Genome-wide analysis of protein-protein interactions by using a two-hybrid array

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Introduction

Genome-wide two-hybrid methodologies are powerful and efficient global approaches used to catalogue the extensive set of protein-protein interactions that occur in a cell. These methodologies rely on knowledge of a large fraction of the predicted genes in an organism and the use of high-throughput techniques coupled with robotic equipment. Large-scale data sets of protein-protein interactions have been generated for several model organisms, including the budding yeast (Uetz et al. 2000; Ito et al. 2001), *Drosophila* (Giot et al. 2003) and *C. elegans* (Li et al. 2004). The data generated by these efforts has influenced many areas of biological research by providing investigators with a framework of interactions between proteins that participate in the specific processes of interest. It has also been fertile ground for computational studies, such as investigations into the topology of protein networks (Han et al. 2004; Yook et al. 2004; Yu et al. 2004). Overall, these global two-hybrid studies are having a significant impact on our understanding of the biological processes within the cell.

The information generated by genome-wide two-hybrid screens has increased our knowledge concerning the properties of two-hybrid interactions. While a significant proportion of the interactions are biologically relevant, interpretation of large-scale two-hybrid data should proceed with caution. A fraction of the data produced by two-hybrid
assays is either not reproducible, or is physiologically unlikely, based on the biological context of the proteins involved. These putative interactions are termed "false positives". The confidence in individual interactions can be increased substantially by integrating information from other genomic data sets. In addition to false positives, one must also consider the interactions that are expected but not observed. A substantial number of such interactions, which are termed "false negatives", are characteristic of genome-wide two-hybrid data sets (Ito et al. 2001; von Mering et al. 2002). Another characteristic of current data sets is a lack of overlap between different two-hybrid sets, potentially indicating that screens are far from saturating the total number of interactions that exist (Hazbun and Fields 2001). This lack of saturation extends to other protein-protein interaction technologies including mass spectrometry, suggesting that our knowledge of protein-protein interactions, even within yeast, is far from comprehensive (von Mering et al. 2002).

Comparison of genome-wide two-hybrid data and mass spectrometry data is proving that each technology tends to identify different areas of the "interaction space". The interaction space is defined as the total of all interactions that physiologically occur between proteins. Two-hybrid and mass spectrometry are both well-suited to detecting soluble protein interactions, but are less successful at detecting the interactions of integral membrane proteins. Each technique is also disadvantaged for particular areas of the interaction space: the two-hybrid approach cannot be used on transcription-stimulating proteins that activate reporter genes on their own, while mass spectrometry is biased against identifying interactions with proteins that are expressed at very low levels. Thus
the two approaches can provide complementary datasets that together will represent a larger fraction of the interaction space for proteins of the cell.

A recent study that used these technologies on a common set of yeast proteins of unknown function indicated the technologies agree on predicted functions that were ascribed based on protein-protein interactions, despite a low overlap in those interactions (Hazbun et al. 2003). Assignment of the cellular role of each protein was achieved using the controlled vocabulary of the Gene Ontology (GO), which has structured networks of terms in three categories, describing the attributes of a protein in terms of a biological process, a cellular component or a molecular function (Ashburner et al. 2000). Assignment of each protein in the biological process category was based on the annotations of its interacting proteins and this resulted in GO terms upon which the two approaches generally agreed. However, the two-hybrid interactions tended to occur with proteins that were annotated in more broadly related biological processes, resulting in GO term assignments that were lower in resolution than the mass spectrometry-based terms (Table 1). This corroborates earlier reports suggesting that the two-hybrid method can detect protein-protein interactions that are thought to be transient and weak (Estojak et al. 1995). This property of two-hybrid is important in identifying links between biological processes that impinge upon each other (Figure 1). Another aspect to consider, particularly in studies with yeast proteins, is the existence of so-called "bridging interactions" in which one or more endogenous yeast proteins mediate the interaction observed between the two-hybrid fusion proteins. For example, a genome-wide two-hybrid screen using Dam1 identified six subunits of the Dam1 complex, whereas
comprehensive *in vitro* binding assays indicated only three direct interactions between Dam1 and particular subunits (Shang et al. 2003). Based on these properties, genome-wide two-hybrid technology is a powerful method to survey possible interacting protein partners. As with other current technologies aimed at delineating protein-protein interactions, it is apparent that we still have far to go, even in a well-characterized model organism such as the budding yeast, *Saccharomyces cerevisiae*. The goal of the remainder of this chapter is to introduce the reader to current protocols for carrying out high-throughput two-hybrid screens. We employ an array method to perform simultaneous analysis of the pair-wise interactions of one protein with all of the other proteins in yeast, as described below.

**Description of the Array method**

The array method we discuss here was applied to $\sim$6000 open reading frames (ORFs) of the *Saccharomyces cerevisiae* genome, but could be applied to any collection of ORFs, including the genome complements of other organisms or smaller sets of functionally related ORFs. The array approach was first used on a small scale in analyzing protein-protein interactions involved in cell cycle regulation (Finley and Brent 1994), but has been extended to a genome-wide level. As the name implies, the array method relies on a spatially ordered set of yeast strains, each expressing a different protein fused to the Gal4 activation domain (AD). A mating strategy is used where a strain expressing a protein fused to the Gal4 DNA binding domain (BD) is mated to each array strain (Cagney et al. 2000). The strain expressing the Gal4 BD fusion protein is termed the “bait” and the
array strains expressing the Gal4 AD fusion protein are termed the “prey”. Diploid yeast strains expressing both of these protein fusions are selected using auxotrophic media and subsequently two-hybrid positives are selected for the activation of a reporter gene that allows their growth on the appropriate media (Figure 2).

