Preface

Many, if not all, essential biological processes require selective interactions between proteins. Complex signaling systems require sequential, ordered protein–protein interactions at essentially all levels of the signaling cascade. For example, peptide hormones interact with selective membrane receptor proteins, and autophosphorylation of the receptor then recruits other key regulatory proteins that initiate kinase cascades in which each phosphorylation event requires selective recognition of the protein substrate. The ultimate signaling effect, in many cases, is the regulation of RNA polymerase II-directed transcription in the nucleus, a process that involves numerous, multiprotein complexes important for transcription initiation, elongation, termination, and reinitiation. Defining, characterizing, and understanding the relevance of these protein–protein interactions is an arduous task, but substantial inroads have been made over the past 20 years. The development of more recent methodologies, such as mammalian expression systems, immunopurification schemes, expression cloning strategies, surface plasmon resonance (BiaCore), and nanosequencing technologies, has contributed a wealth of new insights into these complex multiprotein mechanisms and clearly accelerated the discovery process. Arguably, the yeast two-hybrid system has been one of the predominant and most powerful tools in this discovery process.

On a personal note, my specific interest in the yeast two-hybrid system developed in a manner probably not terribly different from that of many other investigators who were interested in the early 1990s in identifying and characterizing interactions between two proteins. While working in the laboratory of Mark R. Haussler, our interests centered on the vitamin D receptor (VDR), a member of the nuclear receptor family, and the mechanisms involved in VDR binding to DNA. Specifically, I was interested in identifying a nuclear factor that interacted with and conferred high-order binding of the VDR to DNA. We and other larger groups in the nuclear receptor field chose a traditional biochemical approach that focused on purifying and identifying the unknown nuclear accessory factor. Other laboratories used expression cloning strategies with purified radiolabeled proteins to screen cDNA expression libraries for clones encoding the interacting factor. Both approaches were comparatively large efforts at the time, requiring a tremendous number

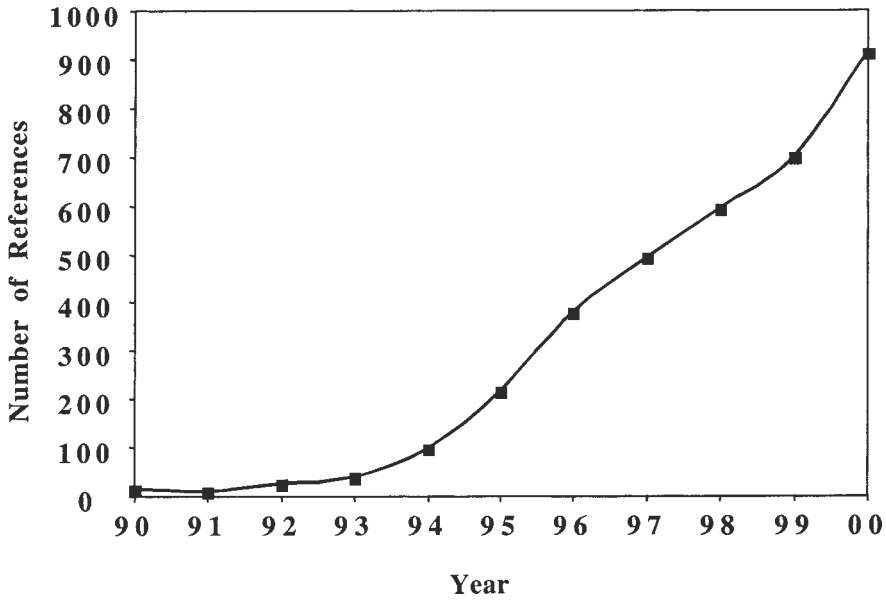

Fig. 1. The number of publications over the past 10 years that were found in a search of PubMed using "two-hybrid" in the search window. The year 2000 value is projected based on the number of references found at the time of the search (September, 2000) and the number of remaining months in the year.

of person-hours. Both approaches eventually resulted in the successful identification of the factor as retinoid X receptor, a common heterodimeric partner for many of the class II nuclear receptors. Unfortunately, we were not one of the groups to first report the identification of RXR as the partner. Our smaller effort was, in no uncertain terms, "scooped."

