Yeast two-hybrid system

Debra L. Parchaliuk, Robert D. Kirkpatrick, Sharon L. Simon, Ronald Agatep and R. Daniel Gietz
gietz@cc.umanitoba.ca

Department of Human Genetics, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, R3E 0W3, Canada

Introduction

The two-hybrid system (THS), first introduced by Fields and Song (1989) (Ref. 1), is a powerful technique for identifying new proteins involved in specific biological processes. It allows for the rapid isolation of the gene that codes for a protein that interacts with a specific protein of interest. Here, we present not a review of the current THS technology but, rather, a comprehensive guide designed to take the reader through a THS screen. Readers not familiar with the THS are asked to review (Ref. 1, 2, 3, 4, 5). Currently, there are a number of different versions of the THS available. This article describes procedures useful for the `Fields' THS, based on the *GAL4* transcription factor. Many of the following protocols can be used with other versions with some modification; however, they might not be directly applicable to the Brent Interaction Trap version because of some distinct differences (Ref. 6).

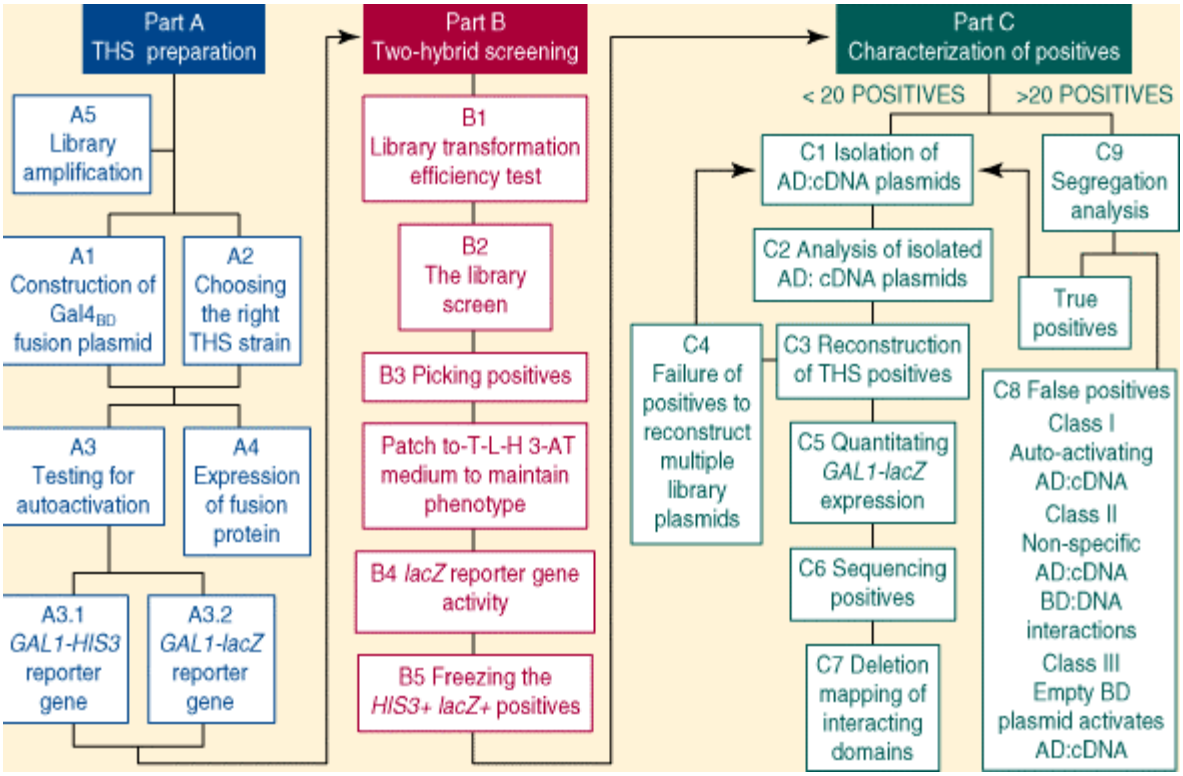

Figure 1. Two-hybrid system flow chart displaying the steps required for a THS screen. Begin at part A for preparation of the library, bait gene and yeast strain. The constructed BD:bait plasmid/yeast strain can then be used in part B to screen a variety of AD:cDNA libraries. Part C lists the steps involved in the characterization of putative THS positives. If less than 20 putative positives are obtained from part B, proceed through parts C1 to C5. When more than 20 putative positives are obtained from part B, or when non-typical THS positives are encountered, begin with part C9: segregation analysis, then proceed to analyze true positives employing the protocols outlined in parts C1 to C5. Part A refers to the protocol described in Ref. 19 and Part C refers to that described in Ref. 20.

1 Part A: screen preparation

This article (part A) is the first of three parts and describes the preparation for a screen; part B describes the screen itself and part C describes the analysis of the THS positives. If beginning a two-hybrid screen with an untested bait gene, start with part A to test the bait plasmid and yeast strain combination (see Figure 1 for a flow chart). This

plasmid–strain combination can be used to screen a variety of libraries following the steps outlined in part B. Part C is then used to characterize THS positives.

1.1 A1: Construction of the *GAL4*$_{BD}$:bait gene fusion plasmid

The first step is to construct the *GAL4*$_{BD}$:bait gene fusion plasmid (BD, binding domain). `Your Favorite Gene' (YFG) encoding the protein of interest (considered the bait) is cloned into a suitable THS vector in-frame with the *GAL4*$_{BD}$ (amino acids 1–147). Table 1 and Figure 2 Figure 3 Figure 4 Figure 5 show a list of THS vectors that include the *GAL4*$_{BD}$ vectors for making the BD:bait fusion plasmid.

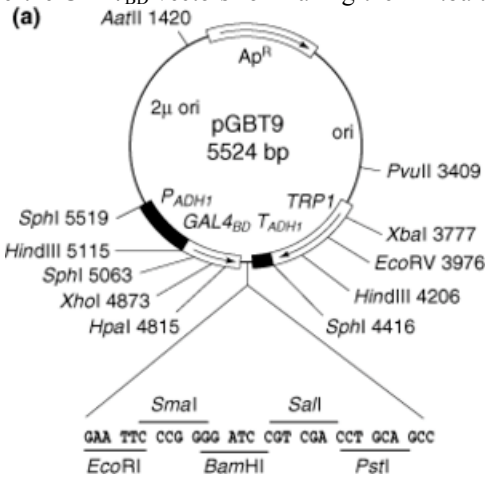

Figure 2. Plasmid maps of two-hybrid system plasmids—DNA *GAL4*-binding domain plasmids: pGBT9 (Ref. 9). The plasmid contains the *GAL4* DNA-binding domain (*GAL4*$_{BD}$ 1–147aa) bounded by the constituitively expressing yeast alcohol dehydrogenase 1 promoter (P_{ADH1}) and terminator (T_{ADH1}). The multi-cloning site is listed to indicate the frame of the *GAL4*$_{BD}$. All plasmids contain the yeast *TRP1* gene, which allows for selection. Useful restriction sites are listed.

1.1.1 A1.1: Cloning strategies

There are a number of different cloning strategies that can be used to clone YFG into a *GAL4*$_{BD}$ vector (see Table 2). The first two strategies use compatible restriction sites found in the bait gene and the multi-cloning site (MCS) of the *GAL4*$_{BD}$ vector (see Figure 6 a and Figure 6b). For the blunt-end ligation strategy (see Figure 6c) each *GAL4*$_{BD}$ vector listed has a number of different options to allow the cloning into each frame.

Table 2. BD:bait plasmid cloning strategies

1 PCR amplification of YFG ORF placing unique *GAL4*$_{BD}$ vector restriction sites 5′ and 3′ to construct the BD:bait plasmid. See Figure 3a

2 Using existing *GAL4*$_{BD}$ vector MCS restriction sites within YFG ORF to construct the BD:bait plasmid. See Figure 3b

3 Blunt-end ligate a restriction fragment containing YFG ORF into the appropriate frame to construct the BD:bait plasmid. See Figure 3c

