High-throughput cell-based assays in yeast

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With the sequencing of many genomes now completed, biologists are faced with the challenge of deciphering the function and association of an immense number of predicted proteins. Comprehension of the networks of proteins and chemicals that comprise the cells and tissues of an organism, and the specific roles of proteins in these networks, will be a necessary next step to understanding cellular function in healthy and diseased states. In the past decade, the budding yeast *Saccharomyces cerevisiae* has emerged as an important tool for large-scale functional genomics analyses. This review describes the use of yeast cell-based assays in the post-genomic era, focusing on high-throughput functional genomics and drug discovery.

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▼ The most useful tools and technologies are often those that can be adapted to carry out new functions. The Internet, for example, originally designed for the US government, is now used for vastly different activities, including entirely new modes of commerce and communication. Similarly, the yeast *Saccharomyces cerevisiae*, traditionally important for brewers and bakers, and more recently used by geneticists, is gaining relevance as a tool in large-scale biomedical research. This encompasses genomic and proteomic technology development, high-throughput functional genomic analysis, and drug discovery.

Yeast has become a prominent model organism for two reasons: its simplicity and its proven relevance to the human genome. Possessing few introns, and with a genome encoding only ~6000 proteins, *S. cerevisiae* provides a starting point for the analysis of more complex eukaryotic organisms. Because of its small size, the yeast genome was the first sequenced eukaryote. As a unicellular organism with a rapid growth rate, yeast can be used for whole-cell studies that would be prohibitively difficult or costly in multicellular organisms. Yeast also has a long history of elegant genetic studies that have proven its relevance to human disease. That two of the 2001 Nobel prize recipients in Physiology or Medicine are yeast cell-cycle researchers is an acknowledgement of the immense contribution the yeast field has had on medicine in general, and cancer research in particular.

Its ease of manipulation means that yeast has also emerged as the model system of choice for the testing of new technologies and approaches for genomic and proteomic studies. Pioneering genome-wide technologies that have been first tested with yeast include genome-wide transcriptional array studies [1], mass-spectrometry analysis of protein–protein interactions [2,3], two-hybrid analysis of protein–protein interactions [4,5], protein arrays [6] and synthetic lethal screens [7]. While these studies provided novel information about yeast biology, they also led to the development of high-throughput methodologies for working with yeast. These highly parallel methods can be used for functional genomic analyses of heterologous proteins expressed in yeast, as well as high-throughput drug discovery using a yeast platform.

Properties of yeast platforms

Yeast cells are advantageous for high-throughput analyses because they are robust, fast growing, and easy to manipulate robotically. Growth assays can be carried out in parallel by robotically pinning yeast into 96-well liquid cultures or onto solid plates with selective media. Unlike mammalian cell culture, culturing yeast does not require elaborate sterile technique or complex media, and yeast can be stored temporarily (weeks to months) in readily usable refrigerator stocks.

An arsenal of strains [8], expression vectors [9] and elegant genetic techniques provides a framework on which yeast assays can be quickly developed. Proteins can be expressed in yeast cells via genes contained on plasmids or integrated

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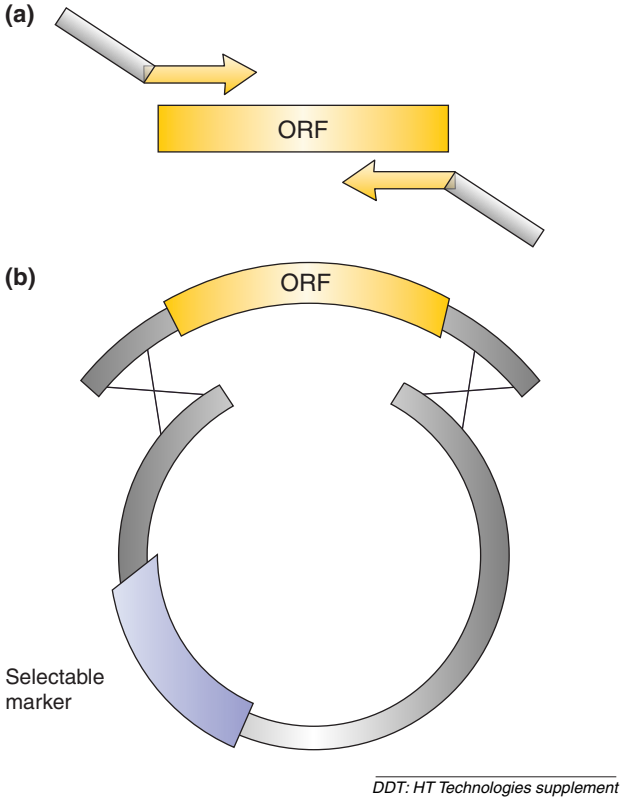

