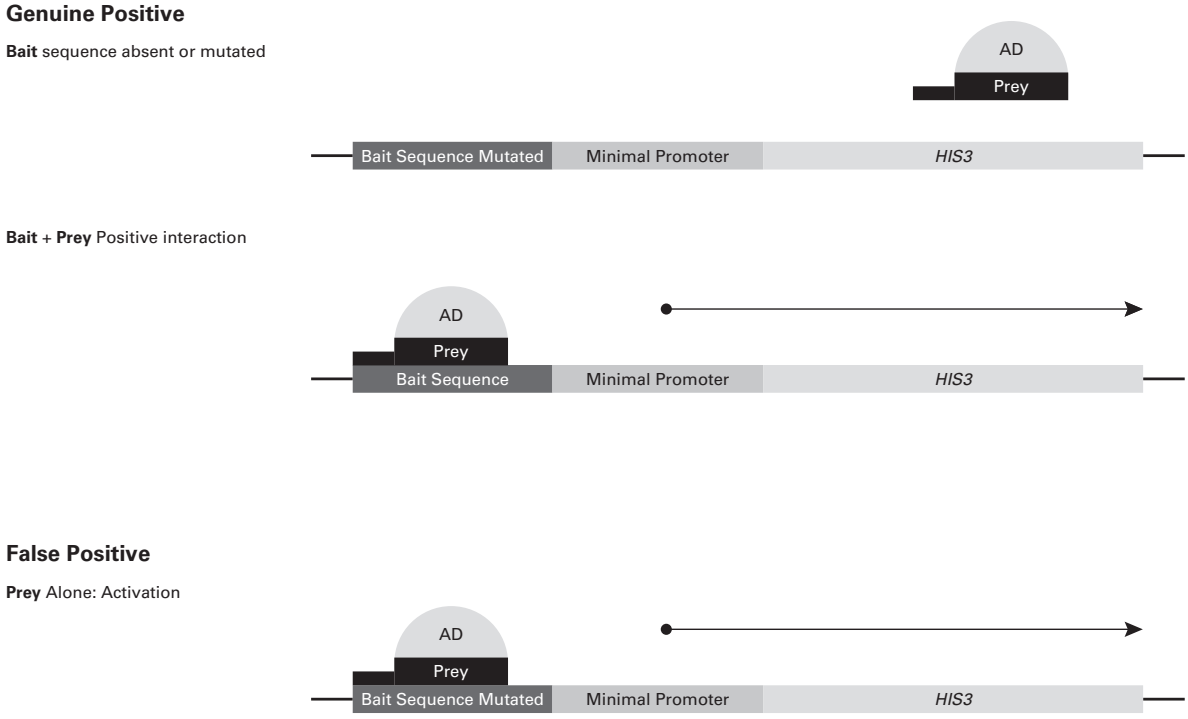

Figure 6. Illustration of the activation of reporter gene expression in genuine and false positives.

Protocol
5 days

You can confirm one-hybrid interactions in yeast on selective media (see Appendix D for recipes) using the following co-transformation procedure (Figure 7).

1. Materials:
   - Competent Y187 cells (Section XII.A)
   - SD/-Leu/-Trp (Appendix D) DDO
   - SD/-His/-Leu/-Trp (Appendix D) TDO + 50–100 mM 3-AT
2. Using the small-scale transformation procedure (Section XII.B), cotransform 100 ng of each of the following pairs of vectors:
   - pHIS2.1/Bait + Prey (in pGADT7-Rec2)
   - pHIS2.1/Mutant sequence + Prey (in pGADT7-Rec2)

   NOTE: We recommend that you perform the experiment side by side with the positive and negative controls (Section VII).
3. Spread 100 µl of 1/10 and 1/100 dilutions of the transformation mix on the following plates:
   - DDO
   - TDO + 3-AT (50–100 mM)