Global two-hybrid methods can be classified on the basis of whether they use an array or library approach (Figure 3). Library methods are based on complex pools of yeast strains expressing different AD-fusion proteins that are probed by an individual BD strain or small pool of strains. The first use of the library approach was demonstrated by searching a library of fragmented genomic DNA (Chien et al. 1991), and has been used recently in several large-scale two-hybrid studies (Uetz et al. 2000; Ito et al. 2001; Giot et al. 2003; Li et al. 2004). One inherent advantage of the array over the library method is that its parallel nature allows the comparison of a large number of individual assays under identical growth conditions. In this way, each individual pair-wise assay between bait and prey can be compared with every other pair-wise assay occurring simultaneously in the array. Among other advantages, this facilitates the identification of false positives, as discussed in Protocol 3. The array approach also has greater sensitivity than a library screen, since weakly growing positives are less likely to be competitively lost, compared to a pooled experiment. In addition, each positive is readily identified due to its known location in the array, and the multiple sequencing of positives and false positives that complicates library searches can be avoided. However, sequencing is not entirely eliminated since it is necessary to sequence positives to ensure quality control for the array elements, but only a sampling of the positives has to be sequenced.
In addition to the above positive factors, several negative factors need to be considered when using a genome-wide array (Table 2). The array method can be laborious, even when robotic equipment is used, because it involves testing thousands of pair-wise interactions on an individual basis. A recent promising method that reduces the work load combines pooling and array approaches using a limited pooling scheme (Zhong et al. 2003). This strategy increases throughput while maintaining high representation of each clone. However, the question of competition between clones even in a limited pool must still be considered. Another pair of problems encountered when relying on arrays of live yeast is loss of viability and occurrences of contamination. The recommendations in our protocols can help minimize such problems. The array we describe is static and dependent on the accuracy of genome annotation. For example, our array was constructed to represent the *S. cerevisiae* genome as it was annotated in 1998, but the gene annotations have since been revised based on comparative sequencing of related fungal genomes (Cliften et al. 2003; Kellis et al. 2003). Thus, the composition of the array will not reflect the most current knowledge of the proteome without being constantly updated with new ORF constructs.

**Outline of procedure**

Screening for protein-protein interactions using the array approach requires a spatially ordered set of yeast strains expressing proteins fused to the Gal4 AD. Construction of the array is achieved by PCR and recombinational cloning (Figure 4), in conjunction with
high-throughput transformation of the appropriate yeast strain. To capitalize on the advantageous use of the mating procedure, the array strains must be haploid yeast. This allows the introduction of the Gal4 BD and AD fusions into the same diploid cell by mating of the haploid array strains with a haploid strain of the opposite mating type containing the BD fusion (Figure 5). Subsequent selection for diploids is achieved using auxotrophic media, and the selection of two-hybrid positives is possible by testing for growth on media that requires the activation of reporter genes under the control of Gal4-binding sites.

We provide protocols for the construction, screening and maintenance of yeast-based arrays, as applied to genome-wide two-hybrid experiments. Many of these protocols have been outlined before (Cagney et al. 2000; Gera et al. 2002), but we have refined and updated them for this chapter. In principle, most of the techniques we provide here could be modified to maintain and screen any live yeast array, such as the viable yeast deletion set (Winzeler et al. 1999) or a set of strains harboring plasmids with different cDNAs. We provide protocols that rely on robotics and the use of multiple high-density plates to represent the yeast genome.

Protocols

**Protocol 1: Amplification of ORFs**

The first step in constructing an array is to amplify the predicted ORFs that are to be included in the array. Gene-specific primers containing vector-specific flanking
sequences that facilitate recombinational cloning are used to amplify each ORF. The flanking sequences we used were homologous to sequences present in both two-hybrid vectors, pOAD and pOBD2 (sequences and construct information available at: http://depts.washington.edu/sfields/yp_interactions/index.html). These allowed fusion of the ORF to the Gal4 AD or the Gal4 BD, respectively. A secondary amplification can be used to extend the length of the homologous vector sequence flanking the ORF (Figure 4).

Buffers, Solutions and Reagents

Set of gene-specific primers for yeast ORFs with vector homologous ends (100 pmol/µl)

Common primers for secondary amplification (100 pmol/µl)

Template DNA (see Step 1 for more information)

Polymerase buffer

MgCl₂, 150 mM stock solution

Thermostable polymerases (Taq – Promega, Corp; Pfu - Stratagene®)

Special Equipment

Thermal cycler

Additional reagents

This procedure also requires equipment and reagents for agarose gel electrophoresis
Method

1. Setup primary PCR reaction for amplification of ORFs with gene specific primers: 50-200 ng of template genomic DNA (10-100 ng of cDNA; for yeast genomic DNA we have successfully used 30 ng as template), 1.5 mM MgCl₂, 5 units of Taq polymerase, 0.003 units of Pfu polymerase, and 20 pmol of each gene-specific primer.

<note> We used genomic DNA since introns are rare in *S. cerevisiae*. When generating an array for higher eukaryotic organisms, use full-length cDNA libraries or other sources such as cDNA clone collections.

2. Generate the yeast array using the following primers;

   Forward primer: 5’- G GAA TTC CAG CTG ACC ACC ATG XXX₂₀₋₃₀-3’

   Reverse primer: 5’-GA TCC CCG GGA ATT GCC ATG XXX XXX₂₀₋₃₀-3’

<note> An X represents gene-specific sequences within the primers, with the first XXX triplet in the reverse primer (reading 5’ to 3’) representing the reverse complement of a stop codon. For other experiments, optimize PCR conditions for the chosen primers.