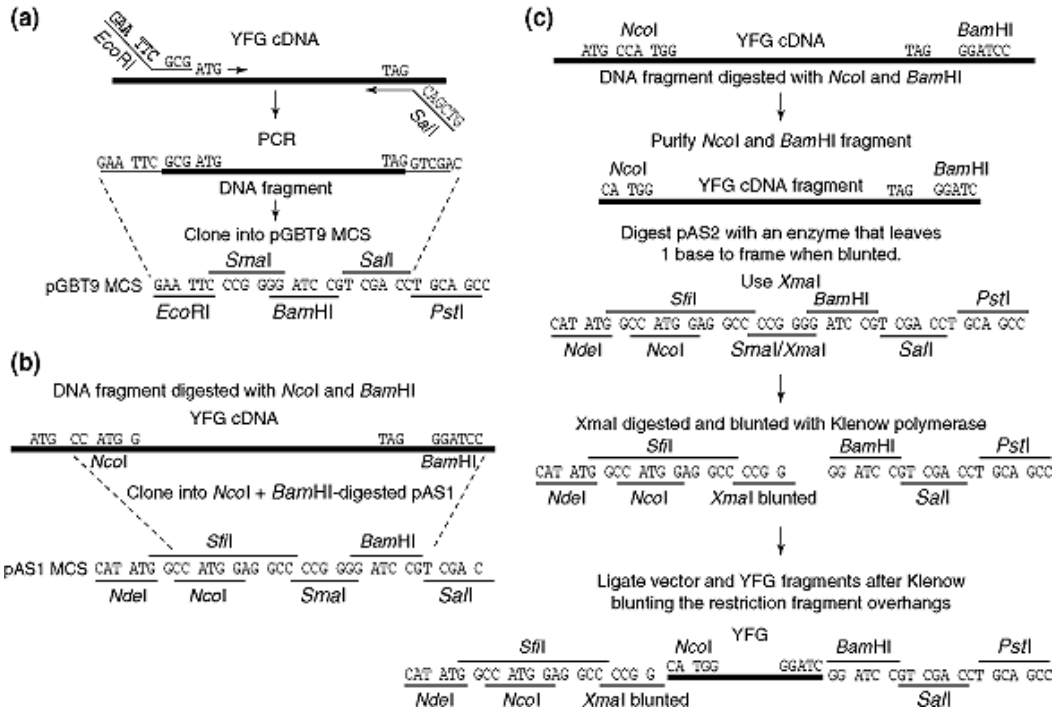

FIGURE 6. Cloning strategies for the *GAL4*$_{BD}$:bait fusion. (a) PCR modify your open reading frame (ORF). PCR primers are designed to amplify the ORF of `your favourite gene' (YFG) and place unique restriction site(s) in-frame at the 5′ and 3′ ends of the ORF, as indicated. The amplified ORF is then digested with the desired restriction enzyme(s) and subsequently purified. The purified fragment is ligated into the multiple cloning site (MCS) of pGBT9. A number of clones should be fully sequenced to confirm that mutations have not been produced in your amplified ORF. (b) Natural restriction sites. Restriction sites that naturally occur in YFG can be used to clone it into the MCS of your *GAL4*$_{BD}$ vector. Note that the *Nco*I site in the YFG ORF has the same frame as the *Nco*I site in the MCS of the vector. This example uses a naturally occurring *Bam*HI site in the 3′ untranscribed region of YFG. Alternatively, a single restriction enzyme can be used. Both the YFG DNA fragment and the *GAL4*$_{BD}$ vector are double digested with *Nco*I, *Bam*HI and purified. The vector is treated with calf intestinal alkaline phophatase (Boehringer-Mannheim) to reduce self-ligation. (c) Blunt-end ligation. Most restriction fragments containing 5′ overhangs can usually be cloned in-frame, by blunt-end ligation, into the *GAL4*$_{BD}$ vectors listed in Table 1. Note that the *Nco*I site at the 5′ end of the YFG ORF has a different frame than the *Nco*I site in the MCS of the pAS2 vector. To produce an in-frame gene fusion, the *Xma*I site in the vector is digested and the resulting 5′ overhang filled in with Klenow polymerase. The ends of the *Nco*I–*Bam*HI DNA fragment are also filled in with Klenow polymerase, followed by ligation into the blunted *Xma*I digested pAS2 vector. This will generate the correct frame for gene fusion. Note that both the *Xma*I and *Nde*I sites in the MCS of the *GAL4*$_{BD}$ vectors listed require two base pairs to restore frame. The *Nco*I, *Bam*HI, and *Sal*I sites in these *GAL4*$_{BD}$ vectors do not require any base pairs to maintain frame, while the *Sma*I site requires one base pair to restore frame.

If YFG has identifiable protein domains or motifs these can be fused to the *GAL4*$_{BD}$ for independent study. However, for uncharacterized proteins it is advisable to use as much of the open reading frame (ORF) as possible to create your fusion. The most important aspect of this cloning is to ensure that YFG is in-frame with the *GAL4*$_{BD}$, so that a fusion protein can be produced. Therefore, we recommend that your cloning strategy is checked by a knowledgeable colleague. Finally, the fusion junction of any plasmid constructed should be sequenced before performing any screen to confirm the ORF fusion. The *GAL4*$_{BD}$ sequencing primer, 5′-TCA TCG GAA GAG AGT AG-3′, can be used for *GAL4*$_{BD}$ vectors such as pGBT9, pAS1 and pAS2.

Tips

- Some vectors, such as pAS1 and pAS2, contain the haemagluttinin (HA) tag, which allows immunological detection of the fusion protein with the 12CA5 monoclonal antibody (Pharmacia) or equivalent monoclonal antibody (Babco), see below.
- The ATG codon of YFG does not have to be present in your fusion construct. Translation is initiated at the start codon of the *GAL4*$_{BD}$.
- If cloning any portion of the 5′ untranslated region of a gene into a *GAL4*$_{BD}$ vector, ensure that there are no in-frame stop codons.

- Ensure that cloning the bait gene into the vector does not create an in-frame stop codon, especially if blunt-end ligation was used.

1.2 A.2: Choosing the right THS reporter strain

There are many reporter yeast strains now available for the `Fields' THS. These strains vary in their reporter constructs. The `second generation' of THS reporter strains usually includes both the *lacZ* and the *HIS3* reporter genes. The *HIS3* reporter allows the direct selection of THS positives, however, some strains contain `leaky' derivatives that require the addition of the chemical 3-amino triazole (3-AT) to the medium to quench background expression of the *HIS3* gene product (Ref. 7). The *HIS3* reporter gene also selects for the optimal ratios of fusion proteins to produce reporter-gene expression for growth on selective medium (Ref. 10). The *lacZ* reporter can be used to verify positives through co-expression as well as to generate quantitative measurements of gene expression.

Another important quality of a THS yeast strain is its transformation characteristics. The ability to generate large numbers of transformants using current transformation protocols is essential for THS screening (Ref. 11). The strains listed in Table 3 all have good transformation characteristics. In addition, PJ69-4a and KGY37 contain reporter genes that are integrated into their genomes, ensuring their maintenance in the absence of selection. Finally, the reporter genes in KGY37 were constructed to produce low levels of basal activity.

1.3 A.3: Testing the BD:bait plasmid for auto-activation

It is important to test each BD:bait plasmid construct for auto-activation of both reporter genes before any screen. The activation of reporter genes by the BD:bait plasmid in the absence of an activation-domain plasmid is defined as auto-activation. Transform the BD:bait plasmid into the appropriate reporter yeast strain using the `Quick and Easy' transformation technique described in Ref. 11, then proceed to test both reporter genes for auto-activation as outlined below.

1.3.1 A.3.1: GAL1-HIS3 reporter gene

To test for auto-activation of the *GAL1-HIS3* reporter, yeast cells containing the BD:bait plasmid should be plated, not streaked, onto synthetic complete, minus histidine (SC-H) medium containing increasing concentrations of 3-AT (1, 5, 10, 25 and 50 mM). In addition, these yeast cells should be plated onto synthetic complete, minus tryptophan (SC-W) medium to select for the BD:bait plasmid as a control of growth. 3-AT is used in the medium to suppress the `leaky' nature of this reporter in most strains. The concentration of 3-AT needed to eliminate background growth is plasmid and strain dependent. We have found that when screening pAS1 or pAS2 BD:bait plasmid constructs, or when using the strain Y190, higher levels of 3-AT might be required to suppress background *GAL1-HIS3* expression.

Protocol

- Grow your yeast transformant containing the verified BD:bait plasmid overnight in SC-W medium to select for maintenance of the $GAL4_{BD}$ plasmid. Alternatively, a 10 µl blob of cells can be scraped from a freshly grown SC-W plate and resuspended in 1 ml of sterile water.
- Titer the liquid culture using a spectrophotometer (OD_{600} 0.1=□1×10^6 cells/ml) or a hemocytometer.
- Plate at least 1000 cells/plate onto a pair of SC-W plates as well as pairs of SC-H plates containing 0, 1, 5, 10, 25 and 50 mM 3-AT.
- Incubate at 30°C for 5 days.

Examine the SC-H +3-AT plates for growth. Most BD:bait plasmids will not produce colonies on the medium once the appropriate concentration of 3-AT is reached. The SC-W control plates should contain approximately 1000 colonies. If you cannot inhibit all growth on SC-H + 3-AT, even at a 50 mM concentration, consider either cloning a different gene fragment into your BD:bait plasmid, or cloning YFG into another $GAL4_{BD}$ vector.

Tip

- Auto-activation by the BD:bait construct does not necessarily mean the end of your screen. Cloning your bait gene into a different vector, such as pGBT9, might reduce the auto-activation if pAS1 or pAS2 were used previously. Alternatively, the construct can be modified by deletion to remove the region responsible for the auto-activation. Another option reduces auto-activation by `dampening' (Ref. 14).

