

# Systematic and large-scale two-hybrid screens

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The increasing rate at which complete genome sequences become available necessitates rapid and robust methods for investigating the functions of their encoded proteins. Efforts have been made to study protein function by systematically screening large sets of proteins using the two-hybrid method. Analyses of the complete proteomes of bacteriophage T7, the mammalian viruses hepatitis C and vaccinia, as well as of several protein complexes including RNA splicing proteins and RNA polymerase III from yeast, have been undertaken. *Saccharomyces cerevisiae* has been studied extensively by two-hybrid methods, with more than 2500 protein–protein interactions described. Systematic studies on metazoan proteomes are, however, still in their infancy.

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## Abbreviations

<b>HCV</b>	hepatitis C virus
<b>MS</b>	mass spectrometry
<b>ORF</b>	open reading frame
<b>pol III</b>	RNA polymerase III
<b>T7</b>	bacteriophage T7
<b>YPD</b>	Yeast Protein Database

## Introduction

By the end of the year 2000 the genomes of more than a hundred bacterial or eukaryotic species will have been completely sequenced. Even in the best-studied model organisms, however, the functions of the majority of proteins remain poorly understood, if not completely mysterious. For example, of roughly 6100 predicted yeast proteins, about 2000 have no known function assigned to them, and the functions of another 800 have only been

inferred from similarity to other proteins with known functions [1,2]. Roughly 50% of yeast genes encoding proteins are considered ‘known’ in the sense that some direct genetic or biochemical observation has been used in assigning a function.

One way to characterize the function of a protein is to identify other proteins that bind to it. If the function of one of the two proteins is known, then the function of the partner is likely to be related and can thus be inferred. In this way, through an iterative process of protein–partner determination, one can build out from a relatively small number of functionally characterized proteins to assign function to a large number of uncharacterized ones. To accomplish this on a genomic scale will require processes that are performed in a highly parallel and automated fashion. However, few large-scale protein–protein interaction studies have been reported so far. Here we review the two major methods for detecting protein–protein interactions — two-hybrid systems and mass spectrometry — and recent progress in genome-scale two-hybrid maps of protein–protein interactions.

## Methods for detecting protein–protein interactions

There are a number of methods available to study protein–protein interactions that are at least somewhat amenable to high-throughput applications. Each has its particular strengths and weaknesses; these are summarized briefly in Table 1.

In the yeast two-hybrid system, pairwise interactions between protein fusions are detected *in vivo* through protein–protein interaction-dependent reporter gene activation (Figure 1) [3–5]. This has typically been done by screening a protein of interest against cells expressing a random library of potential protein partners, and applying a genetic selection for interaction. Plasmid DNA is recovered from cells expressing interacting proteins and their identities are determined by DNA sequencing. However, two-hybrid screening can also