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

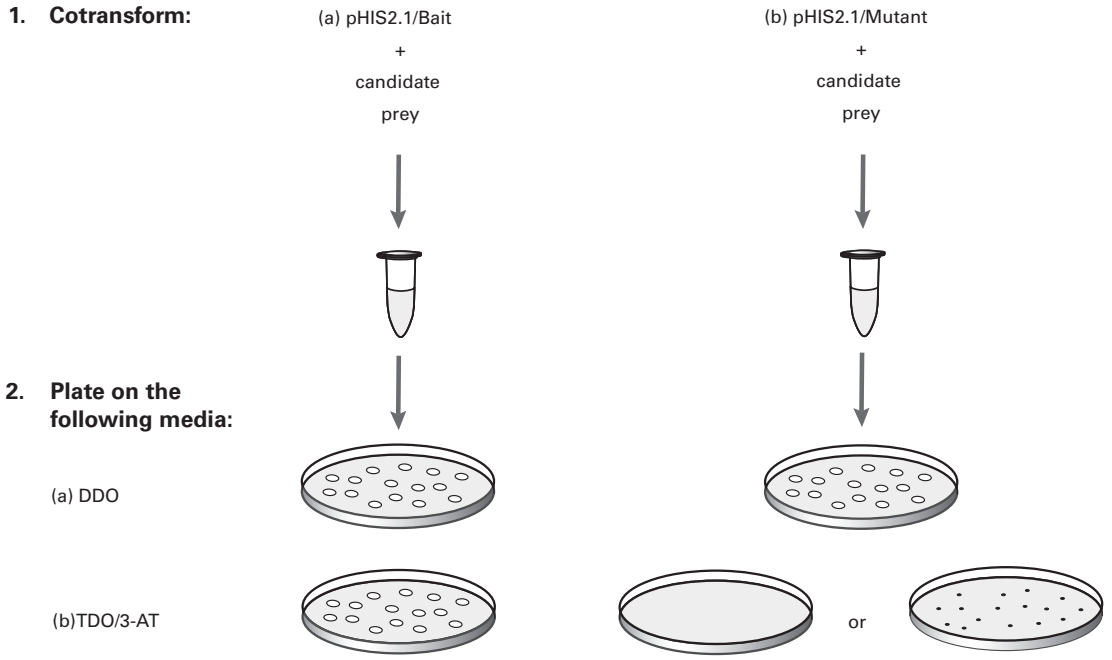

Figure 7. Using cotransformation on selective media to verify interactions. Expected results from genuine interactions.

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

a. Genuine Positive:

Sample	Selective Agar Plate	Distinct 2 mm Colonies
pHIS2.1/Bait + Prey	DDO	Yes
	TDO/3-AT	Yes
pHIS2.1/Mutant + Prey	DDO	Yes
	TDO/3-AT	No (or very small)

b. False Positive:

Sample	Selective Agar Plate	Distinct 2 mm Colonies
pHIS2.1/Bait + Prey	DDO	Yes
	TDO/3-AT	Yes
pHIS2.1/Mutant + Prey	DDO	Yes
	TDO/3-AT	Yes

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

E. Sequence Analysis of a Genuine Positive

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

- Matchmaker AD LD-Insert Screening Amplimer Set (Cat. No. 630433),
- T7 Sequencing Primer,

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

NOTES:

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

- Most library clones will contain some 3′ untranslated region, be sure to scan the entire sequence to find any portion of coding region fused in-frame to the *GAL4* AD (see Appendix A, Section A).
- Yeast tolerate translational frameshifts. A large ORF in the wrong reading frame may correspond to the protein responsible for the interaction. To verify this, re-clone the insert in-frame (this can be easily done using Clontech's In-Fusion PCR Cloning System (see www.clontech.com) and determine if the *HIS3* reporter is 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 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 occurence.

XII. Yeast Transformation

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

The following protocol assumes that you are using Clontech's **Yeastmaker Yeast Transformation System 2**. When using the components from this kit, high transformation efficiencies of > $3x10^5$ per µg of plasmid are readily achieved.