Figure 1. Gap-repair homologous recombination in yeast. **(a)** Sequences homologous to the ends of a cut (gapped) plasmid are added to an insert using PCR. **(b)** Insert and plasmid are co-transformed into yeast cells. Yeast repair the gapped plasmid by the process of homologous recombination, and cells containing the plasmid are selected by the presence of an auxotrophic marker on the plasmid.

into the genome. A range of RNA expression levels can be achieved, and expression can be made inducible, often by the simple addition of galactose to the media. Yeast modify their proteins in a similar way to higher eukaryotes and contain many of the same pathways. Foreign proteins can thus be coupled to homologous yeast pathways and characterized by simple growth or color phenotypes. Similarly, proteins from organisms such as parasites, which are difficult to study *in vivo*, can be expressed and analyzed in the more tractable yeast cell.

One of the most useful methodologies enabled by yeast in high-throughput analyses is the ability to assemble plasmids directly in the yeast using the process of gap-repair by homologous recombination [10]. In this highly efficient process, a cut plasmid is mixed with a DNA fragment, often generated by PCR, that has ends complementary to the gapped ends of the plasmid (Fig. 1). When transformed into yeast, the plasmid and fragment are joined by homologous recombination, bypassing the need for restriction digestion of the fragment or a separate ligation step. This process can also be used to replace normal yeast genes in the chromosome with mutated or engineered DNA sequences.

It should be noted that drugs have traditionally been difficult to deliver into yeast cells, and studies suggest this is primarily caused by activities of multidrug transporters that rapidly transport drugs out of the cell [11,12]. However, this problem can, in large part, be overcome by the use of yeast deletion strains that limit compound efflux, such as *erg6* or *pdr5* [13–15].

High-throughput protein interaction determination

Since the introduction of recombinant DNA technologies in the early 1970s, practically all modern biologists have used bacteria as a tool in their day-to-day experiments for cloning, protein expression and other processes. By contrast, until the past decade, few scientists other than yeast researchers had encountered yeast, and fewer still considered using yeast as a tool in their research. This attitude changed in the 1990s, mainly because of the development of yeast technologies such as the two-hybrid system [16] and its variations.

The yeast two-hybrid system (Fig. 2a) is described in detail in several review articles [17,18]. Improvements to the initial procedure enabled optimization for high-throughput analysis. These improvements included the use of multiple reporter genes, some of which enabled growth-dependent selection, to identify interactions [19–21]. Another improvement took advantage of the fact that yeast can exist in haploid or diploid states, and that haploid yeast of opposite mating types (MATa and MATα) can efficiently mate. The DNA-binding domain (BD) constructs (baits) could be expressed in cells of one mating type, and the activation domain (AD) constructs in cells of the opposite mating type [22,23]. Upon mixing, the yeast form diploid cells containing both constructs, which can be tested for interaction.

The first genomic two-hybrid screen was carried out on the bacteriophage T7 [24]. Although this was not a high-throughput screen (there were a total of 55 proteins involved, yielding 25 interactions), it was a preview of genome-wide screens to come. The first large-scale systematic two-hybrid screen used a random yeast genomic library to identify factors involved in yeast mRNA splicing [25]. A library of approximately 3,000,000 yeast genomic fragments fused to an AD vector was mated to individual bait proteins fused to the Gal4 BD.

Genome-wide high-throughput two-hybrid screens have used either array or library formats. Activation domain arrays were used to screen vaccinia virus [26] and *S. cerevisiae* [4]. The arrays consisted of a grid of yeast, each expressing a separate open reading frame (ORF) fused to an AD. As the array is specifically ordered, positives are identified by their location in