At about this same time, reports from the Fields laboratory on the successful use of the yeast two-hybrid system began to emerge and more beneficial yeast strains and vectors were being developed. The power of the system was inspiring to anyone working on trying to identify protein interaction partners. Here was a simple, direct screening assay that could uncover novel factors that interacted with your protein of interest. Millions of cDNAs could be screened in a single experiment, in a relatively short time, and with comparatively less effort. Following the initial screen, the cDNA clones encoding the putative interactors were already in hand and they could be directly sequenced and identified. The playing field seemed somehow leveled a bit by the two-hybrid system. More than twelve years have passed since the original description of the yeast two-hybrid system was reported, and few would disagree that this system has had a

tremendous impact on virtually every field of modern biology. Continuous refinements and novel innovations of the original systems over the past decade have only strengthened the utility of the approach. As illustrated in **Fig. 1**, it is obvious that many groups continue to adopt the two-hybrid system as a new approach in their laboratories and this trend will only continue to expand in the future as the era of functional genomics unravels over the next century.

Therefore, the overall goal for *Two-Hybrid Systems: Methods and Protocols* is to introduce the yeast two-hybrid system to students, research assistants, research associates, and other more senior investigators considering this as a new approach in their laboratories and research projects. Toward this end, I have assembled a collection of detailed descriptions of basic protocols and a compendium of experimental approaches in different biological systems that I hope reflects the utility of the system and its variations in modern biomedical research. My hope is that this will also serve as a useful reference for those laboratories that have extensive experience with the two-hybrid system. Thus, I invited several authors to discuss in more general terms some of the problems and strategies involved in the yeast two-hybrid assay as well as some of the alternative systems that have evolved from the original system that may prove useful to those more experienced two-hybrid laboratories.

Two-Hybrid Systems: Methods and Protocols is divided into four main sections. The first section is a compendium of general methodologies that are used in the two-hybrid system. Here, the reader will find in-depth discussion and detailed methodologies that serve as the foundation on which successful yeast two-hybrid experiments rest. Since many laboratories beginning two-hybrid approaches have not worked with yeast to a significant extent, this first section begins with a general introduction to handling yeast, a detailed compendium of media formulations, as well as an overview of the common strains of yeast and plasmid vectors that are used for two-hybrid work. This section ends with three chapters that describe the basic methodologies involved in introducing plasmids into yeast, interaction assays, and recovering the plasmids from yeast. This first section was intentionally designed to be somewhat repetitive in nature with components of the subsequent application chapters. The intent was to provide more in-depth methodological detail and variations of these fundamental techniques that serve as the backbone of any two-hybrid assay as well as to illustrate how these techniques are incorporated into individual applications. One well-known, recurring drawback of the two-hybrid system is the potential for artifacts and false positives. Thus, Section II provides a discussion of the various classes of false positives and the common mechanisms through which false-positives arise. This section also includes two chapters that focus on general strategies and detailed

protocols to confirm the authenticity of the interaction using in vitro protein–protein interaction assays. Part III includes four application chapters that describe how the yeast two-hybrid system was applied in various systems to identify interacting partners in important biological systems including the Smad and nuclear receptor pathways. Finally, Part IV describes various alternative strategies that have arisen out of the original yeast two-hybrid paradigm. These alternative strategies include the one-hybrid, split two-hybrid, three-hybrid, membrane recruitment systems, and mammalian systems. These alternative systems serve to illustrate the flexibility and refinements that are possible with the basic two-hybrid approach.

The authors and I hope that *Two-Hybrid Systems: Methods and Protocols* will prove a valuable addition to any laboratory that is interested in studying macromolecular interactions between proteins.

I would like to express my sincere gratitude to all the authors for their valuable, insightful contributions and for their patience in seeing this project to fruition. This book is a testament to their breadth of knowledge on the topic and the power of the two-hybrid approach. It is evident that both the basic system, as well as its many variants, will continue to play a predominant role in the characterization and identification of protein–protein interactions in the genomic and proteomic arenas of the 21st century.