Protocol
5 days

A. Protocol: Preparation of Competent Yeast Cells

1. Materials:
   - Yeastmaker Yeast Transformation System 2
     [provided with the One-Hybrid Kit or available separately (Cat. No. 630439)]
   - 1.1x TE/LiAc
   - YPDA agar plates (Appendix D)
   - YPDA liquid medium (Appendix D)
   - Appropriate SD selective medium
   - Frozen stock of Y187 cells
   - Sterile, deionized water

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

2. Streak a YPDA agar plate with Y187 cells from a frozen yeast stock. Incubate the plate upside down at 30°C until colonies appear (~3 days).

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

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

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

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

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

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

XII. Yeast Transformation continued

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

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

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

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

B. Protocol: Transformation of Competent Yeast Cells

Protocol
3 hr

1. Materials:
   - Yeastmaker Yeast Transformation System 2 [provided with the One-Hybrid Kit or available separately (Cat. No. 630439)]
   - Competent Yeast Cells (Section XII.A)
   - PEG/LiAc
   - 0.9% (w/v) NaCl
   - DMSO

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

	Small-Scale	Library Scale
2. Combine the following in a **prechilled, sterile tube**:	1.5 ml tube	15 ml tube
• **Plasmid DNA**	100 ng	3 µg linear pGADT7-Rec2 + 20 µl ds cDNA
• **Herring Testes Carrier DNA** (denatured; 10 µg/µl)	5 µl	20 µl

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

3. Add **competent cells** and gently mix.	50 µl	600 µl
4. Add **PEG/LiAc** and gently mix.	500 µl	2.5 ml
5. Incubate at **30°C**.	30 min	45 min

NOTE: Mix cells every 10–15 min.

XII. Yeast Transformation continued

	Small-Scale	Library Scale
6. Add **DMSO** and gently mix.	20 µl	160 µl
7. Incubate in a **42°C water bath**.	15 min	20 min

NOTE: Mix cells every 5–10 min.

8. Centrifuge to pellet yeast cells, discard supernatant.	high speed for 15 sec	700 g for 5 min
9. Resuspend cells in **YPD Plus liquid medium**, incubate shaking for 90 min.	1 ml	3 ml

NOTE: YPD Plus is specially formulated to promote high transformation efficiencies and is strongly recommended for library scale transformations. For small-scale procedures that do not necessarily require the highest transformation efficiencies, YPD liquid medium can be substituted for YPD Plus.

10. **Centrifuge** to pellet yeast cells, discard supernatant.	high speed for 15 sec	700 g for 5 min
11. Resuspend **cell pellet** in 0.9% (w/v) NaCl	1 ml	15 ml

NOTE: This volume (1 ml or 15 ml) is the suspension volume (see Section C).

XII. Yeast Transformation continued

Protocol 3–5 days

C. Protocol: Plating and Determination of Transformation Efficiency

1. Spread 100 µl of 1/10 and 1/100 dilution onto a 100 mm plate containing the appropriate SD selection medium (Section VII.B, Table V). For example:
   - For pHIS2.1, use SD/-Trp
   - For pGADT7-Rec2, use SD/-Leu
   - For cotransformations, use SD/-Leu/-Trp

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

Example Calculation

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

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

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

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

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

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

A. Problems with Patching or Streaking Too Many Cells

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

- Dense plating or patching can result in some growth by cells obtaining nutrients from surrounding dead cells, rather than from the medium. Plating more than the recommended volumes can give the illusion of growth.
- For many protocols described in this user manual you will note that we recommend that you perform serial dilutions prior to plating. If you plate undiluted transformations, for example, you may see a lawn of background growth after 1–2 days. It may be difficult to discern the transformed colonies appearing later over this background growth.
- Be particularly aware of this when verifying phenotypes after screening (see Section XI.A). If, for instance, you patch too many cells, even untransformed yeast may give the appearance of some growth on TDO/3-AT selection medium.