Carry out PCR under the following conditions;

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>denaturation</th>
<th>annealing</th>
<th>extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C for 3 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>95°C for 60 sec</td>
<td>50°C for 45 sec</td>
<td>72°C for 210 sec</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>72°C for 8 min</td>
</tr>
</tbody>
</table>

3. Verify the length of amplification products by agarose gel electrophoresis.
4. Carry out a secondary amplification of the primary PCR product;

<note> It is crucial to optimize this secondary PCR step since mutation rates can be very high in multiple PCR amplification steps.

<table>
<thead>
<tr>
<th>Primary PCR product</th>
<th>5 ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq polymerase</td>
<td>0.6 units</td>
</tr>
<tr>
<td>70-mer primer</td>
<td>25 pmol of each</td>
</tr>
</tbody>
</table>

Primer sequences used for this reaction are as follows:

Forward primer: 5’C TAT CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC GAA TTC CAG CTG ACC ACC ATG 3’

Reverse primer: 5’C TTG CGG GGT TTT TCA GTA TCT ACG ATT CAT AGA TCT CTG CAG GTC GAC GGA TCC CCG GGA ATT GCC ATG 3’.

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>denaturation</th>
<th>Annealing/extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C for 45 sec</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>94°C (15 sec)</td>
<td>72°C (2-12 min, depending on ORF size)</td>
</tr>
</tbody>
</table>

<note> The secondary PCR is carried out to add homologous vector sequence and thereby increase recombinational cloning frequency. However, we have found that if the primary PCR product is robust (~ 20 ng/µl), the secondary amplification is not necessary, and efficient recombinational cloning can be achieved with as little as 20 bases. Previous reports show that it is feasible to use 30-40 bases of homology between vector and insert (Oldenburg et al. 1997).
**Protocol 2: Cloning by Homologous Recombination and High-throughput Transformation**

Although described here as a means of producing baits and preys, the generation of an array of ORFs fused to any moiety can be achieved using this protocol. This high-throughput transformation protocol is based on a previous report (Gietz and Woods 2002). The major change is that the transformation is carried out in 96-well plates with adjusted volumes. One person can handle up to 4 plates per day using this procedure. In the case of the yeast genome, sixty-four 96-well plates would be required to generate ~6000 AD-fusion strains.

Our array procedure capitalizes on the asexual/sexual reproductive cycle of yeast cells. Laboratory yeast strains can be maintained indefinitely by asexual reproduction as one of two haploid mating types, MATa or MAT<α>. However, overlaying MATa and MAT<α> cells allows them to fuse and form diploid cells. Hence, our array strategy involves transforming MAT<α> cells with the Gal4 BD (bait) fusion plasmids, and transforming MATa cells with the Gal4 AD (prey) fusion plasmids. The transformants are then mixed on a plate, or in liquid, and diploids are selected by plating them onto media lacking the marker nutrients for both of the plasmids. In this way, the <--> 6000 prey transformations need only be performed once, and each bait protein can then be tested against the array of prey-containing strains using only one transformation per bait. We describe the large-scale transformation used to generate an array of prey
transformants. The protocol is easily adapted to small-scale transformation of single microcentrifuge tubes for bait transformations.

Note: Expression of some heterologous proteins in the two-hybrid context can be poor and the proteins may be susceptible to proteolysis in yeast. Consequently, a quality control step that checks for expression level and protein size by western blot is advisable, at least in the bait fusion strains.

**Strains**

**THE PREY ARRAY STRAIN**

PJ69-4A [MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4delta gal80delta

*LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* (Cagney et al. 2000)]

**THE BAIT STRAIN**

PJ69-4<α> [MATalpha trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80delta

*LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* (James et al. 1996)]

**Buffers, Solutions and Reagents**

Yeast extract-peptone-dextrose (YEPD) plus 20 mg/L adenine

Yeast synthetic dropout (SD) media (Yeast Nitrogen Base-amino acids-dextrose) plus 40 mg/L adenine

Linearized vector DNA- pOAD for generating prey strains with Gal 4 activation domain fusions, or pOBD-2 for generating bait strains with Gal4 binding domain fusions (Cagney et al. 2000).

Insert DNA (PCR product from Protocol 1)
Salmon sperm DNA (Sigma), 5 mg/mL in H₂O and autoclaved (15 min, 121°C). Stored in aliquots at –20°C.

Lithium acetate, 1 M

Polyethylene glycol (PEG) (MW = 3350) solution 50%, filter-sterilized.

Glass beads, 5-mm diameter (autoclaved before use)

96-well flat bottom plate, sterile e.g., Costar 3595, Corning, Inc.

Polyethylene sealer tape – e.g., Thermowell, Costar 3595, Corning, Inc. Non-sterile

Petri plates, 100 mm, sterile

Toothpicks, autoclaved

**Special Equipment**

Shaking incubator, preset to 30°C

Incubator, preset to 42°C

Robot capable of handling high-density replicating tools (see Protocol 3 for more detail).

**Method**

1. Inoculate PJ69-4A yeast cells (for pOAD) into YEPD with 20 mg/L adenine. 
   <note> Because the strains are ade2 mutants, we add extra adenine to all of the media to maximize growth and viability; however, the adenine may be omitted if desired. Use 1 ml medium for each different transformation (i.e., 100 ml for 96-well transformation plus four controls).

2. Grow the culture at 30°C overnight with shaking at 270 rpm, until the yeast are at mid-log phase (0.5-0.6 OD₆₆₀).
3. Pellet the cells by centrifugation at 2500 g for 5 minutes at room temperature.

4. Resuspend the pellet in 100 ml of double distilled H₂O at room temperature.
   Repeat centrifugation (Step 3).

5. Resuspend the pellet in 100 ml of lithium acetate (100mM) at room temperature.
   Incubate at room temperature for 1 hour.
   <note> The cells can be stored for 3 days at 4°C, but the transformation efficiency drops with time.