1.3.2 A.3.2: GAL1-lacZ reporter gene

In addition to testing for *GAL1-HIS3* auto-activation it is also prudent to test for *GAL1-lacZ* auto-activation in those strains that contain this reporter gene. This can be accomplished following the protocol listed below. You can use the pair of SC-W plates that were plated for the *GAL1-HIS3* auto-activation test above.

Protocol

- Carefully place a sterile 75 mm circle of Whatman No. 1 filter paper on top of the colonies or patches growing on selective medium. Ensure that the filter paper makes good contact with the colonies. Mark the orientation of the filter paper relative to the plate using an 18-gauge needle to punch through the filter in an asymmetric pattern.
- Remove the filter from the plate with sterile forceps after it has become fully absorbed to the colonies and immerse in liquid nitrogen for 10 to 15 s.
- Carefully remove the filter from the liquid nitrogen and thaw by placing on a piece of plastic wrap colony side up. Repeat the freeze-thaw cycle twice.
- Place another 75 mm sterile Whatman No. 1 filter into an empty Petri plate (100×150 mm) and dispense 1.25 ml of Z buffer/β-mercaptoethanol/X-GAL (see A) onto the filter.
- Place the filter colony side up, onto a filter paper soaked with Z buffer/β-mercaptoethanol/X-GAL, taking care that the filters line up to distribute the solution evenly.
- Place the lid on each plate and transfer to a plastic bag and incubate at 37°C.

Strong activation of the *lacZ* gene will give a blue color within 1–2 h. If color does not develop, continue to incubate the filters overnight. Note the time needed for color production. A faint blue color after overnight incubation is considered minimal *lacZ* activation.

1.4 A.4: Expression of your fusion protein

Before screening we recommend that the steady state expression of the BD:bait fusion protein is assayed by western blotting. This can be accomplished if the appropriate reagents are available, such as a specific antibody for the product of YFG. Some vectors, such as pAS1 and pAS2, contain the haemagluttinin (HA) tag (Ref. 15), which can be recognized by the commercially available 12CA5 monoclonal antibody (Pharmacia) or equivalent monoclonal antibody (Babco). The $Gal4_{BD}$ antibody (Santa Cruz Biotechnology Inc.) can also be used. In addition to indicating the expression levels of the fusion protein, western blotting can verify the in-frame cloning strategy, because the size of the fusion protein detected should be comparable with the predicted value.

Protocol

Yeast lysates are prepared for electrophoresis following a modified method of Rocchi *et al*. (Ref. 16).

- Inoculate the yeast strain containing the BD:bait plasmid into 50 ml of SC-W medium. Incubate at 30°C with shaking until a titer of 1.0×10^7 cells/ml is reached. This might take 16–24 h. Alternatively, a 10 ml overnight culture can be used to inoculate 50 ml to 2.5×10^6 cells/ml and incubate until a titer of $1–2\times10^7$ cells/ml is reached. This will take 4–6 h in SC-W medium.
- Collect the yeast cells by centrifugation at 5000×g for 5 min and wash the cells with a 0.5 volume of sterile water. Determine the volume of the cell pellet by adding a specific volume of water and then measuring the total volume of the cell slurry.
- Resuspend the cells in two volumes of ice-cold extraction buffer (see A).
- Add one volume of glass beads (Sigma, Catalogue no. G-8772) and place each sample on ice.
- Vortex each sample vigorously for 30 s and return to ice to cool. Repeat six times for each sample.
- Centrifuge samples at 14 000×g at 4°C for 1 min to pellet unbroken cells and cell debris.
- Transfer the supernatant to another 1.5 ml microcentrifuge tube and cool each sample in an ice slurry for 1 min.
- Centrifuge each sample again at 14 000×g at 4°C for 1 min to further clarify the extract.
- Carefully remove supernatant and mix 1:1 with SDS loading buffer (see A) and heat in a boiling water bath for 2 min. Protein extracts can be stored at −70°C until needed.

1.5 A.5: Amplifying the AD:cDNA library

A library screen can consume up to 300 µg of plasmid DNA depending on the yeast strain and BD:bait plasmid. Thus, it might be necessary to transform and/or amplify your AD:cDNA plasmid library. A list of libraries can be found in Ref. 17; however, many AD:cDNA libraries are currently available commercially from companies that include ClonTech Laboratories Inc., InVitrogen and Stratagene.

In order to amplify all clones within a library most efficiently, transformed bacterial cells are grown on plates to allow individual colonies to form. We typically plate about 10 times the library complexity for amplification. Most AD:cDNA libraries have complexities of $1–2\times10^6$ independent clones; therefore, for good library coverage, $10–20\times10^6$colonies should be amplified.

Protocol

Prepare 100 large (150 mm) Petri plates containing the appropriate medium [Lauria Bertani (LB) + ampicillin at 50 mg/ml]. Also, prepare 500 ml sterile saline solution (150 mM NaCl), to be used to resuspend the bacterial colonies. Library plasmid DNA should be transformed into a suitable strain of *Escherichia coli*.

- Titer the *E. coli* library culture by plating 2, 20 and 200 µl of a 10^{-2} dilution onto duplicate LB + Amp (50 µg/ml) plates (100 mm) and incubate overnight at 37°C. Store remaining culture at 4°C.
- Plate 2×10^5 cells of library culture onto each large (150 mm) LB + Amp plate and incubate until the colonies are fully formed (usually 16–24 h).
- Harvest the bacterial colonies by flooding a plate with 10 ml of sterile saline and scraping the colonies from the agar surface using a rubber policeman or bent glass rod. Be careful not to damage the agar while harvesting bacteria. When complete, dump the liquid onto another plate and repeat. Each 10 ml aliquot can

be used for up to 5 plates. After the solution is saturated with bacteria, transfer to a centrifuge tube. Start the next set of plates with a fresh 10 ml of sterile saline. Repeat until all the plates have been scraped.

- Mix all aliquots of bacteria together then distribute into six 50 ml centrifuge tubes. Collect the cells by centrifugation at 10 000 rpm for 10 min.
- Proceed to plasmid DNA extraction as described in Ref. 18.

This procedure will give good amplification of your AD:cDNA library. There is no need to purify the plasmid from the endogenous RNA because it does not affect yeast transformation (R.D. Gietz *et al*., unpublished). You are now ready to begin your screen.

Tips

- Completely resuspend the bacterial pellets before adding the SDS:NaOH reagent to ensure good lysis.
- A single phenol:chloroform extraction produces good-quality plasmid DNA for transformation into yeast.
- Use molecular biology grade phenol or distill your own!
- An exact DNA concentration is not important because a library efficiency test is performed before screening.

1.6 References

[1] Fields S. and Song O. (1989) *Nature*, **340**:245-246.
[2] Li B. and Fields S. (1993) *FASEB J.*, **7**:957-963.
[3] Bartel P.L., Roecklein J., SenGupta D. and Fields S. (1996) *Nat. Genet.*, **12**:72-77.
[4] Wang H. *et al.* (1995) *J. Biol. Chem.*, **270**:23322-23329.
[5] Wang D. *et al.* (1997) *J. Biol. Chem.*, **272**:19383-19392.
[6] Gyuris J., Golemis E., Chertkov H. and Brent R. (1993) *Cell*, **75**:751-803.
[7] Durfee T. *et al.* (1993) *Genes Dev.*, **7**:555-569
[8] Harper J.W. *et al.* (1993) *Cell*, **75**:805-816
[9] Bartel, P.L., Chien, C.T., Sternglanz, R. and Fields, S. (1993) in *Cellular Interactions in Development: A Practical Approach* (Hartley, D.A., ed.), pp. 153–179, Oxford University Press
[10] Fields S. (1993) *Methods: A Companion to Methods in Enzymology*, **5**:116-124.
[11] Agatep, R., Kirkpatrick, R.D., Parchaliuk, D.L. and Gietz, R.D. (1998) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01525
[12] James P., Halladay J. and Craig E.A. (1996) *Genetics*, **144**:1425-1436.
[13] Graham, K.C. (1996) MSc.Thesis, University of Manitoba
[14] Cormack R.S. and Somssich I.E. (1997) *Anal. Biochem.*, **248**:184-186.
[15] Kolodziej P. and Young R.A. (1991) *Methods Enzymol.*, **194**:508-519.
[16] Rocchi S. *et al.* (1996) *Endocrinology*, **137**:4944-4952.
[17] Bartel P.L. and Fields S. (1995) *Methods Enzymol.*, **254**:241-263.
[18] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning—a laboratory manual*, Cold Spring Harbour Laboratory Press
[19] Parchaliuk, D.L. (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01713
[20] Parchaliuk, D.L. (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01714

2 Part B—screening procedure

This article is the second of three parts (see Figure 1 for a schematic), describing the screen itself, after the preparation steps outlined in part A. Part C describes the characterization of putative THS positives.