XIV. Troubleshooting Guide

PROBLEM	CAUSE	SOLUTION
Inability to suppress basal *HIS3* expression with 100 mM 3-AT	• Improper media preparation (see Section IV)	Repeat experiment with the control vectors, to confirm, and remake media, if necessary.
	• You are plating too many cells per plate (see Section XIII). An indication of high basal *HIS3* expression is that **single colonies** appear in 5 days. If you do not see single colonies but a lawn instead, it is possible that there are too many cells on the plate.	Repeat with diluted transformation mix.
	• Your target sequence is strongly recognized by endogenous yeast transcription factors (see Section X)	The one-hybrid system may not be suitable in this particular case.
Too few or too many positives	See Section X	See Section X
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 faster growing culture. • Perform the control transformation with pGBT9 supplied in the Yeastmaker transformation kit.
	• Problems with Herring Sperm Carrier DNA quality	Denature and cool your Herring Sperm 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	Ensure that the quality of your cDNA is good (see Section VIII), and that you have >2 µg.
	• Problems with DMSO quality	Purchase a fresh bottle of DMSO, since we find that some batches of DMSO result in low transformation efficiencies.
	• pH of growth medium is not optimal	Ensure that you checked the pH of your growth medium; 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 you adjusted the pH of the media to 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 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, which is why we recommend supplementing YPD with additional L-adenine hemisulfate (Appendix D).

XIV. Troubleshooting Guide continued

PROBLEM	CAUSE	SOLUTION
Failure to detect known protein-DNA interactions	• Only one or two repeats of the target sequence are present.	Try increasing the number of repeats of your target sequence. At Clontech we find that three sequence repeats often results in stronger interactions than one or two sequence repeats. Presumably the central repeat is simply acting as a spacer between two binding sites.
	• If expression of the AD hybrid protein is toxic to the cell, transformants will not grow or will grow very slowly on the selection plate.	Sometimes truncation of the AD hybrid protein will 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 AD hybrid proteins to interact with the target element: – The hybrid proteins are not stably expressed in the host cell. – The fused 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.

XV. References

- An extensive list of Matchmaker System citations can be obtained from our website (**www.clontech.com**).

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Appendix A: Cloning & Screening a Single Gene via Cotransformation

The following protocol provides instructions for cloning and screening a single gene in pGADT7-Rec2 by cotransformation of yeast.This procedure may be used to screen known prey sequences rather than a library. You can blunt-end ligate your sequence into the linear pGADT7-Rec2 via traditional cloning in *E.coli.* However, it is much easier to utilize the highly potent homologous recombination system of *S.cerevisiae*. Simply amplify your prey of interest with additional sequence homologous to the insertion site of pGADT7-Rec2 and cotransform the PCR product and linear pGADT7-Rec2 into competent yeast cells as described below.

1. Materials:
   - cDNA of your prey(s) that you wish to clone
   - pHIS2.1/Bait construct
   - Yeastmaker Yeast Transformation System 2 (Cat. No. 630439)
   - SD/-Leu Agar plates
   - SD/-Trp Agar plates
   - SD/-Leu/-Trp Agar plates
   - SD/-Leu/-Trp/-His/50–100 mM 3-AT agar plates.
   - Primers specific for your gene of interest with additional sequence (see Step 3)
   - Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207)
2. Determine the optimum 3-AT concentration for your bait construct (Section VII.B).
3. Amplify the cDNA of your prey construct via PCR so that it contains additional flanking SMART sequence that is homologous to the insertion site of pGADT7-Rec2. Gel purify the amplified band.

Primer Design:

Forward Primer: 5′-GAATTC CAC CCA AGC AGT GGT ATC AAC GCA GAGTGG- xxx xxx xxx xxx xxx -3′

Reverse Primer: 5′-ATC GAT GCC CAC CCT CTA GAG GCC GAG GCG GCC GAC- yyy yyy yyy yyy yyy -3′

xxx = Codons at the start of your gene of interest

yyy = Reverse complement of the end of your gene of interest

4. Using the small scale transformation procedure (Section XII.B) to cotransform the following:
   - 125 ng amplified cDNA (from Step 2)
   - 250 ng pGADT7-Rec2
   - 250 ng pHIS2.1/Bait or pHIS2.1/Mutant
5. Plate 100 µl of 1/10 and 1/100 dilutions on the following 100 mm dishes
   - SD/-Leu agar plates
   - SD/-Trp agar plates
   - SD/-Leu/-Trp agar plates (DDO)
   - SD/-Leu/-Trp/-His/50–100mM 3-AT agar plates. (TDO/3-AT)