6. After the 1 hour incubation, pellet the cells by centrifugation at 2500 g for 5 minutes and resuspend them in 1/10th of the starting volume (10ml) of 100 mM lithium acetate.

7. Dispense 100 µl of the resuspended yeast into each well of a 96-well plate.

8. Centrifuge the plate at 2500 g for 5 minutes at room temperature. Remove the supernatants.

9. Make up the following transformation mix:
   For each 96-well plate:
   
   PEG solution, 50%  8 ml
   H₂O  2 ml
   Lithium acetate, 1 M  1 ml
   0.5 ml Sheared salmon sperm DNA, 5 mg/ml  0.5 ml

   Total =11.5 ml
   <note> Only 10 ml per plate is actually used.

10. Pipet 100 µl of the transformation mix onto each cell pellet. Vortex to resuspend the yeast in the PEG solution.
11. Add 5-50 ng of linearized vector to each well.  
<note> The exact amount is vector preparation dependent, but a molar ratio between 1:5 to 1:10 of vector to insert is optimal. 

12. Add 5 <µ>l of a 50 <µ>l PCR insert obtained in Protocol 1 to each well. 

13. Mix by pipetting up and down with an eight-tip pipettor. Alternatively, seal the plate wells with tape and vortex to mix, taking care to avoid cross-contamination of wells. 

14. Seal plates with tape and incubate at 30°C for 45 minutes. 

15. Incubate at 42°C for 20 minutes. 

16. Dispense the contents of each well onto a separate 100-mm Petri plate containing yeast SD media lacking the appropriate nutrient (leucine for pOAD, or tryptophan for pOBD2) and with 40 mg/L adenine. 

17. Add 3-8 glass beads (5-mm diameter) to each plate and shake to spread the cell suspension over the plate. Pour off the beads and incubate the plates upside down at 30°C for three-four days, until colonies are ~2mm-3mm in diameter. 

18. Once the colonies have grown, transfer individual transformants or pools of transformants with sterile toothpicks into a 96-well plate containing 100 <µ>l of the appropriate SD media in each well. Grow the cultures, and then transfer the cells to solid media using robotic pinning (see Protocol 3).  
<note> It is important to pre-test the linearized vector preparation to minimize vector only transformants. If linearized vector alone gives rise to transformants, this problem can be reduced by digesting with two restriction enzymes followed by agarose gel purification. 

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For a higher density array, the yeast can be condensed by robotic pinning of four staggered 96-spot plates onto each 384-spot plate.

19. To obtain larger spots of yeast, grow the array of prey-containing yeast on SD-Leu media (plus 40 mg/L adenine) to maintain selection for the plasmid and pin the spots onto YEPD (plus 20mg/L adenine) prior to pinning for screens (see Protocol 3).

**Protocol 3**

**High-density Robotic screening procedure**

This protocol focuses on how to handle arrayed yeast using robotics. There is a steep learning curve associated with robotics, but once that is overcome the procedure is straightforward. We do not explain how to use a robot because that is beyond the scope of this protocol and the available robotic equipment will vary for each researcher.

**Buffers, Solutions, and Reagents**

AD-ORF array – from Protocol 2

BD-ORF strain (PJ69-4<α> transformed with the bait fusion in pOBD)

Empty sterile Omni Trays- 2 (Nunc™)

Omni Trays (32) filled with 40 ml of solid YEPD media with 20 mg/ml of Adenine

YEPD liquid medium

YEPD solid medium containing 20 mg/L Adenine

SD medium containing 40 mg/L Adenine, lacking tryptophan (Trp) and leucine (Leu)
SD medium containing 40 mg/L Adenine lacking histidine (His), tryptophan and leucine
SD medium containing 40 mg/L Adenine, lacking tryptophan (SD- Trp + Ade)
Adenine hemisulfate salt (added to a final concentration of 20 mg/L for YEPD and 40 mg/L for SD)
Plastic bags, re-closable, 25.4 cm x 25.4 cm
3-AT 3-Amino-1,2,4-triazole) (Sigma)
Ethanol, 95%
Bleach, 20%

**Equipment**

Robotic 96-pin and 384-pin replicating tools (Beckman Coulter, Inc) or manual tool (Nunc™, V&P Scientific, Inc)
Bio mek® FX or 2000 robot (Beckman Coulter, Inc) or other robot capable of handling high-density replicating tools
Incubator set for 30°C

**Method**

**Robot configuration and use:** Detailed explanations of the use of a Bio mek 2000 robot (Beckman Coulter, Inc.) for transferring yeast with high-density replicating tools have been outlined previously (Cagney et al. 2000; Gera et al. 2002). The basic principle relies on metal pins that, in a 96 or 384 array format, are used to transfer cells from liquid
or colonies to new plates. The pins must be sterilized by sequentially dipping them into 20% bleach, sterile water, and 95% ethanol. The conditions for the sterilization cycle should be tested to ensure there is no cross-contamination. Residual ethanol can be dried from the pins using a fan or heat source, or dipping in sterile water. It is important to have the robot pre-wet the pins by dipping them in sterile water before pinning from a colony because this increases the uniformity of transfer. The size of pins used in the 384-format can vary from 1 mm to 1.3 mm.

**pOBD2-ORF transformation:**

1. Transform the BD-ORF strain (PJ69-4<α>) with the pOBD2-ORF plasmid and select transformants on SD-Trp +Ade. If yeast strains containing pOBD2-ORF were constructed by recombinational cloning, they should be streaked out to obtain fresh colonies. Incubate at 30°C until the colonies are 2-3 mm in size (<~3 days).