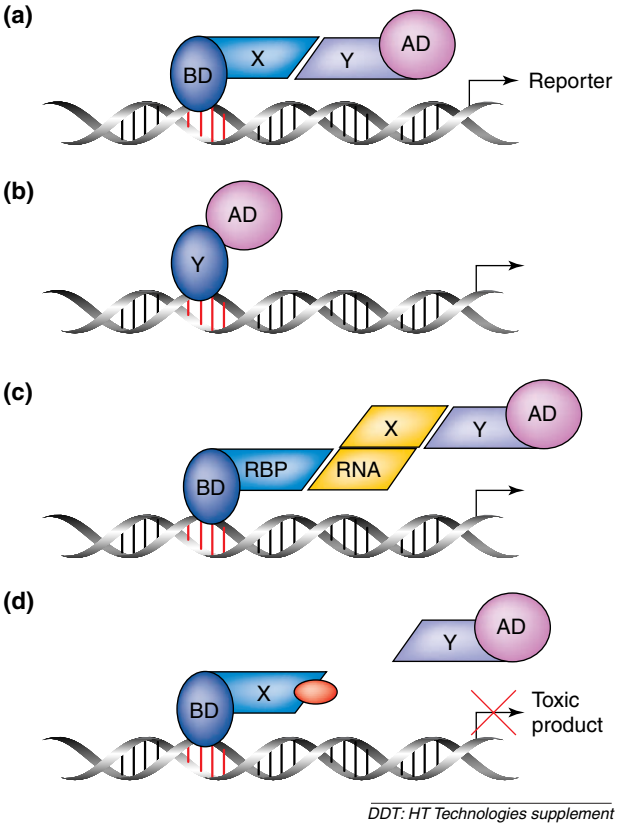

Figure 2. (a) The yeast two-hybrid system. A protein to be tested for interaction (X) is fused to a DNA-binding domain (BD), and a library of protein inserts (Y) fused to an activation domain (AD) is made. The DNA-BD binds to a sequence on the promoter region of a reporter gene (highlighted in red). If protein X interacts with Y, the transactivation domain is brought into the locality of the reporter gene promoter, enabling transcription. **(b)** The one-hybrid system. The library of AD fusions, Y–AD, is assayed for binding to a specific DNA sequence located in a reporter gene promoter. **(c)** The RNA-based three-hybrid system. A BD is fused to an RNA-binding protein (RBP), and this hybrid binds a known RNA fused to an RNA of interest (X). If X interacts with Y–AD, transcription occurs. **(d)** The reverse two-hybrid system. If a protein or small molecule interferes with the X–Y interaction, transcriptional activation of a toxic gene is reduced, enabling the cell to grow better.

the array. This format enables rapid assessment of the reproducibility of a screen by looking at the pattern of positives. To generate arrays, ORFs were individually PCR-amplified and cloned into AD vectors using *in vivo* gap-repair, then transformed into a two-hybrid reporter strain. To test for protein interaction, a single bait was mated against the AD-ORF array by robotically pinning the yeast on top of each other, and then onto selective media. A second comprehensive approach for analyzing protein–protein interactions in yeast used a similar method to generate ORF hybrids, but pooled AD-ORF libraries during screening [4]. A total of 6000 BD-ORF fusions were individually screened in 96-well plates against the pooled AD library. A pooled-library versus pooled-library approach was taken in another comprehensive screen of protein–protein interactions in yeast [5]. Each yeast ORF was amplified by PCR and ligated into BD and AD vectors. BD or AD vectors were individually transformed into MATa or MATα haploid two-hybrid strains, respectively, then pooled into groups for mating.

Two-hybrid variations

Variations of two-hybrid methods have been developed that can be used in high-throughput assays. In the one-hybrid screen (Fig. 2b), a binding site for a sought-after DNA-BD is placed in the promoter of one-or-more reporter constructs, such that expression of these genes is regulated by this binding site. Yeast are transformed with a library of inserts fused to an AD with an aim of identifying a DNA-binding protein that associates with the binding site.

The three-hybrid assay to detect protein–RNA interactions is used to discover an RNA-binding protein (RBP) that associates with an RNA [27]. The assay is similar in nature to the two-hybrid technique, but contains an extra component: a 'bridging' RNA (Fig. 2c). The three hybrids that comprise the assay are: (1) a DNA-BD fused to the RBP; (2) an RNA that binds the RBP fused to an RNA of interest; and (3) a library of inserts fused to the AD. The small-molecule three-hybrid assay [28] is similar in design, containing a DNA-BD fused to a known ligand-binding protein, the known ligand fused to a small molecule of interest, and a library of inserts fused to the AD. This assay can be used to identify a protein target for a particular small molecule. A limitation of this assay, however, is that the small molecule of interest must be synthesized so that it is covalently attached to the known ligand.