2.1 B1: library transformation efficiency test

The first thing that should be done before embarking on a large-scale screen is to perform a AD:cDNA plasmid library transformation efficiency test. This is accomplished by transforming increasing amounts of library plasmid DNA into the two-hybrid yeast strain containing the BD:bait plasmid at a 1×transformation scale. This experiment will allow you to use the library plasmid DNA efficiently, as well as to target a specific number of transformants for THS screening. If the DNA concentration used for the transformation is too high, multiple AD:cDNA library plasmids will be transformed into a single yeast cell, making the subsequent analysis of two-hybrid positives more difficult (see Section C2).

2.2 B1.1: Determining library transformation yield and efficiency

Protocol

- Using the `standard high-efficiency transformation protocol' listed in Agatep *et al.*, (Ref. 7), transform increasing amounts of the AD:cDNA library plasmid DNA into your THS yeast strain containing the BD:bait plasmid at the 1×transformation scale (e.g. 0.1 µg, 1 µg, 2 µg, 5 µg and 10 µg of AD:cDNA library plasmid DNA). Plasmid DNA preparations containing RNA can be estimated for concentration from agarose gels. Dilute transformation reactions 1:10 and then plate 10 µl and 100 µl in duplicate for each library plasmid quantity transformed. Incubate the plates for 3 to 4 days at 30°C.
- Count the colonies on each set of plates to determine the transformation yield (total number of transformants) as well as the transformation efficiency (transformants/µg) for each transformation (see Table 1 for calculation formulae).

Table 1. Transformation efficiency and yield example

µg DNA	Average no. of colonies/plate	Transformation yield[1]	Transformation efficiency[2]×10^6/µg
0.1 mg	255	255000	2.6
1.0 mg	1545	1545000	1.5
2.0 mg	1765	1765000	0.8
5.0 mg	1894	1894000	0.3
10.0 mg	2019	2019000	0.2
20.0 mg	2208	2208000	0.1

[1]*Transformation yield (total transformants)=[(colonies/plate)÷(volume/plate)]×[(volume □ µl of total reaction)÷(dilution factor)]*
In this example, for the 0.1 µg transformation the colonies/plate were 251 and 259, giving an average of 255. A 10 µl volume of a 10:1 dilution was plated and the final volume of the transformation reaction was 1000 µl.
[(255 colonies/plate)÷(10µl/plate)]×[(1000µl/reaction)÷(10^{-1})]=255000 colonies/reaction
[2]Transformation efficiency (transformants/µg)=(transformation yield)/(amount of DNA in µg)

The example in Table 1 shows that, as the DNA concentration increases in the transformation reaction, the transformation yield increases but the transformation efficiency decreases. It is best to scale up the transformation reaction, rather than to increase the amount of DNA transformed, to limit the production of transformants that contain multiple library plasmids. From this data, the DNA concentration to use for a library screen is either 1 or 2 mg for each 1×scale transformation. Performing a 30× scale-up should produce 46×10^6 to 52×10^6 transformants with 30 to 60 mg of AD:cDNA library plasmid DNA.

2.3 B2: The library screen

Once the tranformation yield test has been completed, a large-scale library screen can be performed. Typically a 30× or 60× transformation scale-up is used. However, the protocol has been scaled successfully up to 120×. As plating density affects transformation negatively (R. D. Gietz, unpublished), we recommend using at least 50–100 large (150×15 mm) petri plates containing SC-W-L-H+3-AT medium (A). Freshly made plates should be allowed to dry for a few days at room temperature to eliminate excessive condensation. Media should also be stored in the dark to prevent a reduction in plating efficiency owing to exposure to fluorescent lighting. Using the appropriate amount of AD:cDNA library plasmid DNA, transform the THS yeast strain containing the BD:bait plasmid using the methods outlined in (Ref. 7) and plate onto medium that selects for reporter gene activation. Incubate the plates for 4–21 days at 30°C.

Tips

- Plating a 30× or 60× transformation onto 100 large plates can take up to 30 min. Spread the plates out on a counter top and dispense 400 µl of transformed cells onto each plate. Using a sterile glass spreading wand, start from the first plate and move to the last, carefully spreading the inoculum onto the surface of the entire plate. There is no need to re-sterilize your spreading wand between each plate.
- The use of a Petri plate turntable (Fisher Scientific, Calog no. 08-758-10) will greatly aid plating.
- Incubate plates in loosely taped Petri plate bags to reduce drying during growth.

2.4 B3: Picking THS positives

Transformation plates should be checked for colonies after four days of incubation at 30°C. Continue to check the plates and pick positives every day for the first week and then every two days for up to three weeks. When colonies become visible they should be patched to fresh selection plates (SC-W-L-H+3-AT) in a grid pattern. These patched plates should be incubated at 30°C until sufficient growth occurs. Colonies that do not produce growth on the patched plate after 5–7 days can be eliminated.

Tips

- When picking positives be sure to select large colonies that are actively growing. To be certain, observe the colony growth over a number of days. Depending on the strain and BD:bait plasmid, small colonies can usually be found in areas of the plate containing heavy inoculum. Avoid these colonies as they are usually not true positives.
- Be aware that other bacterial and fungal contaminants will probably occur on the screening plates. Use caution when picking from plates containing colonies with a different coloration or texture. Plates heavily contaminated with filamentous fungi producing conidia should be discarded. In many cases, attempts to rescue colonies from such plates will only further contaminate the laboratory air space.
- Positives should be kept on medium that selects for reporter gene activation and all plasmids at all times (e.g. SC-W-L-H+3-AT plates). This ensures that the BD:bait and AD:cDNA library plasmids encoding the interacting fusion protein are maintained. In cases where a yeast transformant contains multiple AD:cDNA library plasmids, this will ensure the maintenance of the correct plasmid.
- Yeast colonies maintained on medium containing 3-AT have a reduced viability. Streak or patch to fresh plates weekly and/or cryo-preserve your positives as soon as possible.

2.5 B4: *lacZ* reporter gene activity

A good indication of a true THS positive is co-activation of all reporter genes. The *lacZ* reporter can be used for this purpose. Once positives are patched and replicated, *lacZ* gene activation can be assayed. It is important to maintain positives on medium that selects for *GAL1-HIS3* reporter activation. This will optimize the expression of fusion proteins to give good levels of reporter gene activity (Ref. 8). Assay for *lacZ* reporter activity using the method in Part A3.2 (Ref. 9).

2.6 B5: Cryo-preserving the His+ *lacZ*+ positives.

Patched colonies that activate the *lacZ* reporter should be cryo-preserved. Streak the *His*+ *lacZ*+ positives onto fresh SC-W-L-H+3-AT plates and incubate at 30°C for 24–48 h. Scrape a blob of fresh inoculum using an inoculating loop or a sterile toothpick and resuspend in 1 ml of sterile 20% glycerol in a 1.5 ml microcentrifuge tube or cryo-tube. Store at −70°C. Alternatively, large numbers of positives can be patched in a grid pattern onto 150 mm SC-W-L-H+3-AT plates and cryo-preserved using a 96-well microtiter plate replicator (Fisher Scientific, Calog no. 05-450-9). The replicator is sterilized with ethanol and flame and cooled. It is placed onto the grid of patched colonies to make contact with the inoculum. The cells are scraped from the plate by pulling the teeth of the replicator along the surface of the agar without breaking into it. This can be repeated until sufficient inoculum is deposited onto each tooth. Care must be taken not to cross contaminate different patches. The teeth of the replicator containing the inoculum are then carefully lowered into a sterile microtiter plate (Fisher Scientific, Catalog nos 07-200-104 and 07-200-376), containing 150 µl of sterile 20% glycerol in each well. The cells are washed from the replicator teeth using a gentle rotating mixing action. After the inoculum has been resuspended, the lid is replaced and the plate sealed in a plastic bag and stored at −70°C.

Tips

- To mark the patching grid onto a plate, carefully place a flame-sterilized replicator onto a fresh plate. This will leave an impression of each prong for patching.
- It is important to always use the original freezer stock of your THS positives while further tests are being performed.

2.7 References

[1] Fields S. and Song O. (1989) *Nature*, **340**:245-246.
[2] Li B. and Fields S. (1993) *FASEB J.*, **7**:957-963.
[3] Bartel P.L., Roecklein J., SenGupta D. and Fields S. (1996) *Nat. Genet.*, **12**:72-77.
[4] Wang H. *et al.* (1995) *J. Biol. Chem.*, **270**:23322-23329.
[5] Wang D. *et al.* (1997) *J. Biol. Chem.*, **272**:19383-19392.
[6] Gyuris J., Golemis E., Chertkov H. and Brent R. (1993) *Cell*, **75**:751-803.
[7] Agatep, R., Kirkpatrick, R.D., Parchaliuk, D.L. and Gietz, R.D. (1998) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01525
[8] Bartel P.L., Chien C.T., Sternglanz R. and Fields S. (1993) *Bito.*, **14**:920-924.
[9] Parchaliuk, D.L. *et al*. (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01616
[10] Parchaliuk, D.L. *et al.* (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01714

3 Part C—characterizing positives

This third article of the three on the yeast two-hybrid system describes the characterization of THS positives.