Appendix A: Cloning & Screening a Single Gene via Cotransformation continued

6. Expected results, if there is a genuine interaction, after 3–5 days at 30°C.

Sample	Selective Agar Plate	Distinct 2 mm Colonies
pHIS2.1/Bait + Prey	DDO	Yes
	TDO/3-AT	Yes
pHIS2.1/Mutant + Prey	DDO	Yes
	TDO/3-AT	No (or very small)

Appendix B: Plasmid Information

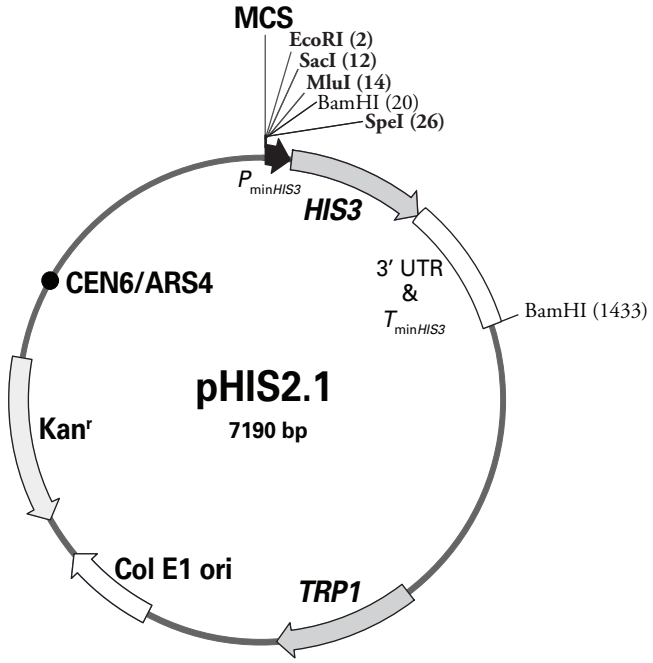
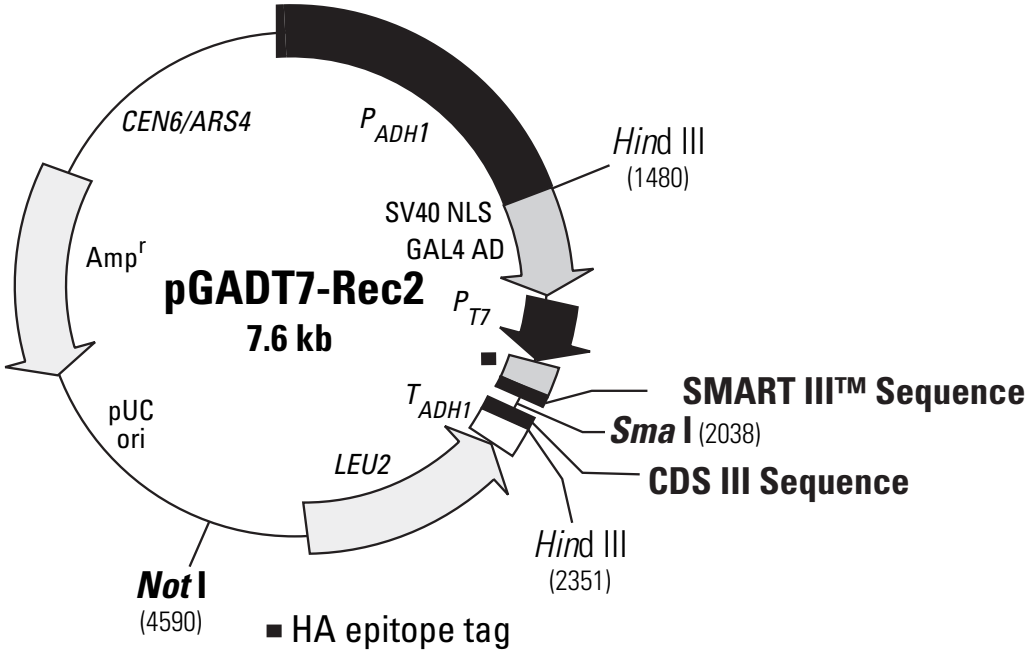