Two-hybrid assays can also be used to identify factors that disrupt a particular interaction. In the reverse two-hybrid screen (Fig. 2d), the interaction of two proteins is linked to the production of a toxic product [29]. Disruption of the interaction by a chemical or protein reduces or eliminates production of the toxic product, enabling cells to grow. The Ura3 protein is often used as the reporter in such assays, as yeast that express Ura3 are sensitive to 5-fluoroorotic acid (5-FOA). A miniaturized version of this assay has been developed that could increase throughput by using nanodroplets of yeast and media, and photoreleasable compounds attached to beads [30].

Other methods for detecting protein interactions

Non-transcription factor-based methods for detecting protein interactions, including a ubiquitin-based two-hybrid assay [31,32] and the Sos recruitment system [33], have also been developed in yeast. These have proved to be useful for identifying protein interactions between molecules, such as membrane

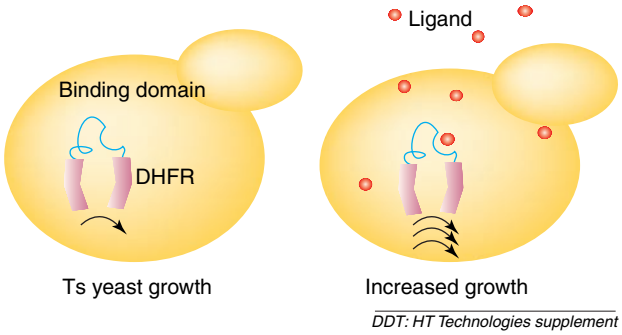

Figure 3. Yeast-based ligand sensor. Yeast show temperature-sensitive (Ts) growth owing to a binding-domain insertion in dihydrofolate reductase (DHFR) that causes instability. In the presence of ligand, this temperature-sensitivity is reduced owing to ligand-induced conformational or stability changes in the hybrid protein.

proteins, which cannot be assayed by traditional two-hybrid methods because of nuclear localization requirements.

Several methods have been developed to identify small-molecule compounds that bind to heterologous proteins expressed in yeast. One of the most widely used is the yeast estrogen screen. Although yeast do not contain endogenous steroid hormone receptors, heterologously expressed steroid receptors are responsive to their cognate ligands and can induce reporter gene activity [34]. In the yeast estrogen screen, a mammalian estrogen receptor is expressed in yeast along with a reporter gene containing an estrogen response element [35]. Chemicals, xenobiotic compounds, food, and water samples can be screened for estrogenicity in this sensitive, simple and inexpensive yeast assay.

A generic yeast assay for detection of protein–small-molecule-ligand interactions was recently developed [36]. A ligand-binding protein or domain of interest is inserted into an essential yeast metabolic protein, dihydrofolate reductase (DHFR), and the chimeric protein is expressed in yeast in place of the endogenous DHFR (Fig. 3). However, the chimeric DHFR is unstable because of the protein insertion, resulting in temperature-sensitivity of the yeast. Binding of a ligand to the inserted domain causes a change in the protein that reduces the temperature-sensitivity. This technique is adaptable to HTS in 96-well plates to identify compounds that interact with a particular protein, and might be useful in identifying compounds that bind proteins with unknown ligands.

Drug discovery in yeast: tying into yeast biology

With the exception of the two-hybrid system and variations, most assays that use yeast to characterize heterologous proteins take advantage of similarities in pathways between yeast and higher eukaryotes. Yeast have many of the same pathways found in humans and other higher eukaryotes, enabling assays to be developed that couple foreign proteins to yeast proteins in ways that yield growth phenotypes. For example, heterologous proteins expressed in yeast can cause phenotypic effects caused by overexpression, or can complement a yeast strain that is missing a particular gene, enabling identification of proteins that have similar activities (Fig. 4).