Primary THS positives that activate both the *HIS3* and the *lacZ* reporter genes can now be subjected to further analysis. Due to the *in vivo* nature of this system, unforeseen obstacles might be encountered that require you to return to a previous step. If less than 20 positives are obtained, follow section C1 (see Figure 1 for a schematic) through each step until all are characterized. If greater than 20 positives are identified, proceed to section C9 and use segregation analysis to eliminate false positives. Continue the analysis with remaining positives by returning to section C1. In those cases where it is difficult to isolate the AD:cDNA library plasmid responsible for reporter-gene activity (in steps C1–C4), segregation analysis (C9) will allow you to discern true positives from non-typical THS positives.

3.1 C1: Isolation of AD:cDNA plasmid

To isolate the AD:cDNA library plasmid, nucleic acids are extracted from the yeast cells of each THS positive. A quick and effective method described by Hoffman and Winston (Ref. 7) uses glass beads and phenol:chloroform to extract nucleic acids. Alternatively, the method of Cryer *et al*. (Ref. 8), which uses lyticase to produce spheroplasts, can also be used. These nucleic acid preparations will include both *TRP1* and *LEU2* plasmids and should be transformed into an *Escherichia coli* host containing a *leuB* mutation to specifically select for the yeast *LEU2* gene harbored on the AD:cDNA library plasmid.

3.1.1 C1.1: Isolation of yeast DNA

This protocol, modified from (Ref. 7), can be used to isolate DNA from yeast cells grown in either liquid culture or harvested from a plate.

Protocol

- Inoculate individual THS positives from SC-W-L-H+3-AT plates into 2 ml of SC-H or SC-W-L medium and incubate at 30°C overnight. Alternatively, scrape a 50 µl blob of cells from an SC-W-L-H+3AT plate and resuspend in 500 µl of sterile water in a 1.5 ml microcentrifuge tube.
- Collect the yeast cells from the liquid culture by centrifugation at 13 000×g for 30 s.
- Remove the supernatant and add 200 µl of yeast lysis buffer (see A) and gently resuspend the cell pellet using a micropipette tip to avoid the generation of bubbles.
- Add an approximately 200 µl volume of 425–600 µm glass beads (Sigma, Catalog no. G 8772) and 200 µl of buffer-saturated phenol:chloroform.
- Vortex each sample vigorously for 30 s and then place on ice. Repeat twice, leaving samples 30 s on ice between treatments.
- Centrifuge tubes at 13.000×g for 1 min.
- Remove the aqueous phase (□200 µl) to a fresh tube and precipitate the nucleic acids by adding 20 µl of 3.0 M sodium acetate (pH 6.0) and 500 µl of 95% ethanol. Incubate at −20°C for 30 min and collect the precipitate by centrifugation at 13 000×g for 5 min at 4°C. Wash the pellet with 100 µl of 70% ethanol (room temp) and dry the pellet for 5 min at room temperature.
- Dissolve the pellet in 25 µl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA).

This DNA should be transformed into an *E. coli* strain containing a *leuB* mutation, such as KC8 or HB101, which allows for direct selection of the yeast *LEU2* gene contained on the AD:cDNA library plasmid.

3.1.2 C1.2: Electroporation of *E. coli* and selection of LEU+ colonies

The most effective method of transforming a yeast DNA extract into *E. coli* is the electroporation method of Dower *et al*. (Ref. 9). The protocol listed below gives electroporation conditions that work with *E. coli* in our hands. However, you should determine the conditions for your strain experimentally. Alternatively, the method of Hannahan (Ref. 10) can be used to transform *E. coli*.

Protocol

- Mix a 2 µl aliquot of extracted yeast DNA with a 25 µl aliquot of electrocompetent KC8, or other *leuB*-containing *E. coli* strain, and place carefully into an cold electroporation cuvette. Keep loaded cuvette on ice.
- Place electroporation cuvette into electroporation device and pulse the DNA bacterial mixture with the following settings; 25 µF, 1.25 kV, with a pulse controller in parallel with the samples set at 400 Ω.
- Immediately after pulse, add 1 ml of warm SOC medium to the electroporation cuvette and resuspend the cells. Transfer to a sterile tube and incubate at 37°C for up to 30 min.
- Plate samples of 25–100 µl onto 2–4 LB-Amp (50 µg/ml) plates and incubate at least 16 h at 37°C.
- The Ap® colonies are then replica plated, using a replicating block (Fisher Scientific, Catalog no. 09-718-1) covered with sterile velveteen, onto M9 minimal media minus leucine (M9-L; see A) and incubated for another 16 h at 37°C.
- Inoculate 4–5 Leu+ colonies per putative positive into 2 ml LB-Amp liquid medium and incubate at 37°C overnight with shaking.
- Extract the plasmid DNA from these cultures using the method of Maniatis *et al.* (Ref. 11) and dissolve plasmid DNA in 50 µl of TE.

Tips

- Plating onto LB Amp medium followed by replica plating onto M9-L plates will save two days over plating directly onto M9-L media. Overnight incubation of the Leu+ replicas will clearly show good growth while the Trp+ replicas will not.
- Replica plate those plates that contain 300–600 isolated Ap® colonies.

3.2 C2: Analysis of isolated AD:cDNA plasmids

The *LEU2* AD:cDNA library plasmids isolated from the *leuB E. coli* strain can now be characterized by restriction enzyme digestion and agarose gel electrophoresis. Restriction enzymes that digest on the 5′ and 3′ ends of the cDNA are vector and library specific; check the AD:cDNA library plasmid information. This analysis will group the plasmids by insert size and restriction pattern. Restriction enzyme analysis should be carried out on 4–5 library plasmid isolates from each THS positive. If more than one type of library plasmid is isolated from a single THS positive, further analysis should be carried out with each unique isolate. Independent positives with similar-sized inserts should not be considered duplicates until sequence information can be produced. Figure 2 shows that with most THS positives each of the four plasmid isolates have identical restriction digest patterns, indicating the presence of a single AD:cDNA library plasmid. However, in some cases (Figure 2, lanes B1–4) the plasmid isolates have different restriction digest patterns showing the presence of multiple AD:cDNA library plasmids in this THS positive.

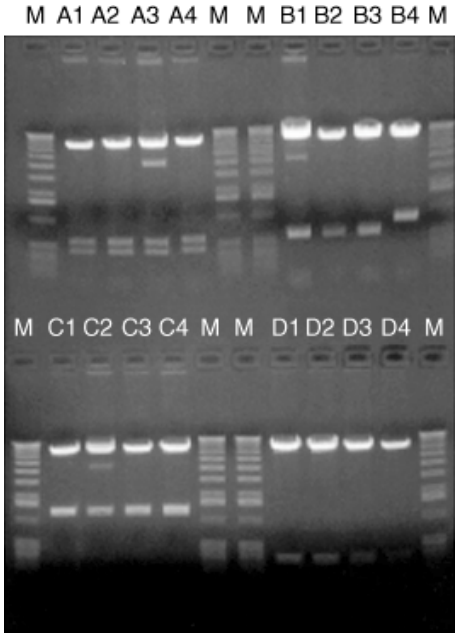

FIGURE 2. Plasmids isolated from LEU+ *Escherichia coli*. Nucleic acids were extracted from four independent THS library positives (A–D), and transformed into *E. coli* KC8. Approximately 500 ng of plasmid DNA, from four Leu+ Ap® isolates (1–4) originating from each positive (A–D), were digested with *Eco*RI and then analyzed on a 0.7% agarose gel. The plasmid isolates are flanked with 1 kb ladder (Gibco-BRL Life Technologies) (Lane M). Every plasmid isolated from each of the positives A, C and D (Lanes A1 to A4, C1 to C4, D1 to D4) shows a similar restriction pattern and insert size, suggesting the presence of a single library plasmid type within each positive. Conversely, two different restriction patterns are observed in lanes B1 to B4, suggesting the presence of multiple library plasmids in this positive.

3.3 C3: Reconstruction of two-hybrid system positives

The next step in the analysis of the THS positives is their reconstruction. Plasmid DNA isolated from the *leuB E. coli* strain is transformed back into the THS yeast strain containing the BD:bait plasmid. Thus, a representative from each plasmid group is tested for activation of both the *HIS3* and *lacZ* reporter genes when in combination with the original BD:bait plasmid. This is accomplished using the standard high efficiency protocol listed in Agatep *et al*. (Ref. 12). Transformed cells are plated onto SC-W-L as well as SC-W-L-H+3-AT media. Growth on SC-W-L confirms the presence of both the BD:bait and AD:cDNA library plasmids. Colony formation on SC-W-L-H+3-AT demonstrates activation of the *HIS3* reporter gene. These His+ colonies can also be tested for activation of *lacZ* reporter using the β-galactosidase assay listed in Section A 3.2.