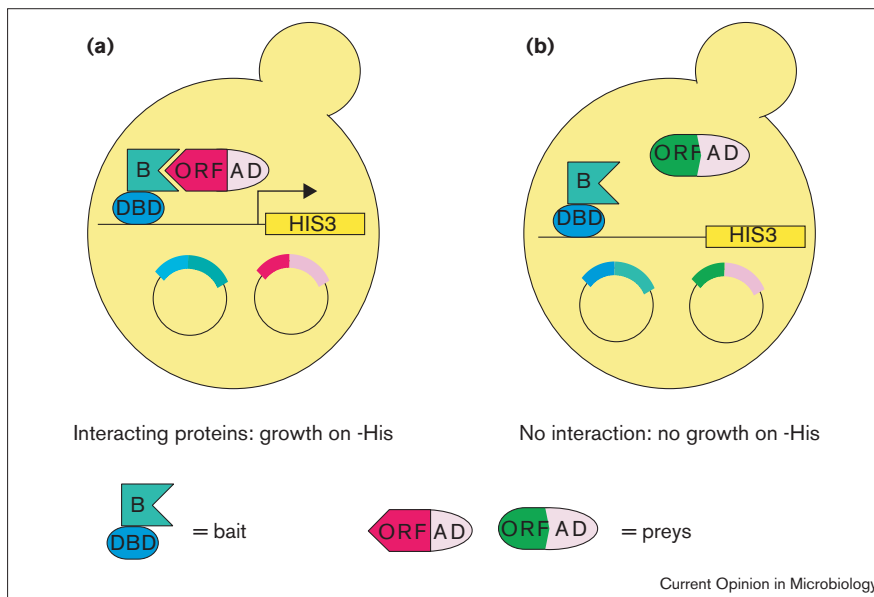
**Table 1**

### Major methods to study protein–protein interactions.

Method	Major properties	Reference
Two-hybrid	Inexpensive; <i>in vivo</i> conditions; yields binary interactions; uses living cells; requires protein fusions; requires localization to the nucleus; simple	[19]
Pull-down ( <i>in vitro</i> assays)	<i>In vitro</i> conditions; semi-quantitative	[13**]
Immuno-precipitation	Allows study of native complexes; requires antibodies	[20]
Mass spectrometry	Allows study of native complexes; requires purification of complex; expensive; technically difficult	[6]
Surface-plasmon resonance	Quantitative; expensive; pure proteins required	[21]

For a detailed review of methods see [20].

Figure 1



Principle of the yeast two-hybrid system. Two hybrid proteins are expressed in yeast. One consists of a DNA-binding domain (DBD) fused to protein 'B' (= bait) whose interactions are being studied. The other hybrid (the 'prey') consists of a transcriptional activation domain (AD) which is fused to a defined protein of interest (ORF) or random protein fragments (i.e. a 'library'). (a) If bait and prey interact, a transcriptional activator is reconstituted that can activate transcription of a reporter gene (here *HIS3*). Expression of *HIS3* allows the cell to grow on histidine-free media. Interaction of two proteins therefore generates an easily visible yeast colony, whereas (b) non-interacting proteins don't.

be done in a colony array format where the identities of cells expressing potential interactors are spatially addressed. Since the particular protein expressed in each colony is encoded in its position in the array, positive interactors are identified by position, thus obviating the need for DNA sequencing. Several two-hybrid studies have used yeast colony 'mini-arrays' for the analysis of particular groups of proteins. Mini-arrays (as opposed to complete genome arrays) comprise sets of cells expressing proteins related by some common function, or the interests of particular investigators.

The interrogation of a two-hybrid colony array usually involves a mating strategy in which one bait is tested against all preys in a grid pattern. For the sake of simplicity, arrays usually use full-length open reading frames (ORFs) or fragments found in a previous library screen. They allow one to systematically test all possible protein pairs of interest in an internally controlled fashion (see also discussion below).

In contrast, mass spectrometry (MS) methods generally involve purification of protein complexes from living cells and the subsequent identification of their components based upon highly accurate mass determinations [6]. This approach can be limited by the abundance of the proteins studied, and often requires that significant amounts of proteins be purified. This is the reason that most MS studies have concentrated on relatively abundant complexes like ribosomes or spliceosomes [7,8].

In addition to the technically difficult purification and analysis of such complexes, the pairwise connectivities of the protein components remain unclear by MS analysis; in contrast, two-hybrid studies identify pairwise interactions and, therefore, can allow the reconstruction of 'higher resolution' protein interaction maps [9<sup>•</sup>-11<sup>•</sup>,12<sup>••</sup>].

Two-hybrid and MS analysis of the same complexes can yield surprisingly different results. For example, in a two-hybrid screen the yeast Skp1 protein was tested for interactions with all other yeast proteins individually, yielding 11 interacting proteins [12<sup>••</sup>]. Purification of a complex by means of a HA-tagged Skp1 followed by analysis by mass spectrometry yielded eight interacting proteins, of which only two overlapped with the set obtained by two-hybrid analysis (R Aebersold, personal communication). For this reason, the methods should be considered as complementary rather than competing. Both two-hybrid systems and MS can be automated to a high degree, and, therefore, used in large-scale approaches. With the advent of genome-wide purification of glutathione-S-transferase (GST)-fusions, genomic scale *in vitro* assays such as MS analysis are likely to be in common use in the near future [13<sup>••</sup>].

## Two-hybrid screens

### Viral genomes

The first genome-wide two-hybrid study was carried out on bacteriophage T7. Bartel *et al.* [14] screened a library of random T7 protein fragments against random libraries of T7 activation domain fusions. Among the 55 phage proteins, these authors found 25 interactions, including four interactions that had been described previously. Surprisingly, this study uncovered an interaction between proteins 18.5 and 18.7, which are encoded by the same DNA sequence but in different reading frames. In addition, the study found seven intramolecular interactions between domains or sequences of the same protein and an additional seven homotypic intermolecular interactions.