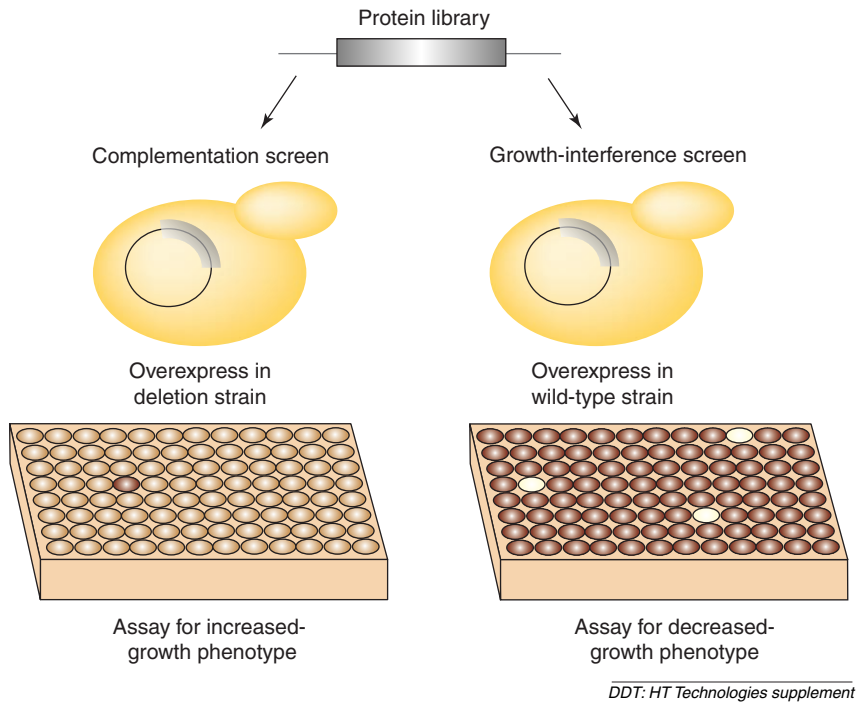

Figure 4. Complementation and growth interference screens. In the complementation screen, proteins are assayed for the ability to complement a strain that has a deletion or mutation in a particular protein. In a growth interference screen, proteins are assayed for their ability to interfere with normal yeast growth.

A recent study [37] describes a high-throughput method of identifying heterologous proteins that cause growth-inhibitory phenotypes in yeast. Human cDNAs were cloned in yeast under the control of a galactose-inducible promoter. The transformants were characterized in high-throughput liquid growth assays in 96-well plates for a difference in growth between the induced and non-induced states. Twelve out of 38 cDNAs of diverse functional classes (including some encoding

protein kinases, small G-proteins, and nuclear receptors) resulted in significant growth interference. Mutagenesis of the active site or addition of active-site inhibitors reversed growth inhibition in most cases.

Complementation of a deficient yeast strain provides another method of characterizing heterologous genes. Knock-out of a single yeast gene is a simple process and, indeed, the entire set of *S. cerevisiae* deletion strains is now available [8]. A protein of interest from another organism can be expressed in an appropriate yeast deletion strain, and the strain can be assayed for complementation or modulation of activity. For example, the G-protein-coupled yeast pheromone response pathway has been used to study mammalian G-protein-coupled receptors, a class of proteins of general interest in drug discovery (see [38] for a review). In one screen, mammalian genes were assayed for their involvement in modulating G-protein-coupled receptor signalling [39]. A yeast strain was constructed that contained a substituted mammalian-yeast G_{α}-protein linked to the pheromone response pathway. Activation of this pathway was linked to a reporter that enabled growth of the yeast in selective media. Mammalian cDNAs under galactose-inducible control that activated the downstream pathway were detected by their growth in the presence of galactose.

Proteins from organisms that are difficult to cultivate and culture can be expressed in yeast and screened for complementation of activity. Dihydrofolate reductase from *Plasmodium falciparum*, the major parasite that causes malaria, can complement yeast strains lacking endogenous DHFR [40]. Dihydrofolate reductase from *Plasmodium* isolates were expressed in yeast, and simple growth assays were used to investigate resistance to various antimalarial DHFR inhibitors [41].

The identification of a protein that affects a pathway and causes a phenotype in yeast almost always enables the development of a yeast-based assay for drug screening. For example, the *Plasmodium* DHFR complementation assay described previously was used to screen yeast expressing *P. falciparum*, *Cryptosporidium parvum*, *Toxoplasma gondii*, *Pneumocystis carinii*, or human DHFR, to identify compounds that inhibit a pathogenic DHFR to a greater extent than the human DHFR [42]. Yeast cell-based assays have previously been used to study the action of topoisomerase II enzymes [43], and complementation of a mutation in yeast topoisomerase II by a human topoisomerase II was the basis for a high-throughput screen to characterize inhibitors of human topoisomerase II α and β [44].

Concluding remarks

Although yeast platforms are obviously not ideal for all studies, in many cases they provide an alternative, complementary, robust, and inexpensive approach to gene function and drug discovery. Indeed, in the area of protein interactions, yeast platforms have proved themselves to be tremendously capable, enabling the discovery of thousands of potential protein interactions from diverse organisms. Such high-throughput yeast cell-based assays have great potential for elucidating gene function and misfunction, detecting novel disease targets and enhancing early stages of drug discovery.

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