Paul N. MacDonald

2

Growth and Maintenance of Yeast

Lawrence W. Bergman

1. Introduction

On many occasions, baker's yeast (*Saccharomyces cerevisiae*) has been referred to as the *Escherichia coli* of the eukaryotic world. Yeast has been extensively characterized genetically and a complete physical map is now available. Much of the comparison to *E. coli* is based on the observations that culturing yeast is simple, economical, and rapid, with a doubling time in rich medium of approx 90 min. Cells divide mitotically by forming a bud, which is subsequently pinched off to form a daughter cell. Yeast can also be grown on a completely defined medium, which has allowed the isolation of numerous nutritional auxotrophs. This type of analysis has provided manly mutations useful for genetic analysis and as selectable markers for plasmid manipulation.

Physiologically, yeast can exist stably in either haploid or diploid states, with the haploid cell being either of two mating types called **a** and α. Diploid **a**/α cells, formed by the fusion of an **a**-cell and an α-cell, are stable mitotically. However, under conditions of carbon and nitrogen starvation, the diploid cell will undergo meiosis to produce four haploid spores. It is possible to recover all four haploid products of the meiosis individually, which may facilitate many types of studies.

The genome sequence of yeast is now known and the genome contains 16 linear chromosomes, ranging from approx 200 to 2200 kb. The functional units of the chromosomes have been identified, cloned, and characterized: origins of replication (ARS elements), centromeres (CEN elements), and telomeres. The combination of these elements with the auxotrophic selectable markers has led to the construction and utilization of numerous plasmids that vary in a number of properties (integrating vs extra chromosomal, high copy vs single copy, circular vs linear). Several of these plasmids are now commercially available, and

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procedures for the high-efficiency transformation of yeast with plasmid vectors and gene libraries have been available for more than 20 yr. Thus, the utility of the yeast as a vehicle for the two-hybrid system is evident.

The purpose of this chapter is to discuss the general laboratory principles used to grow and maintain yeast for use in the two-hybrid protein interaction assay. The goal is to provide a working knowledge of the general principles involved in working with yeast cells. Many of these general principles are highlighted throughout the more detailed protocols and formulations discussed in subsequent chapters.

2. Growth of Yeast Strains

2.1. Growth in Liquid or Solid Medium

Yeast can be grown in either liquid medium or on the surface of a solid agar plate. Yeast cells will grow on a minimal medium containing dextrose (glucose) as a carbon source and salts that supply nitrogen, phosphorus, and trace metals. Yeast cells grow much more rapidly in the presence of rich medium that contains reagents such as yeast extract and bactopeptone. These provide many of the metabolites that the cells would synthesize when growing under minimal growth conditions. During log-phase growth in rich medium, yeast cells divide once approximately every 90 min. Early log phase is the period when cell densities are $<10^7$ cells/mL. Mid–log phase is the period when densities are between 1 and 5×10^7 cells/mL. Late log phase occurs when cell densities are between 5×10^7 and 2×10^8 cells/mL. The measurements of cell density are discussed later.

Detailed recipes for media that are commonly used for yeast are provided in Chapter 3. The rich medium yeast extract, peptone, dextrose (YPD) is most commonly used for growing yeast under nonselective conditions (e.g., when maintaining plasmid slection is not required). Note that some transformation procedures suggest a 4- 6-h period of growth in rich medium (despite the presence of plasmids in the cells) prior to the transformation process itself. In some cells, particularly those strains containing an *ade2* mutation, adenine may be added to YPD. Cultures of an *ade2* or *ade1* mutant will turn pink in 2 to 3 d. The formulas for synthetic complete media vary in the amount of adenine, such that some have amounts of adenine so high that colonies never turn pink or red. Autoclaving is usually carried out for 15 min at 15 lb/in.^{2} but should be increased when larger volumes are prepared. It may be preferable to use a 20% solution of dextrose that has been autoclaved separately or filter sterilized, because this prevents caramelization or darkening of the medium and promotes optimal growth.