Interactions within the much larger vaccinia proteome were studied by McCraith *et al.* (personal communication).

Table 2

## Systematic and large-scale two-hybrid studies.

System	Number of proteins	Number of baits screened	Number of two-hybrid* interactions observed	Bait and library format†	References
T7	55	55	25 (4)	Random vs. random	[14]
Vaccinia	266	266	37 (9)	ORF vs. ORF	(a)
HCV	~10	10	5 (2)	Random vs. random	[15]
Splicing	>60	15 [10], 11 [11]	170/31 <sup>§</sup> [10], 277/66 <sup>§</sup> [11]	ORF vs. random <sup>‡</sup>	[10*,11*]
RNA pol III	17	19**	20 <sup>‡</sup>	ORFs vs. random <sup>‡</sup>	[9*]
Yeast	~6200	192/432 <sup>§</sup>	957 (1700)	ORF vs. ORF	[12**]
<i>Drosophila</i> Cdks, Cdis	?	14	19	ORF vs. ORF	[18]
<i>C. elegans</i> vulva	≥47	29	148	ORF vs. random	[17*]

See text for details. \*Number of interactions known before are given in parentheses. †Baits and libraries can be random fragments or full-length ORFs. ‡Total number of preys/A1 preys (see text and table 3 for explanations). §These two studies used the same library. \*\*Including four non-pol III subunits (TFIIB70, TBP, A12.2 and B12.5) and three

proteins that acted as transcriptional activators and were not screened. TFIIB70 is a pol III-associated protein. ‡Only interactions with known pol III components or transcription factors. §192 baits were screened using an array, 432 baits were screened using a full-length ORF library (see text). (a) S McCraith, personal communication.

Vaccinia, a double-stranded DNA virus that infects mammalian cells, has about 266 ORFs. McCraith *et al.* systematically tested all 266 full-length ORFs against all full-length ORFs in a colony array format, and observed about 40 interactions, most of which were previously unknown. It will be of interest to use this technique to determine which viral proteins interact with which host proteins.

Flajolet *et al.* [15] studied interactions among proteins of the hepatitis C virus (HCV), a plus-stranded RNA virus. This virus represents a special case because its genome encodes a single polyprotein that is processed into about 10 mature proteins. In this study, all mature proteins were tested pairwise against each other in 'full-length' DNA-binding domain fusion ('bait') and transcriptional activation domain fusion ('prey') combinations. Surprisingly, no interactions were found this way. The authors conclude that protein products from this "full-length" pre-protein don't work in their two-hybrid assay, most likely due to folding problems. In order to circumvent these problems, they generated random libraries from the HCV genome in both bait and prey vectors. After eliminating auto-activators (i.e. DNA-binding domain fusions which themselves activate transcription), they screened 200 randomly chosen bait clones. In this configuration, five interactions were found, of which three had not been previously reported.

### Protein complexes

The first large-scale analysis of a functional complex by two-hybrid methods was done by Fromont-Racine and co-workers in 1997 [10\*]. These authors used 15 yeast proteins involved in mRNA splicing to screen a random yeast genomic library, and found a total of 170 putative interactions corresponding to 145 different prey ORFs. Among these were nine known splicing factors and another five proteins with similarity to known splicing proteins.

However, 50% of the interacting proteins corresponded to proteins of unknown function.

Among the 145 ORFs that Fromont-Racine *et al.* [10\*] selected as preys, 29 were represented by overlapping protein-coding fragments and therefore allowed the mapping of a particular domain that interacted with the bait. Interestingly, among the 29 overlapping sets of ORFs, nine included inserts with out-of-frame fusions. For some of these, Fromont-Racine *et al.* showed independently that the fusions produced functional proteins. Therefore, at least under the conditions chosen, translational frameshifting seems to be a significant mechanism of protein expression in yeast, and should be taken into account when analyzing two-hybrid preys by DNA sequencing.

In a follow-up study, Fromont-Racine *et al.* [11\*] again screened their random yeast library with another 11 yeast proteins involved in splicing (one of them, LSM8, had been screened before [10\*]). These 11 screens yielded a total of 277 interactions of which 66 belonged to their 'A1' class — that is, interactions of high confidence that had been found in several independent screens as overlapping fragments (see discussion below).