<note> It is important to compare the size of transformant colonies to those obtained after transformation with empty vector (pOBD2) or other bait plasmids. Small or highly heterogeneous colonies are indicative of possible toxicity from expression of the bait protein and such colonies may not mate well in subsequent steps. In rare instances, we have observed that freshly transformed colonies perform better for some strains than colonies that have been stored at 4°C on plates for more than a week. These strains should use the modified Protocol in step 3. Yeast proteins that are very toxic when over-expressed should be used with a two-hybrid vector containing an inducible promoter and the two-hybrid selection done under inducing conditions.

**Bait Self-Activation Test**
Bait strains can vary in basal reporter gene activation so they need to be tested for activation:

2. Obtain a diploid strain by mating the MAT<α>bait strain (PJ69-4<α>) with a MATα strain containing pOAD (the empty prey vector). Generate the diploid by overlaying the haploid strains on solid YEPD +Ade medium and growing overnight at 30°C.

Optimal mating efficiency is seen when both haploids are grown in liquid to logarithmic phase, but saturated cultures, or even colonies taken from plates will still provide sufficient successful matings.

3. Select diploids by replica-plating onto SD media +Ade, lacking leucine and tryptophan, and incubating for 2-3 days at 30°C. Then streak the diploid yeast onto SD +Ade, lacking tryptophan, leucine, and histidine, supplemented with varying levels of 3-AT (3 mM, 10 mM, 25 mM and 50 mM).

The use of 3-AT helps to reduce background levels of expression of the reporter gene HIS3. This is via the stoichiometric inhibition of His3 enzymatic activity by 3-AT. Thus, the lowest level of 3-AT that completely inhibits growth of the cells in the absence of histidine is used in subsequent experiments. Non-activating baits are screened at 3 mM 3-AT, while the highest level of 3-AT we have used successfully is 150 mM.

**Bait growth:**

4. Inoculate two separate flasks containing 25 ml of YEPD plus adenine (20 mg/L), each with a single, large transformant colony and grow overnight for a minimum of 15 hours (OD₆₀₀ = 0.7-1) at 30°C.

Two cultures of the same bait are prepared because the screen is done in duplicate. For slow growing or problematic strains, grow overnight in 100 ml of YEPD
plus 20 mg/L adenine and concentrate to 25 ml. This concentration step helps collect enough yeast to allow sufficient mating in step 5.

**Copying the AD array:**

5. Pre-wet pins in H$_2$O and then use a 384-pin HDR replicating tool to pin colonies from the AD array onto 2 sets of 16 Omni Trays containing YEPD solid media. The spots should be slightly visible, but not too dense and it is acceptable to have some variability in spot density.

**Transfer bait onto AD array:**

6. Pour 25 ml of bait culture (or concentrated culture from Step 4) into an empty sterile Omni Tray, and place it on an orbital shaker on the robot. (Shake at 600 rpm on the Biomek FX). Program the robot to dip the pins into the bait culture and transfer it onto the freshly pinned AD array from Step 5. Make sure the bait pinning overlays the previous AD array pinning and ensure mixing of the spots by pinning up and down several times in the same position.

7. After transfer of the bait strain to the array plate, sterilize the pins by robotic pinning into plates of 20% bleach (45s), water (5s), then 95% ethanol (10s), as described above. Perform this cycle 16 times for one copy of the array then pin the second copy of the array with the second bait culture.

8. Enclose the Omni Trays in plastic bags to ensure they do not dry out and incubate for 2 days at 30°C.

<note> The Plastic bags ensure sufficient aeration and allow plates to expand. Sealing plates with Parafilm or tape is not recommended.

**Selection of diploids:**
9. Using the pin tool, transfer colonies from the 2 sets of 16 Omni Trays with mated yeast to new Omni Trays containing SD, supplemented with adenine, but lacking leucine and tryptophan.

10. Incubate for 2 days at 30°C.

<note> This step indicates the success of the mating. If the growth of diploids is low or spotty, then it is likely that there was a problem with pinning or that the mating frequency was low, possibly due to a problematic bait strain. If this happens, the screen can be continued, and positives may still be identified, but the completeness of the screen is highly suspect.

Selection of two-hybrid positives:

11. Use the robot to transfer the diploid colonies to Omni Trays containing SD –Leu –His –Trp, supplemented with the appropriate amount of 3-AT. Incubate for 1-2 weeks at 30°C.

<note> Strong positives will appear within 3 days, but there will often be additional positives that appear over longer incubations.

12. Score the screens using a 384 spot grid generated by any graphics software program. Colonies that grow above the background level of growth of the rest of the array are scored as positives.

<note> We make use of a Filemaker Pro script that is integrated with our database of two-hybrid results. Screens with some baits result in many non-reproducible two-hybrid positives through ill-defined mechanisms. This problem can be circumvented by considering only the positives that are confirmed by the duplicate screen. These double positives are usually reproducible upon re-testing. It is still worth considering interesting
single positives because there may have been a pinning error on one set of the duplicate plates. Single positives should be re-tested manually or in a low-density plate to confirm that they are reproducible.

**Low-density alternative:** Low-density screens using a 96 spot format can be used for i) re-testing single or double positives from the high-density screen, ii) testing a group of functionally related ORFs, or iii) comparing two-hybrid interactions between a set of related baits. A 96-pin high-density replicating tool (pin diameter of 2 mm) can be used either with a robot or manually. These pins will deliver approximately 1 µl of liquid. The above high-density procedure of sterilization and pre-wetting of pins should be used when replicating.