Minimal medium, also known as synthetic defined (SD) medium, supports the growth of yeast, which has no nutritional requirements. It contains yeast nitrogen base, ammonium sulfate, and dextrose. Minimal medium is commonly used when testing the mating type of yeast cells using specific mating tester strains (of both mating types). Minimal medium is most often used as a basal medium to which mixtures of amino acids and nucleoside precursors are added. SD dropout medium lacks a single (or several) nutrient that allows selection for maintenance of particular plasmids or selection for induction or repression of specific gene promoters. These two particular properties are the basis of the two-hybrid system.

In instances in which it is necessary to sporulate a diploid yeast cell, a nitrogen-deficient starvation medium containing acetate as a carbon source to promote respiration is used. A general formula for this medium is 1.0% potassium acetate, 0.1% yeast extract, and 0.05% dextrose, and sporulation of diploid cells can be carried out in liquid medium or on plates.

Wild-type yeast can use a variety of carbon sources other than glucose to support growth. In particular, raffinose and galactose are used under conditions to relieve glucose repression (in the case of raffinose) or to induce expression from a Gal4p-dependent promoter such as *GAL1* and *GAL10*. All are used at a concentration of 2.0% (wt/vol) (20 g/L) and are used to replace dextrose in either rich or defined medium.

As mentioned previously, yeast can grow on solid medium. For all plates, agar is added to a final concentration of 2.0% (20 g/L). To prevent agar breakdown during autoclaving, it is possible (although not necessary) to add a pellet of sodium hydroxide (per liter) to the medium-agar suspension. Care should be taken to avoid autoclaving the medium-agar suspensions longer than necessary, because this will cause agar breakdown leading to "soft" plates.

Specialty plates containing either 5-fluoro-orotic acid (5-FOA) or cycloheximide are used in negative selection experiments against the wild-type *URA3* or *CYH2* genes, respectively. Note that 5-FOA is quite expensive, it should be filter sterilized, and plates that contain 5-FOA should also contain uracil. Cycloheximide may be prepared as a filter-sterilized 10 mg/mL solution with the final working concentration in plates being 10 μg/mL.

Wild-type yeast grows well at 30°C with good aeration and glucose as a carbon source. Erlenmeyer flasks work well for growing liquid cultures, and baffled-bottom flasks are good but not necessary. Although small cultures may be grown in culture tubes, in many cases the cells will settle out from suspension. For optimal aeration and growth, the medium should constitute no more than 20% of the total volume of the flask, and growth should be carried out in a shaking incubator at 250–300 rpm. On solid YPD medium at 30°C, single colonies

may be seen after 24 h, but generally growth for at least 48 h is required prior to picking of colonies or replica plating. Growth on dropout medium is approx 50% slower than that observed in YPD.

2.2. Determination of Cell Density

The approximate number of cells in a culture can be determined with a spectrophotometer by measuring the optical density (OD) at 600 nm. Cultures should be diluted such that the observed reading (OD_{600}) is <1.0. In this range, an OD_{600} = 1.0 is approximately equal to 3×10^7 cells/mL. However, there is strain variability in this measurement, or it may be affected by overexpression of a particular gene product within a strain (such as a two-hybrid bait). It is best to determine this function by graphing the OD_{600} as a function of actual cell number that has been determined by counting in a hemocytometer or plating for viable colonies. Many transformation procedures utilize growth of the yeast culture to a certain cell density prior to harvesting.

3. Strain Preservation and Revival

Yeast strains can be stored at –70°C in 15% glycerol and are viable for more than 3 yr. Alternatively, they can be stored at 4°C on slants of rich medium for 6 mo to 1 yr. To prepare glycerol stocks, make a sterile solution of 30% glycerol (w/v). Pipet 1.0 mL of the solution into sterile 4-mL screw-cap vials. Add 1.0 mL of a late log or early stationary phase culture, mix, freeze on dry ice, and store at –70°C. Revive the strain by scraping some cells off the frozen surface and streak onto plates. It is not necessary to thaw the entire vial. Cells can also be stored in a similar manner using 8% (v/v) dimethylsulfoxide (DMSO); however, the quality of the DMSO is critical. Yeast strains can be conveniently mailed as slants. Also, cells may be mailed after transfer to a piece of sterile Whatman 3MM paper. Dip the paper into a yeast culture or press onto a yeast colony using sterile forceps. Then wrap the filter paper in sterile aluminum foil and mail. The strain is revived by placing the paper onto the surface of an agar plate and incubating the plate at 30°C for several days.