The yeast RNA polymerase III (pol III) is a protein complex comprised of 17 subunits. Flores *et al.* [9\*] screened 15 cloned pol III subunits (of 17) and four non-pol III proteins against the same yeast genomic library used by Fromont-Racine *et al.* [10\*,11\*]. Three baits acted as transcriptional activators and were not analyzed further. Another three proteins did not yield any positive interactions, among them the two large subunits of pol III. Of the remaining 13 subunits, all but one yielded at least one component of the pol III complex.

**Table 3****Comparison of array and random library screens.**

Bait	Hits with high confidence*			Hits of low confidence*		
	Library	Array	Overlap	Library	Array	Overlap
LSM2	11	10	2	43	24	4
LSM4	7	9	2	27	38	4
LSM8	10	12	6	47	69	8

Array screens are full-length ORFs, whereas the library screens described here use random fragments. \*Library screens classify preys as A1–A4, whereas array screens classify preys as reproducible or non-reproducible. High confidence: A1 (library screens) and reproducible (array screens). Low confidence: A2–A4 (library screens) and non-reproducible (array screens). A1 = several independent overlapping clones, A2 = fusions start close to the initiation codon of a yeast ORF and encode amino-terminal interacting domains, A3 = fusions consisting of large coding inserts, A4 = other candidates (classification [10], data from [11]). Reproducible = found in two out of two array screens. Non-reproducible = found in only one of two array screens (data and classification from [12\*] and Uetz *et al.*, unpublished data). Numbers correspond to different preys found. Overlap numbers correspond to preys found in both library and array screens.

**Yeast**

By the beginning of 2000, the Yeast Protein Database (YPD) listed about 1200 interactions among *Saccharomyces cerevisiae* proteins [1]. Approximately half of these have been identified using two-hybrid methods [16]. Recently, our laboratory has started a genome-scale two-hybrid screen using yeast colony arrays and robotics to systematically identify protein interactions in *S. cerevisiae* [12\*\*]. Initially, we cloned all 6000 yeast ORFs into an activation domain ('prey') plasmid and transformed these plasmids into yeast to generate an array of 6000 colonies. This array could then be interrogated by mating its colonies to yeast cells of the opposite mating type expressing a bait construct. This was done for 192 bait proteins, and 287 putative interactions were detected. In addition, we [12\*\*] describe an alternative approach where all prey cells are pooled and screened in a more conventional two-hybrid screen format. This was done with several thousand bait strains, but only 432 yielded interactions. Interestingly, the array approach yielded about 3.4 interactors per bait, whereas the library approach yielded only about 1.8 [12\*\*]. Clearly, the latter number depended also on the number of colonies analysed by sequencing, but by sequencing 12 colonies per bait almost 7,000 PCR products were sequenced to generate 692 putative interactions. Altogether, both approaches generated 957 interaction pairs of which only 109 were known before.

***Caenorhabditis elegans***

One of the first attempts to study interactions of *C. elegans* proteins in a systematic fashion [17\*] focused on a set of 29 proteins involved in vulval development — probably the best-studied developmental process in worms. First, 29 proteins known to be involved in vulval development were tested against each other for interaction; 11 interactions were already known before and six of these were found in

the matrix experiment. In addition, two novel interactions were found. Second, Walhout *et al.* [17\*] carried out library screens with 27 of the vulval proteins as baits and found 148 potential interaction partners for 17 of them. The average number of interactions reported in these screens was about nine per bait — a similar number to that found for yeast splicing proteins. However, the screens also contained six bait proteins that generated more than 10 hits each, and it remains to be seen how many of these will be confirmed by other methods.

***Drosophila***

Finley and Brent [18] reported the first small-scale array experiment to study interactions among cyclin-dependent kinases (Cdks) from *Drosophila* and other species with potential interactors, mainly cyclins and related proteins (cyclin-dependent kinase interactors, Cdis). Finley and Brent [18] discovered 19 interactions showing a striking specificity of these closely related proteins. With the recently published *Drosophila* genome sequence, plans have been made for systematic two-hybrid screens of the *Drosophila* proteome both in academia and corporate settings (R Finley, J Rothberg, personal communication).