Figure 8. Map and Multiple Cloning Site (MCS) of pHIS2.1 Vector. Unique restriction sites are in bold. pHIS2.1 is a reporter vector that can be used in yeast one-hybrid assays to identify and characterize DNA-binding proteins. The vector was specifically designed for use with the Matchmaker™ One-Hybrid Library Construction & Screening Kit (Cat. No. 630304). It contains a HIS3 nutritional reporter gene, located downstream of a multiple cloning site (MCS) and the minimal promoter of the HIS3 locus (PminHIS3). Cis-acting DNA sequences, or DNA target elements, can be inserted into the MCS and used as baits to screen GAL4 AD/cDNA fusion libraries for proteins that interact with the target sequence. A protein-DNA (or one-hybrid) interaction can be detected by performing the assay in a yeast strain such as Y187 that is auxotrophic for histidine. Positive one-hybrid interactions drive expression of the HIS3 reporter gene, which enables the host cell to grow on histidine-deficient media.

In the absence of activation, the constitutive HIS3 expression from PminHIS3 is very low. During library screening, basal expression of HIS3 is controlled by adding 3-amino-1,2,4-triazole (3-AT) to the medium. The concentration of 3-AT needed to fully suppress HIS3 expression must be determined empirically for each DNA target element.

pHIS2.1 can be maintained in both yeast and bacteria. It contains an autonomous replication sequence (ARS4) and TRP1 nutritional marker for replication and selection in yeast (1, 2); it contains a Col E1 origin and a kanamycin resistance gene (Kanr) for propagation and selection in E. coli. The centromeric sequence CEN6 ensures proper segregation of the plasmid during cell division in yeast (Sikorski et al, 1989; Rose et al, 1991).

Appendix B: Plasmid Information continued



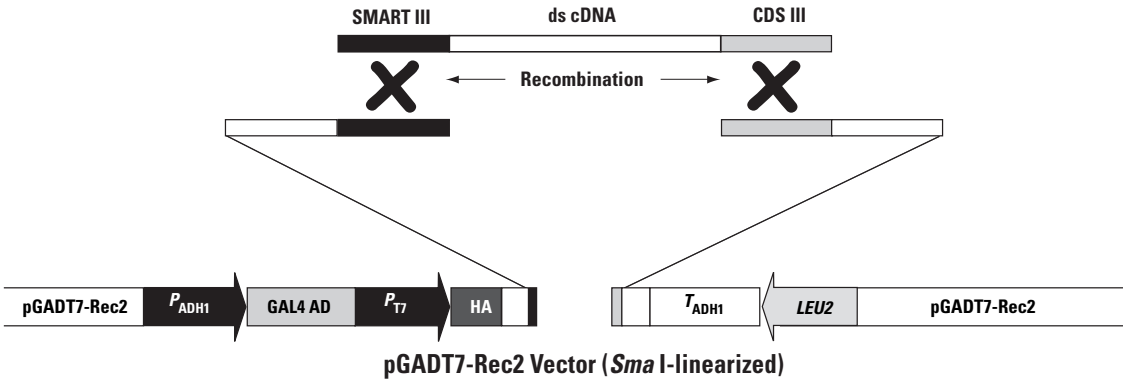


Figure 9. Map of pGADT7-Rec2 Vector. pGADT7-Rec2 is engineered for constructing GAL4 AD/cDNA libraries by homologous recombination in yeast. To construct AD fusions in pGADT7-Rec2, first generate double-stranded (ds) cDNA using SMART DNA Synthesis. Then transform yeast with the cDNA products and Sma I-linearized pGADT7-Rec2. Cellular recombinases will use the ds cDNA to repair the gap in pGADT7-Rec2 (Figure 2). Successful recombination results in a fully functional, circular expression vector, which confers the Leu+ phenotype to Leu auxotrophs such as yeast strain Y187.