3.4 C4: Failure of THS positives to reconstruct

The failure to obtain colonies on SC-W-L-H+3-AT medium while generating colonies on SC-W-L medium suggests that the AD:cDNA library plasmid used in the transformation was not responsible for activation of the reporter genes in the original THS positive. There are two specific situations that are known to give rise to this: the first is the presence of multiple AD:cDNA library plasmids in the original THS positive, caused by transformation with high library plasmid DNA concentrations; the second is alteration of the BD:bait plasmid in the yeast strain. Each situation is discussed below.

3.4.1 Multiple library AD:cDNA plasmids

The presence of multiple AD:cDNA library plasmids in a single yeast THS positive is a relatively common occurrence if high plasmid DNA concentrations were used in the transformation reaction. This situation will be immediately apparent if multiple restriction digestion patterns are identified among the 4–5 AD:cDNA library plasmids originally isolated. Each plasmid type should be tested for reconstruction. If each of the 4–5 AD:cDNA library plasmids have identical restriction patterns and do not reconstruct reporter gene activation, it is likely that your THS positive contains multiple AD:cDNA library plasmids. An additional 20 plasmids should be isolated and analyzed as above to identify others plasmids that might be responsible for reporter-gene activation.
Failure to identify other AD:cDNA library plasmids in a THS positive suggests that it may be non-typical. Depending on the numbers of positives from the screen these non-typical positives can be retired for later analysis. When time permits they can be analyzed using the segregation analysis strategy outlined in part C9.

3.4.2 Rearranged BD:bait plasmids

Some THS false positives are caused by deletions between direct repeats within the bait cDNA, giving rise to an auto-activating BD:bait plasmid (Ref. 13). Rare events such as this can be identified using segregation analysis (see part C9).

Tips

- Cryo-preserve all positives that activate both reporter genes and initially analyze only those plasmids that reconstruct.

3.5 C5: Quantitating *GAL1-lacZ* expression

3.5.1 Liquid β-galactosidase assays

Reconstructed THS positives can be tested for the levels of *GAL1-lacZ* activation using a liquid ONPG assay modified for application to yeast (Ref. 14). It is important to assay fresh His1 yeast cultures to ensure that the *GAL1-lacZ* reporter is induced to optimal levels. Two different protocols are supplied below. The SDS-chloroform method can be used to measure the β-galactosidase activity in positives that turn blue quickly with the filter assay. The liquid-nitrogen assay can be used for positives of various strengths and is especially useful when assaying positives that require more than three hours to turn blue with the filter assay.

3.5.2 C5.1: SDS-chloroform method

Protocol

- Inoculate individual THS positives from SC-W-L-H+3-AT plates into 10 ml of SC-H or SC-W-L medium and incubate at 30°C overnight.
- Titer the overnight culture and subculture into 10 ml fresh SC-H or SC-W-L liquid to a titer of 5×10^6 cells/ml.
- Incubate with shaking at 30°C until the titer is $1–2\times10^7$ cells/ml. This should take about 2–4 h, depending on the positive.
- Dilute 500 µl of the original culture into 500 µl of water and determine the exact OD_{600}.
- Aliquot 2×1.5 ml from each individual positive into a microcentrifuge tube and pellet the cells at 13 000×g for 30 s.
- Remove the supernatant and resuspend each cell pellet in 100 µl of Z buffer (see A).
- Add 700 µl of Z buffer containing Z buffer/BME (see A).
- Add 50 µl of 0.1% SDS and 50 µl of chloroform to each tube and immediately vortex vigorously for 30 s.
- Add 160 µl of freshly made ONPG (4 mg/ml in Z buffer) and vortex to start the reaction.
- Incubate at 37°C until a yellow color develops. Incubate reactions for no more than 15 min as little color development will occur after this time.
- Stop reactions by the addition of 400 µl of 1.0 M Na_2CO_3 and record the elapsed time.
- Pellet cell debris by centrifugation at 13 000×g for 5 min. Carefully remove the supernatant and determine the absorbance at 420 nm (A_{420}).

Calculate units of β-galactosidase activity using the following formula:

$$Units=(A_{420}\times1000)/(t\times V\times OD_{600})$$

where t=elapsed time (in min), V=volume of culture used in ml, and OD_{600}=optical density of the culture used.

Tips

- Most incubation times for the SDS-chloroform assay range from 5–10 min.
- Be sure to stop the reaction when yellow color develops. Incubating reactions too long will reduce unit values.

3.5.3 C5.2: Liquid-nitrogen method

Protocol

- Inoculate individual THS positives from SC-W-L-H+3-AT plates into 10 ml of SC-H or SC-W-L medium and incubate at 30°C overnight.
- Titer the overnight culture and inoculate into 10 ml fresh SC-H or SC-W-L liquid to a titer of 5×10^6 cells/ml.
- Incubate with shaking at 30°C until the titer is between $1–2\times10^7$ cells/ml. This should take about 2–4 h depending on the positive.
- Dilute 500 µl of the original culture into 500 µl of water and determine the exact OD_{600}.
- Aliquot 2×1.5 ml from each individual positive into a microcentrifuge tube and pellet the cells at 13.000×g for 30 s.
- Remove the supernatant and resuspend each cell pellet in 100 µl of Z buffer (see A).
- Snap freeze by placing the samples in liquid nitrogen (see Tip below).
- Thaw the tubes by incubation in a 37°C water bath.
- Repeat the freeze-thaw cycle two times.
- Add 700 µl of Z buffer/BME (see A) and vortex.
- Add 160 µl of freshly made ONPG (4 mg/ml in Z buffer) and vortex to begin reaction.
- Incubate at 37°C until a yellow color develops.
- Stop the reaction by the addition of 400 µl of 1.0 M Na_2CO_3 and record elapsed time. Stop those reactions that do not turn yellow after one hour.
- Pellet the debris by centrifugation at 13000×g for 5 min. Carefully remove the supernatant and determine the A_{420}.

Calculate the units of β-galactosidase activity as described in part C5.1.

Tips

- Pierce the top of each tube with a 26–18 gauge needle before snap freezing tubes. Failure to do so may cause some tubes to explode which can lead to serious injury.

3.6 C6: Sequencing positives

Representative members of each group of AD:cDNA library plasmids that reconstruct should be sequenced to identify those positives that contain ORFs in-frame with the $GAL4_{AD}$. Double-stranded plasmid DNA can be sequenced using various commercial kits. The primers used to sequence any *GAL4* based THS vector can be found in Table 1 .

Table 1. Sequencing primers

Primers	**Vectors**
5′-TCA TCG GAA GAG AGT AG-3′	pGBT9, pAS1, pAS2
5′-TAC CAC TAC AAT GGA TG-3′	pGAD10, pGAD424, pACT, pACT2

DNA sequence information can be analyzed using your favourite DNA analysis software package. Complete or partial THS vector sequence files can be found either at GenBank (http://www.ncbi.nlm.nih.gov/) or the Misener's Vector database (http://vectordb.atcg.com/). The sequences from AD:cDNA library plasmids should be analyzed using the BLAST 2.0 algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) to identify previously cloned genes in the GenBank database. In addition, the amino acid sequence of the predicted in-frame ORF can be used to search for similarities in a protein database

Tips

- We have found that DNA isolated from the *E. coli* strain KC8 or HB101 is usually not suitable for DNA sequencing. It is best to transfer the plasmids to a strain such as DH5α.
- Positives found to encode short fusion proteins of under 20 amino acids can usually be eliminated from further analysis, however this decision should be made with reference to additional criteria. For example, Yang *et al*. (Ref. 15) have successfully used the THS to identify short peptides that interact with a protein of interest.
- THS positives showing no significant homology to known genes can be used to search the EST database found at GenBank. In some cases cDNAs with identity to your DNA sequence can be identified.

3.7 C7: Deletion mapping of interacting domains

Further analysis of THS positives includes the identification of protein motifs responsible for interaction. Deletions of the bait and library cDNA genes can be generated using restriction sites found within each. It is recommended to begin by deleting the 3′ ends of both the bait and library cDNA genes in order to preserve the fusion junctions of the translated proteins. We have found that in some cases, altering the fusion junction can unexpectedly affect the intracellular steady-state level of fusion-protein expression.