1. Aliquot of 100 µl YEPD, plus adenine, into each well of a 96-well plate.
2. Inoculate each well with the individual prey strains which will constitute a mini-array. To prevent the plate drying out, place in an airtight container with a wet paper towel. Grow at 30°C overnight.
3. Inoculate 2 test tubes containing 5-10 ml of YEPD plus adenine with single colonies of bait strain and grow overnight at 30°C with shaking. As described for the high-throughput screens, perform this screen in duplicate using two independent bait colonies against a prey strain.
4. Pipette 50 µl of one bait strain and 50 µl of the prey strain into each well of a sterile 96-well plate. Repeat this with the other bait culture to produce a duplicate 96-well plate containing the pair-wise combinations of bait and prey. Pin 2 or 3 times
from these plates so as to transfer \(2-3 \mu\)l onto solid YEPD plus adenine media.

Incubate overnight at 30\(^\circ\)C.

5. Transfer the array by replicating to SD -Leu-Trp. Incubate for 2 days at 30\(^\circ\)C.

6. Transfer the array again by replicating to SD –Leu-Trp-His +Ade with 3-AT.

   Incubate for 1-2 weeks at 30\(^\circ\)C. Score the growth to identify two-hybrid positives.

**Protocol 4: Yeast Plasmid Miniprep**

This protocol is an efficient method for the recovery of yeast plasmids using the QIAGEN QIAprep\textsuperscript{®} Spin Miniprep Kit (Catalog #27106). The quantity of plasmid DNA isolated using this protocol is lower than the amounts routinely isolated from bacteria, but provides sufficient DNA for *E. coli* transformation by electroporation for further steps. The only change from the manufacturer’s instructions is the inclusion of the glass bead step. This protocol can also be applied to the 96-plate format QIAGEN kit (Catalog #27191), but in our experience the yields from yeast cells are often too low even for electroporation. Hence we prefer a large number of individual tube preps to the less-efficient plate format.

**Materials**

**Buffers, Solutions, and Reagents.**

YEPD liquid medium
Adenine hemisulfate salt
**Special Equipment**

Incubator preset to 30°C

Rotating wheel or horizontal shaker

QIAGEN QIAprep® spin miniprep kit (Cat. # 27106)

Acid-washed 425-600 micron glass beads (Sigma®)

Vortexer with multiple tube attachment(s)

**Method**

1. Supplement 2 ml of YEPD with 40 µg adenine and inoculate with a colony of yeast containing the plasmid of interest. Incubate overnight at 30°C on a rotating wheel or horizontal shaker. Alternatively, grow the yeast in selective media (SD +Ade -Leu -Trp -His).

2. Transfer 1-1.5 ml of the yeast to a microcentrifuge tube and centrifuge to pellet the yeast.

3. Aspirate the supernatant. Add 250 µl of P1 buffer from the Qiagen kit and 100 µl of acid-washed glass beads (425-600 micron) to each tube.

4. Vortex the tubes for 10 minutes to break open the cells.

5. Add 250 µl of P2 buffer. Invert several times to mix.

6. Add 350 µl of N3 buffer and invert several times to mix. Centrifuge in a microcentrifuge at maximum speed for 10 minutes.

7. Apply the supernatant to a Qiaprep column. Centrifuge the column in a microcentrifuge at maximum speed for one minute.
8. Empty the collection tube and add 750 µl of PE buffer. Centrifuge at maximum speed for one minute.

9. Empty the collection tube and centrifuge at maximum speed for one minute.

10. Place the Qiaprep column into a fresh microfuge tube.

11. Add 30 µl of EB buffer. Centrifuge at maximum speed for a further minute to elute the plasmid DNA.

**Protocol 5: Storage, Maintenance, and Working With Living Arrays**

The most difficult aspect of using an array approach for a high-throughput study is maintaining the array's viability while avoiding contamination of the plates. Generating frozen stocks of the array strains individually, as well as in the arrayed format, is essential. The individual strains are used to replace lost positions, and the full copies to restore the array if contamination or loss of viability destroys the active versions. Multiple copies (at least 3) of the plates are important to reduce the need to go back to the frozen stocks. Frequent re-plating of the active array copy helps to keep the yeast healthy and prevents the emergence of slow-growing contaminants on the plates. Following the precautions outlined in this protocol will help prevent catastrophic loss of the hard-earned array reagents.

We describe three different storage methods for the array. Frozen stocks are the most permanent method, and are an absolute requirement for maintaining a record of the array. Unfortunately, because thawing is necessary to access the strains from a 96-well format, the repeated use of the frozen stocks as a source is expected to eventually lead to loss of
viability of the stock. Maintenance of a water stock provides a source that is more convenient, and (relative to yeast cells in media) semi-permanent (\(\sim\) 6 months). However, the water stock is fairly precarious in that cross-transfer of strains between wells can occur quite easily if the plates are dropped or disturbed. The third method of storage, that of maintaining extra copies of the solid media array plates, is the most convenient, but it is also the least stable. In short, the best option is to make several frozen stocks. For greater convenience in repeated use of the array, generate water stocks and additional copies of the solid media plates.

**Materials**

**Buffers, Solutions, and reagents**

96-well format array, frozen stocks

Glycerol or DMSO

YEPD plus 20 mg/L adenine Omni Trays

SD-Leucine plus 40 mg/L adenine Omni Trays

**Special Equipment**

Paper towels

Plastic bags, re-closable, 25.4 cm x 25.4 cm
Method

96-Well Plate Freezer Stocks

<note> When vortexing 96-well plates it is important to try and reduce cross-contamination by limiting the volume in each well to less than 140 µl. Gentle vortexing at low speed will prevent splashing across wells or onto the tape covering the well. It is worth practicing this before embarking upon the following protocol.