Appendix B: Plasmid Information continued

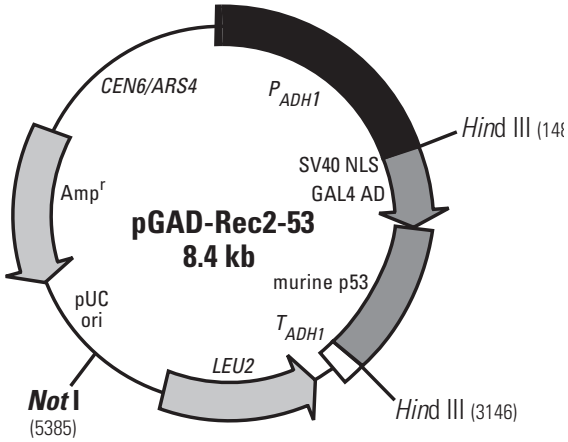


Figure 10. Map of pGAD-Rec2-53 AD Control Vector. pGAD-Rec2-53 encodes a fusion of the GAL4 AD and murine p53, a known DNA-binding protein (Thukral, S. K., *et al.,* 1994). The vector is derived from pGADT7-Rec2 and was constructed at Clontech by homologous recombination in *E. coli*. Specifically, the vector was produced by transforming competent *E. coli* cells with *Eco*R I/*Bam*H I-linearized pGADT7-Rec2 and ds cDNA encoding murine p53 (a.a. 73-391). As a result, this vector does not contain the T7 RNA polymerase promoter or hemagglutinin (HA) epitope tag, nor does it share any homology with the SMART III or CDS III oligonucleotides.

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

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

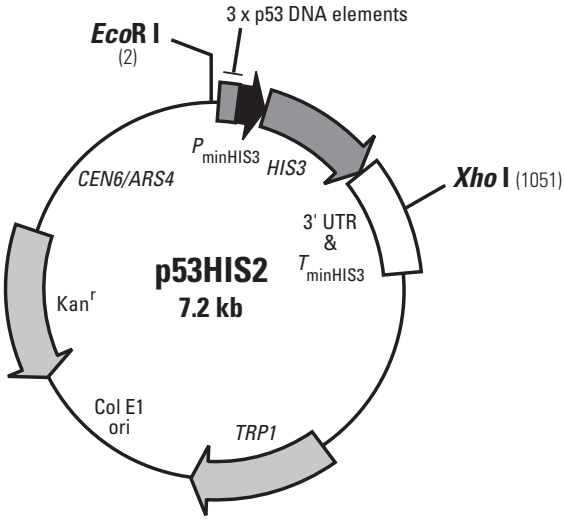


Figure 11. Map of p53HIS2 Control Vector. p53HIS2 is a yeast one-hybrid reporter vector that serves as a positive control in the Matchmaker One-Hybrid Library Construction & Screening Kit (Cat No. 630304). It contains 3 tandem copies of the consensus DNA binding site for p53. The three DNA targets are located upstream of the minimal promoter of the *HIS3* locus ($P_{minHIS3}$) and the *HIS3* nutritional reporter gene. p53HIS2 is designed for use with pGAD-Rec2-53, a plasmid that encodes murine p53 as a fusion to the GAL4 AD. Yeast cells that contain both of these plasmids will display the His^+ phenotype as a result of the interaction between murine p53 and the DNA binding sites in p53HIS2. When the GAL4 AD-p53 fusion interacts with these sites, it stimulates transcription of *HIS3*, giving yeast strains such as Y187, which is normally auxotrophic for histidine, the ability to grow on histidine dropout medium.