Tips

- Deletions from the 3′ end of a gene can usually be made by digesting plasmids with restriction enzymes that will cleave within the bait or library cDNAs as well as in the multiple cloning site that is 3′ of the cloned fragment. The large DNA fragment containing the gene fusion can be purified on an agarose gel and self ligated to produce the 3′ deletion plasmid. The reading frame of each gene does not normally have to be considered in this type of deletion as the 5′ end remains undisturbed.
- Deletions of the 5′ end of the bait and library cDNA genes can be produced in a similar fashion as above, however, in each case, the reading frame between the ORFs must be maintained. In most cases it is difficult to accomplish this, as available restriction sites might not necessarily provide a suitable frame. It is usually more convenient to purify a specific restriction fragment containing the desired gene fragment, followed by blunt-end ligation of it into the appropriate restriction site in a new BD or AD vector.

3.8 C8: False positives

When a large number of positives are recovered from a two-hybrid screen, some are likely to be false positives. A true THS positive will only produce reporter gene activation when in combination with a specific BD:bait plasmid. Listed in Table 2 are three classes of false positives that can occur in a two-hybrid screen.

Table 2. Two-hybrid system false positives		
Class	**Behavior**	**Test**
I	AD:cDNA library plasmids that do not require the presence of a BD:bait plasmid to activate reporter genes.	Transform AD:cDNA library plasmid into a THS yeast strain alone and test for reporter gene activity.
II	AD:cDNA library plasmids that activate reporter genes in the presence of any BD:bait plasmid.	Transform AD:cDNA library plasmid into a THS yeast strain with another unrelated BD:bait plasmid and test for reporter gene activity.
II	AD:cDNA library plasmids that activate reporter genes in the presence of an empty BD:bait plasmid.	Transform AD:cDNA library plasmid into a THS yeast strain with an empty BD:bait plasmid and test for gene activity.

False positives can be determined by transformation of the AD:cDNA library plasmid into three different yeast strains: 1) a yeast strain containing no plasmid; 2) a yeast strain containing an unrelated BD:bait plasmid; and 3) a yeast strain containing an empty $GAL4_{bd}$ vector. All transformations can be performed when reconstructing your putative positives. Alternatively, co-transformation of each plasmid combination can be performed using the `standard high-efficiency protocol' listed in Agatep *et al*., (Ref. 12). This allows a single yeast strain to be used for all transformations. Alternatively, segregation analysis (below) can also be useful in discerning class I false positives as well as identifying THS positives that contain more than one type of AD:cDNA library plasmid. A list of false positives that have been identified in some two-hybrid screens can be found at the Golemis Lab Home page (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html).

3.9 C9: Segregation analysis

In those circumstances where there are a large number of THS positives (>20) segregation analysis might be useful to eliminate class I false positives. In addition, when it is difficult to isolate the AD:cDNA library plasmid responsible for reporter gene activation, segregation analysis can be used for diagnosis. This technique involves growing the original THS positive yeast strain non-selectively, to lose (segregate) the BD:bait and/or the AD:cDNA library plasmid(s) from a portion of the cells. How the loss and/or maintenance of either plasmid affects reporter gene activity is then examined. The phenotype defined by the complement of plasmid(s) within the yeast colony can then be used to identify Type A–E positives (see Figure 3).

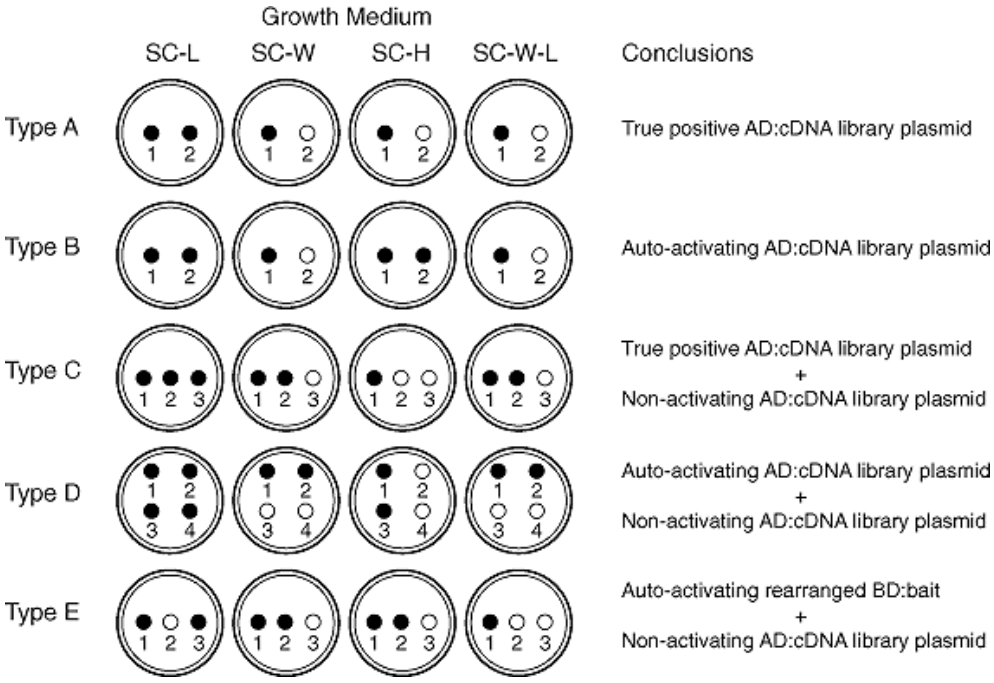

FIGURE 3. Segregation analysis of THS positives. Closed and open circles represent colony replicas that did or did not grow, respectively. The positives are classified as either Type A, B, C, D or E, based on the phenotype that defines the library plasmid. In each case, colony 1 represents the original library positive and will grow on all types of media. Colonies 2, 3, or 4, display the phenotype that define them as one of the five types of positives.Type A positives (true positives) are defined by the phenotype of colony A2. This colony contains only a *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a true positive as it requires the BD:bait plasmid to activate the reporter gene.Type B positives (auto-activating positives) are defined by the phenotype of colony B2. This colony contains only a *LEU2* AD:cDNA library plasmid, and activates the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as an auto-activating positive as it does not require the BD:bait plasmid to activate the reporter gene.Type C positives (true positive with a non-activating library plasmid) are defined by the phenotype of colonies C2 and C3 respectively. Colony C2 contains both the *TRP1* BD:bait plasmid and *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a non-activating library plasmid as it cannot activate the *HIS3* reporter gene even in the presence of the BD:bait plasmid. Colony C3 contains only a *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a true library positive as it requires the BD:bait plasmid to activate the *HIS3* reporter gene.Type D positives (auto-activating with a non-activating library plasmid) are defined by the phenotype of colonies D2 and D3 respectively. Colony D2 contains both the *TRP1* BD:bait plasmid and *LEU2* AD:cDNA library plasmid and fails to activate the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as a non-activating library plasmid as it cannot activate the *HIS3* reporter gene even in the presence of the BD:bait plasmid. Colony D3 contains only a *LEU2* AD:cDNA library plasmid and activates the *HIS3* reporter gene. This defines the AD:cDNA library plasmid as an auto-activating library positive as it does not require the BD:bait plasmid to activate the *HIS3* reporter gene. Colony D4 contains only a *LEU2* AD:cDNA library plasmid. The phenotype of D4 is likely the result of the same non-activating AD:cDNA library plasmid found in colony D2 in the absence of the BD:bait plasmid, and is not a true positive AD:cDNA library plasmid. This is the result of multiple library plasmids within your library positive. Because colony D3 contains an auto-activating AD:cDNA library plasmid, it is assumed that it is this plasmid that is responsible for reporter gene activation in this positive.Type E positives (auto-activating, re-arranged BD:bait plasmid) are defined by the phenotype of colony E2. This colony contains only a BD:bait plasmid and activates the *HIS3* reporter gene. This defines the BD:bait plasmid as an auto-activating plasmid as it does not require a AD:cDNA library plasmid to activate the reporter gene. Because this plasmid had been previously tested for *HIS3* auto-activation, it is assumed this phenotype is likely the result of a bait plasmid rearrangement.

Protocol

Non-selective growth of your THS positive can be accomplished in a number of ways. An effective method is to grow the yeast in YPAD medium for at least ten generations. Alternatively, cells can be grown in SC-L medium to ensure that the AD:cDNA library plasmid is maintained in the yeast cells.

- Inoculate 5 ml of YPAD or 10 ml of SC-L media to a titer of approximately 2×10^4 cells/ml and incubate overnight with shaking at 30°C.
- Dilute the overnight culture in sterile double distilled H_2O to give approximately 1×10^4 cells/ml and plate 100 µl of this onto each of two SC-L master plates and incubate 2 days at 30°C. This should give about 1000 colonies per plate.
- Replica plate the colonies from the SC-L master plates by taking a single impression onto a sterile velveteen replicator and then transfer onto individual plates in the following order; SC-L, SC-W, SC-W-L, and SC-H+3-AT. Incubate the plates at 30°C for 1–2 days.

Be sure to mark the orientation of each plate before replica plating. Replica plating from a single velveteen impression allows the number of cells deposited onto the SC-H+3-AT plate to be reduced. This ensures that growth on SC-H+3-AT plates is due to reporter gene activation and not heavy inoculum.