1. Make multiple (at least 3) copies of the 96-well format frozen stocks. Although freeze-thawing is the most efficient way to re-initiate the working array, over time, it will ruin the viability of the stock. Remake copies as needed to maintain 3 copies of the stocks;
   a. Grow yeast in 96-well omni plates containing 100 µl YEPD with 20mg/L adenine. Seal the wells to avoid evaporation and incubate at 30°C for two nights. Gently vortex the plate(s) to mix the yeast on the first day. Alternatively, pin into the liquid plates from thick colonies grown on YEPD agar, transferring enough yeast to simulate a saturated culture.
   b. Add 50 µl of 50% glycerol (final concentration of 16.7%) or 6 µL of DMSO (final concentration of ~5%) to each well.
   c. Seal the wells with tape designed to maintain adhesion at ~80°C (e.g. costar cat # 6524). Vortex to mix the contents. Place immediately at ~80°C.
96-Well Plate Water Stocks

Note: Yeast are viable in water for months when stored at 4°C. An array of strains in water can be used to generate new arrays or for easy access to individual strains. Strains harboring plasmids, such as the prey array, can be maintained this way without obvious plasmid loss. The advantage over the frozen stock is that repeated accessing of water stocks will not affect viability.

1. Completely thaw frozen 96-well stock plates and vortex gently to resuspend the cells. Use a 96-Pin tool to pin from a 96-well frozen stock of yeast onto solid medium, such as Omni Trays containing YEPD plus 20 mg/L adenine.
2. Generate thick colonies by incubating at 30°C for three days.
3. Pin from the thick colonies grown on YEPD agar to sterile double distilled water. Transfer as much yeast as possible by repeating this step several times.
4. Seal the wells with polyethylene tape to reduce evaporation during storage. Store at 4°C.
Maintenance of the Working Array

1. Maintain three copies of the array on agar plates. Contaminated plates, or those missing colonies due to pinning errors, can be discarded and replaced by good copies. If all copies of a plate are lost, then resort to the freezer or water stocks to restore it.

2. Use plastic bags to prevent the plates drying out. In addition, add a moist paper towel to the bag when putting into the 30°C incubator. As with Petri plates, store the Omni Trays upside down so moisture does not collect on the agar surface.

3. When moving the plates to the 4°C refrigerator, remove the paper towel and, if condensation has accumulated on the lids, place them in the refrigerator without the plastic bag for a day or two. Be sure to return plates to plastic bags for long term storage.

4. If condensation accumulates on the lids at any time, hold the plate upside down, remove the lid and shake off the condensation. Take care to keep the plates level when first returning them to a bag in case enough liquid is present to roll around the lids. Rotating the top of the stack to the bottom and *vice versa* once a week will help to keep the bottom plates from becoming saturated with water and the top plates from drying out.

5. Pin the array to new plates after they have been at 4°C for a maximum of one month.

7. To maintain plasmids, alternate the array between YEPD and SD plus 40 mg/L adenine lacking the appropriate selective marker.
8. Prior to screening, use YEPD plus 20 mg/L adenine as a source plate for the frozen stock, and as the first plate pinned from the frozen stock. The growth of the colonies on this medium is larger, there is increased yeast viability, and the recovery from freezing is more rapid and consistent than on synthetic media.

References


**Figure Legends**

Figure 1. Network map of protein-protein interactions identified by two-hybrid and mass spectrometry-based technologies (Hazbun et al. 2003). Proteins are represented by colored circles. The underlined proteins were used as Gal4 BD-fusions in two-hybrid screens to identify positives, as well as being affinity tagged and purified before being subjected to mass spectrometry to identify co-purifying proteins. The solid lines represent interactions identified by two-hybrid, and the dashed ovals encircle proteins purified with the underlined protein within that oval. The protein-protein interactions observed by mass spectrometry involved proteins predominantly functioning in DNA repair, suggesting these unknown proteins are part of a novel DNA repair complex. In contrast, the function of interacting proteins identified by two-hybrid included DNA repair and other processes that may be more transiently associated with these novel DNA repair complexes. These other processes are functionally grouped by color and labeled with the process. The grey protein (Arl1) is likely a false positive since it is involved in vesicle-mediated transport.

Figure 2. The mating strategy used in genome-wide array screens. The BD-X plasmid is used to transform a haploid two-hybrid yeast strain (MAT alpha), or “Bait”, that carries the reporter gene. The AD-Y plasmid is used to transform a haploid strain of the opposite mating type (MAT a), or “Prey”, by recombinational cloning (see Protocol 2). These two strains are mated to form a diploid strain carrying both plasmids. A two-hybrid interaction will result in the transcription of the reporter, *HIS3*, resulting in the ability of the strain to grow on synthetic dropout (SD) media lacking histidine.
Figure 3. Diagram comparing the array and pooled library approaches. (A) In the array approach, each pair-wise combination of BD-X and AD-Y is tested individually and the resulting two-hybrid positives are known by their location in the array. (B) The library approach pools all the AD-Y strains in one tube and subsequent positives are identified by sequencing. Several other approaches that use the basic principles of the array and library approaches are also actively used.

Figure 4. Diagram of the recombinational cloning strategy employed in Protocol 2. In the primary amplification, a pair of gene-specific primers, each containing 20 nucleotides of vector sequence, is used to amplify each ORF (black or white arrows with orange and yellow tails, respectively). In the secondary amplification, vector sequence homology is extended using primers with 50 nucleotides of additional sequence (orange or yellow arrows with purple or cyan tails). The resulting amplification product contains 70 bp of vector sequence flanking the ORF. This product is used, with linearized vector, to co-transform the appropriate 2-hybrid strains, generating in-frame fusions to either Gal4 BD or AD.