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

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 (Zhu, Y. Y., et al., 2001). This cDNA synthesis and amplification system is particularly well suited for one-hybrid and two-hybrid library construction because it consistently delivers high yields of cDNA while maintaining sequence representation. By maintaining the complexity of the original tissue, the SMART procedure provides you with the best opportunity of detecting rare and potentially novel interactions during yeast one-hybrid and two-hybrid screening.

B. Mechanism of cDNA Synthesis

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

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

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

Appendix D: Yeast Media Recipes

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

- Allow plates to harden at room temperature. Store plates in a plastic sleeve at 4°C.
- Prior to use, allow agar plates to dry (unsleeved) at room temperature for 2–3 days, or at 30°C for 3 hr, prior to plating cells. Moisture droplets on the agar surface can lead to uneven spreading of cells.
- Media should be autoclaved at 121°C for 15 min. Autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the media. Store liquid SD medium at 4°C,
- YPD and SD Base from Clontech contain Glucose.
- Ensure that the pH is adjusted appropriately; pH 5.8 for SD Minimal Media and pH 6.5 for YPD and YPDA.
- **[Optional]** To reduce the incidence of bacterial contamination the antibiotic kanamycin may be added to all media. Prepare and autoclave the media, once the medium has cooled to 55°C, add kanamycin to a final concentration of 50 µg/ml.
- To prepare YPD Agar Medium or minimal SD agar medium from YPD Medium or minimal SD base medium respectively, add agar (18–20 g/L) just prior to autoclaving.
- Continue spreading the inoculum over the agar surface until all visible liquid has been absorbed. This is essential for even growth of the colonies.

B. Rich Media

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

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

Appendix D: Yeast Media Recipes continued

C. Single Dropout Media

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

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

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

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

Appendix D: Yeast Media Recipes continued

D. Double Dropout (DDO) Media

NOTE: Not all Double Dropout supplements are available, but they can be easily made by adding back individual amino acids to other supplements.

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

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

SD/-His/-Trp Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
L-Leucine	100 mg
Deionized water	Up to 1 L
Adjust pH to 5.8, then autoclave. Store at 4°C in subdued light	

SD/-His/-Trp/50 mM 3-AT Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
L-Leucine	100 mg
Deionized water	Up to 950 ml
Adjust pH to 5.8, then autoclave. After cooling to 65° and adding 3-AT. store at 4°C in subdued light.	
50 mM 3-AT [1]	50 ml (cool autoclaved agar to 65°C before adding)

[1] For 1 M 3-AT, make a stock of 84.08 g/l of 3-AT using deionized water.

Appendix D: Yeast Media Recipes continued

SD/-His/-Trp/100 mM 3-AT Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
L-Leucine	100 mg
Deionized water	Up to 900 ml
Adjust pH to 5.8, then autoclave. After cooling to 65° and adding 3-AT. store at 4°C in subdued light.	
100 mM 3-AT [1]	100 ml (cool autoclaved agar to 65°C before adding)

[1] For 1 M 3-AT, make a stock of 84.08 g/l of 3-AT using deionized water.

E. Triple Dropout (TDO) Media

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

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

SD/-His/-Leu/-Trp/50 mM 3-AT Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
Deionized water	Up to 950 ml
Adjust pH to 5.8, then autoclave. After cooling to 65° and adding 3-AT. store at 4°C in subdued light.	
50 mM 3-AT [1]	50 ml (cool autoclaved agar to 65°C before adding)

[1] For 1 M 3-AT, make a stock of 84.08 g/l of 3-AT using deionized water.

Appendix D: Yeast Media Recipes continued

SD/-His/-Leu/-Trp/100 mM 3-AT Agar (1 L)	
Reagent	**Amount**
Minimal SD Agar Base	46.7 g
-His/-Leu/-Trp DO Supplement	0.62 g
Deionized water	Up to 900 ml
Adjust pH to 5.8, then autoclave. After cooling to 65° and adding 3-AT. store at 4°C in subdued light.	
100 mM 3-AT [1]	100 ml (cool autoclaved agar to 65°C before adding)

[1] For 1 M 3-AT, make a stock of 84.08 g/l of 3-AT using deionized water.

Notes