**What do we learn from these studies?**

More than 2500 protein interactions have been reported from yeast. A preliminary analysis shows that more than 2500 of these proteins are connected to each other by about 2300 protein interactions in a huge protein network (P Uetz, B Schwikowski, unpublished data). Such studies show that there is considerable crosstalk between proteins in a cell, even between proteins belonging to different functional groups.

Furthermore, interactions between well-studied proteins and proteins of unknown function allows the prediction of protein function. For example, Uetz *et al.* [12\*\*] have found more than 200 proteins of unknown function that interact with proteins of at least partially characterized function. It may be possible to deduce functions for most unknown proteins when information about protein interactions is combined with expression or localization data that is currently generated by large functional genomics studies.

**Technical aspects: arrays and random libraries**

A few systems have been studied with both arrays and random libraries and, therefore, allow comparison of the two strategies (Table 2; see also the section above about HCV). Uetz *et al.* [12\*\*] screened their yeast array with the three splicing proteins LSM2, LSM4, and LSM8, and Fromont-Racine *et al.* [11\*] screened random genomic libraries with the same proteins (Table 3). Although both screens are claimed to be close to saturating, they identified different sets of preys, with only a small number of preys found in both screens. Overall, both types of screens yielded a significant percentage of highly plausible hits (i.e. proteins that have been associated with splicing or RNA metabolism).

The relative advantages and disadvantages of using full-length proteins versus protein fragments in two-hybrid assays are specific to any given interacting pair, and can often only be determined empirically. One general case in which full-length proteins are a significant disadvantage is when one of the interactors is an integral membrane protein. It is highly likely that interactions involving integral membrane proteins will not be efficiently detected in a full-length protein array format. However, simplicity and high-throughput considerations strongly favor the use of full-length ORFs in the construction of genome-scale colony arrays. Large scale analyses of two-hybrid interactions involving integral membrane proteins will require the construction of baits and arrays containing soluble domains of these proteins.

### Large-scale two-hybrid screens: large-scale production of false-positives?

False positives arise when yeast cells grow under selective conditions in a manner that is not dependent on bait/prey protein–protein interaction. This can occur through a number of mechanisms. Large-scale two-hybrid screens can effectively identify some classes of systematic false positives because they occur in multiple, independent screens under standardized conditions. For example, many false positives tend to appear repeatedly in screens with unrelated baits and, therefore, can be excluded. Fromont-Racine *et al.* [10•] devised a classification scheme for the reliability of two-hybrid positives. Their most reliable positives are called ‘A1’ and consist of preys that are found as independent overlapping clones (see Table 3). The array screens by Uetz *et al.* [12••] use ‘reproducibility’ as a measure for reliability by testing each individual two-hybrid pairing twice in a highly standardized, parallel fashion. Non-reproducible positives are most likely generated by mutations in the bait or prey plasmids or in the reporter gene including its promoter. When screens are done in duplicate, such mutations are unlikely to occur simultaneously in both screens. Reproducible positives often correspond to highly plausible preys. For example, the array screen with the splicing factor LSM8, mentioned above [12••], had a total of 69 hits of which only 12 were reproducible. Of these 12, 10 were known to be involved in RNA metabolism or RNA binding.

### Conclusions

The systematic two-hybrid studies described here clearly show that single screens rarely capture all proteins capable of interacting with a given bait. Even multiple screens carried out with different two-hybrid variants usually generate only partially overlapping sets of positives. The widely hailed advent of automated mass spectrometry doesn’t replace two-hybrid screens but rather complements them. Obviously several different methods are still needed to identify all interacting proteins and complexes but more studies have to be done to address the strengths and weaknesses of each method.

Recognizing the potential value of protein–protein interaction data for the identification of drug targets, several private interests have embarked on genome-scale two-hybrid analyses (see <http://www.hybrigenics.com> and <http://www.myriad-pronet.com>). Given the value of this information to the academic community at large, the development of proprietary protein–protein interaction databases and the ‘ownership’ of particular protein–protein interactions raises issues similar to those raised by public versus private DNA sequence information. One hopes that the interests of both academia and private industry can be well served by the free distribution of this information for non-commercial purposes.

### Update

Ito *et al.* [22•] have recently published a second large scale two-hybrid screen that identified 183 interactions among yeast proteins of which half were novel. Emili and Cagney [23] have recently reviewed the use of various protein arrays.

The work referred to in the text as (S McCraith, personal communication) is now in press [24].

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