There are five different types of growth patterns that will be identified from these plates. These are illustrated in Figure 3. Type A is the pattern seen for true positives containing a single AD:cDNA library plasmid. Type B occurs for a class I false positive, which activates the reporter genes in the absence of a BD:bait plasmid. If a yeast cell containing a true positive AD:cDNA library plasmid also contains with another non-activating AD:cDNA library plasmid Type C growth pattern will occur. If your THS positive contains a class I false positive and another non-activating plasmid, Type D growth pattern will result. Finally, if your THS positive contains a BD:bait plasmid that has been rearranged, causing it to auto-activate, a Type E growth pattern will be identified.

Tips

- Class I false positives have a defining colony phenotype (see Type B, Figure 3). If the AD:cDNA library plasmid is found to be auto-activating, no further analysis is required.
- The isolation of the AD:cDNA library plasmid responsible for reporter gene activation in a THS positive containing multiple AD:cDNA library plasmids can be performed from a segregated yeast colony displaying the reporter activation phenotype (see Type C colony 1, Figure 3).
- The presence of Type E (Figure 3) positives, BD:bait plasmids that auto-activate can be assayed for using an altered protocol (C9.1). The initial segregation should be done in YPAD (Step 1). The master plate must be SC-T (Step 3).

3.10 References

[1] Fields S. and Song O. (1989) *Nature*, **340**:245-246.
[2] Li B. and Fields S. (1993) *FASEB J.*, **7**:957-963.
[3] Bartel P.L., Roecklein J., SenGupta D. and Fields S. (1996) *Nat. Genet.*, **12**:72-77.
[4] Wang H. *et al.* (1995) *J. Biol. Chem.*, **270**:23322-23329.
[5] Wang D. *et al.* (1997) *J. Biol. Chem.*, **272**:19383-19392.
[6] Gyuris J., Golemis E., Chertkov H. and Brent R. (1993) *Cell*, **75**:751-803
[7] Hoffman C.S. and Winston F. (1987) *Gene*, **57**:267-272.
[8] Cryer D.R., Eccleshall R. and Marmur J. (1975) *Methods Cell Biol.*, **12**:39-44.
[9] Dower W.J., Miller J.F. and Ragsdale C.W. (1988) *Nucleic Acids Res.*, **16**:6127-6145.
[10] Hanahan D. (1983) *J. Mol. Biol.*, **166**:557-580.
[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning—a laboratory manual*, Cold Spring Harbour Laboratory Press
[12] Agatep, R., Kirkpatrick, R.D., Parchaliuk, D.L. and Gietz, R.D. (1998) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01525
[13] El Hounsni H. *et al.* (1998) *Anal. Biochem.*, **262**:94-96.
[14] Miller, J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press
[15] Yang M., Wu Z. and Fields S. (1995) *Nucleic Acids Res.*, **23**:1152-1156.
[16] Parchaliuk, D.L. et al. (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01616
[17] Parchaliuk, D.L. et al. (1999) *Technical Tips Online* (http://www.biomednet.com/db/tto) P01713

4 Appendix A

4.1 Media

4.1.1 Yeast extract–peptone–dextrose medium + adenine (YPAD)

Yeast extract 6 g
Peptone 12 g
Glucose 12 g
Adenine hemi-sulphate 24 mg (liquid), 48 mg (plates)
Distilled water 600 ml
Difco Bacto-agar 10 g (plates)
Sterilize by autoclaving at 121°C for 20 min. We make 600 ml of media in a 1 l flask as it is easier to handle for pouring and makes exactly 20 plates (30 ml/plate×20–100 mm plates).

4.1.2 LB + ampicillin

Tryptone 6 g
Yeast extract 3 g
NaCl 6 g
Distilled water 600 ml
Titrate to pH 7.0 with 10N NaOH
For plates: add 10 g Difco Bacto Agar to per 600 ml volume in each flask prior to sterilization. When cooled, add 300 µl of a 100 mg/ml stock of ampicillin.
For liquid: do not add agar; ampicillin should be added just prior to use.

4.1.3 Synthetic complete medium (SC)

Difco yeast nitrogen base (without amino acids) 4 g
Glucose 12 g
Synthetic complete selection medium mix 0.4 g
Distilled water 600 ml
Difco Bacto Agar 10 g (add for agar medium)
Add ingredients to water, mix and adjust the pH to 5.6–6.0 with NaOH; autoclave at 121°C for 20 min.

4.1.4 Synthetic complete selection medium mix (SC medium)[1]

Supplement Amount Final concentration
Adenine hemisulfate 1.8 g 30 mg/l
Arginine HCl 1.2 g 20 mg/l
Histidine HCl 1.2 g 20 mg/l
Inositol 2.0 g 33 mg/l
Isoleucine 1.8 g 30 mg/l
Leucine 1.8 g 30 mg/l
Lysine HCl 1.8 g 30 mg/l
Methionine 1.2 g 20 mg/l
p-aminobenzoic acid 0.2 g 3 mg/l
Phenylalanine 3.0 g 50 mg/l
Homoserine 6.0 g 100 mg/l
Tryptophan 2.4 g 40 mg/l
Tyrosine 1.8 g 30 mg/l
Uracil 1.2 g 20 mg/l
Valine 9.0 g 150 mg/l
[1]Omit the appropriate component(s), indicated in **bold type**, to prepare SC-H, SC-L, SC-W, SC-W-L, and SC-W-L-H.
Combine the ingredients in a clean 250 ml plastic bottle. Add three or four clean glass marbles and shake vigorously to mix. This quantity will be sufficient for approximately 60 liters or 100×600 ml batches of SC medium.

4.1.5 M9 Minus Leucine prototrophy medium (M9-L)

10×M9 salts 60 ml
distilled water 540 ml
Difco Bacto-agar 10 g
Autoclave this, allow to cool until flask is warm to touch, then add sterile:
$MgSO_4$ (1.00 M) 0.60 ml
$CaCl_2$ (0.10 M) 0.60 ml
Thiamine (4 mg/ml) 0.50 ml
Glucose (20% w/v) 6.00 ml
$FeCl_3$ (0.01 M) 0.15 ml
Vitamin B1 (2 mg/ml) 0.60 ml
Depending on the genetic markers found in your *Escherichia coli* strain, add the appropriate amino acids:
For KC8 add the following:
Histidine (2 mg/ml) 6 ml
Uracil (2 mg/ml) 6 ml
Tryptophan (2 mg/ml) 6 ml

4.2 Solutions

4.2.1 Z Buffer (1.0 litre) pH 7.0

$NaH_2PO_4.H_2O$ 13.79 g
KCl 750.00 mg
$MgSO_4.7H_2O$ 246.00 mg
Titrate with 10 N NaOH to pH 7.0.

4.2.2 X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 20 mg/ml

Dissolve 1.0 g of X-GAL in 50 ml of N,N-dimethylformamide and store at −20°C.

4.2.3 Z buffer/β-mercaptoethanol

This should be made fresh by adding 270 µl of β-mercaptoethanol to 100 ml Z buffer.

4.2.4 Z buffer/β-mercaptoethanol/X-GAL

This should be made fresh by adding 270 µl of 2-mercaptoethanol and 1.67 ml of X-GAL solution (20 mg/ml) to 100 ml of Z buffer.

4.2.5 ONPG (o-nitrophenyl-β-D-galacto-pyranoside), 4 mg/ml

Dissolve 200 mg of ONPG in 50 ml Z buffer.

4.2.6 Extraction buffer

50 mM HEPES pH 7.4
200 mM NaCl
10 mM ethyline diaminetetraacetic acid (EDTA)
2 mM $NaVO_4$
10 mM NaF
5 µg/ml aprotinin
5 µg/ml leupeptin
2 µg/ml E-64
2.5 µg/ml pepstatin A
1 mM phenylmethylsulfonyl fluoride (PMSF)

4.2.7 TBST Buffer

20 mM Tris-HCl pH 7.5
200 mM NaCl
0.1% Tween 20
Add 5% (w/v) skim milk powder just before use.

4.2.8 SDS-loading buffer

3% w/v sodium dodecyl sulfate
62.5 mM Tris pH 6.8
720 mM β-mercaptoethanol
10% v/v glycerol
0.125% (w/v) bromophenol blue

4.2.9 TE buffer

10 mM Tris-Cl pH 8.0
1 mM EDTA pH 8.0

4.2.10 Yeast lysis buffer

10 mM Tris-Cl pH 8.0
100 mM NaCl
1 mM EDTA
2% (v/v) Triton X-100
1% (w/v) sodium dodecyl sulfate (SDS)

4.2.11 M9 Salts (10 ×)

Na_2HPO_4 60 g
KH_2PO_4 30 g
NaCl 5 g
NH_4Cl 10 g
Bring up to 1 l with distilled water and autoclave.