Figure 5. Flowchart depicting the array-based screening procedure outlined in Protocol 3. Each replicating step is achieved by using a high-density replicating tool. The figure depicts 96-spot format plates, although we use 384-spot plates to represent the yeast genome. The screens are performed in duplicate starting with two transformants of the identical bait strain cultured in yeast extract-peptone-dextrose (YEPD) liquid media. The
bait strain harbors a plasmid with the *TRP1* marker and the prey strain harbors a plasmid with the *LEU2* marker. The haploid bait and prey strains are overlaid together on solid YEPD media to allow them to mate. The resulting diploids are then selected on SD medium lacking leucine and tryptophan (SD –Leu –Trp) (diploids will have both plasmids and will therefore survive in the absence of those nutrients). Selection of two-hybrid positives is performed on SD lacking leucine, tryptophan, and histidine (SD –Leu –Trp –His) supplemented with 3-aminotriazole (3-AT). The positives that appear on both plates of the duplicate screen are considered reproducible positives, whereas the single positives usually do not reproduce upon re-testing.
Table 1. Assignment of Gene Ontology (GO) terms to uncharacterized yeast proteins (left column, ORF) based on protein-protein interactions observed by two-hybrid or mass spectrometry methods (Hazbun et al. 2003). GO Biological Process terms were determined by using the GO Term Finder available on the Saccharomyces Genome Database website (http://www.yeastgenome.org). For each protein, the tool was used to find significant shared GO terms among the interacting proteins identified by two-hybrid or mass spectrometry. From the 100 proteins that were analyzed, 8 proteins had sufficient data that allowed the assignment of GO process terms by both methods. The protein-protein interaction overlap between the methods was low but the Biological Process terms generally agreed in that they were related and within the same branch of the ontology network. However, the two-hybrid predictions resulted in generally lower resolution terms because they encompassed interactions that included proteins in several related biological processes. The asterisk indicates that these proteins were part of unknown complex 1 and could not be assigned a Biological Process term since all the complex members at the time had unknown function.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Two-hybrid prediction</th>
<th>Mass spectrometry prediction</th>
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<tbody>
<tr>
<td>YFR003C</td>
<td>Morphogenesis</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>YKL088W</td>
<td>Cellular process</td>
<td>Coenzyme A biosynthesis</td>
</tr>
<tr>
<td>YML023C</td>
<td>Cell growth and/or maintenance</td>
<td>DNA repair</td>
</tr>
<tr>
<td>YDR288W</td>
<td>Response to DNA damage stimulus</td>
<td>DNA repair</td>
</tr>
<tr>
<td>YGR002C</td>
<td>Nuclear division</td>
<td>Histone acetylation</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Complex</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>YLR424W</td>
<td>Nucleobase, nucleoside, nucleotide and nucleic acid metabolism</td>
<td>mRNA splicing</td>
</tr>
<tr>
<td>YDR013W (PSF1)</td>
<td>DNA repair</td>
<td>Unknown complex 1*</td>
</tr>
<tr>
<td>YJL072C (PSF2)</td>
<td>Unknown complex 1</td>
<td>Unknown complex 1*</td>
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Table 2. Advantages and disadvantages of using genome-wide two-hybrid arrays compared to library approaches.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td><strong>Unbiased</strong>: A genome-wide array is not overly biased toward protein categories that are more amenable to the two-hybrid approach.</td>
<td><strong>Slow</strong>: Although the method has a high-throughput capability, it does require testing thousands of pair-wise assays</td>
</tr>
<tr>
<td><strong>Reduced false positives</strong>: Each protein is tested individually and in duplicate so false-positives can be identified and eliminated or ignored.</td>
<td><strong>Maintenance</strong>: Difficult to maintain and handle an array of live yeast. Care is necessary to reduce contamination.</td>
</tr>
<tr>
<td><strong>Sequencing</strong>: Identity of positives is known from their location in the array reducing the amount of sequencing required.</td>
<td><strong>Annotation dependent</strong>: The ORFs present in the array are dependent on the accuracy of genome annotation.</td>
</tr>
<tr>
<td><strong>Sensitivity</strong>: Potential positives are not subjected to growth competition between each other because they are separated and tested individually.</td>
<td><strong>Equipment</strong>: Robotic equipment is essential for high-throughput screening.</td>
</tr>
<tr>
<td><strong>Controls</strong>: The non-interacting positions in the array provide a large number of negative controls, but empty vector can also be added to do this. False-positives in the array provide positive controls that indicate that the media is sufficient to allow growth of positives.</td>
<td><strong>Pitfalls of the array method</strong>: Biologically-irrelevant interactions may be observable in the one vs. one format of the array that would not be competitive in library approaches. As with any two-hybrid approach, some positives, while valid two-hybrid interactions, may not be physiological interactions, and follow-up experiments must be used to address the validity of a given interaction.</td>
</tr>
</tbody>
</table>
Figure 1

DNA repair complex I

DNA repair complex II

Transcription

Chromosome organization

Chromosome cycle

Sumoylation/Ubiquitylation

False positive
Figure 3

A

Identified two-hybrid positive

B

Sequence to identify
Primary amplification of ORF1 with gene-specific primers

Secondary amplification to extend homologous vector sequence

Linearized vector

Clone into two-hybrid vector by homologous recombination \textit{in vivo}
Bait
BD-ORF
YEPD (2 x 25 mL)

YEPD (2 x 16 plates)

2 days

Diploid with
BD-ORF & AD-ORF 1-6144

SD -Leu -Trp (2 x 16 plates)

2 days

Reproducible
two-hybrid positive

SD -Leu -Trp -His + 3-AT (2 x 16 plates)
1-3 weeks

Prey
AD-ORF 1-6144
YEPD (1 x 16 plates)