4. Replica Plating

Cells from yeast colonies grown on one medium can be tested for their ability to grow on another medium by replica plating. There are now several commercial sources for the purchase of both a replica-plating block and velveteen squares. A master plate containing the cells of interest is first printed onto sterile velvet. A copy of these cells on the velvet is then transferred to plates made with all the relevant selective media. In general, three or four copies may be made from a single master plate and up to five or six copies made from a single

square of velvet. This type of plating has application with the two-hybrid system for testing nutritional requirements (either different dropout media or different carbon sources), placing cells onto filter paper for β-galactosidase assays, or in mating studies.

5. Plasmid Segregation from Yeast

It is sometimes useful to generate a yeast strain that has only a single type of plasmid (as compared with multiple plasmids). As discussed, it is possible with certain plasmids to select against the presence of a plasmid (5-FOA or cycloheximide); however, this does not work for all plasmids. Alternatively, the yeast strain containing multiple plasmids is grown for several days in medium that maintains selection for the plasmid of interest but not on the plasmid you wish to lose. Under nonselective conditions, plasmids are estimated to be lost at a rate of 10–30% per generation. A diluted sample is then spread onto agar plates that will select only for the desired plasmid and after subsequent growth; individual colonies are picked and screened to verify loss of the unwanted plasmid and maintenance of the desired plasmid.

6. Mating Analysis and Two-Hybrid System

6.1. Diploid Construction

Diploid strains are constructed by mating strains of opposite mating types on the surface of agar plates. Mix cells from freshly grown colonies of each haploid parent in a small circle of approx 0.5 cm in diameter on an agar plate. The plates should allow growth of both haploid strains. Allow mating to occur for more than 4 h at 30°C. This time frame will allow mating of two strains containing plasmids without significant loss of the plasmid. Then, streak or replica the mating mixture onto a plate that will select for the genotype of the diploid. This type of procedure is particularly adaptable to testing an activation domain (AD) plasmid vs multiple baits.

6.2. Sporulation on Plates and in Liquid Medium

Starvation of diploid cells for nitrogen and carbon sources induces meiosis and the formation of spores. Sporulation can be induced in cells growing on solid or in liquid medium; however, there may be strain specifically as to whether the solid or liquid medium induces better formation of spores.

On plates, cells that have been grown on YPD or selective plates are patched onto a sporulation plate and incubated for 3–5 d at 25°C, because sporulation is usually less efficient at higher temperatures. Generally, formation of spores is monitored microscopically (×250–400) by looking for the presence of tetrads

(clusters of four small spheres within a small sac or ascus). The proportion of cells that undergoes sporulation as well as the fraction of four-spore asci varies from one strain to another.

As stated, some strains sporulate better in liquid culture. Often sporulation in liquid medium is completed within 48 hours and is not inhibited by higher incubation temperatures. Briefly, cells are grown in YPD or selective medium to late log phase or early stationary phase. One milliliter of culture is collected by centrifugation, washed with sterile water, and resuspended in 1.0 mL of sporulation medium. These cells are incubated for 2 to 3 d at 30°C with shaking (300–350 rpm) and examined microscopically for the formation of tetrads.

6.3. Random Spore Analysis

Although it is possible to isolate individual haploid spores, this generally requires a light microscope with a movable stage, a micromanipulator, and a skilled yeast geneticist. As an alternative, haploid spores can be released from the ascus and plated directly onto agar plates. Cells are collected from either the sporulation plate or sporulation liquid culture and washed with sterile water. The preparation is treated with glusalase (an extract from snails) in sterile water for 20 min and then is plated directly on YPD plates or selective plates for the appropriate haploid. It is always necessary to test individual colonies obtained in this procedure to ensure that the cells are haploid. This can be accomplished by mating the selected cells with a pair of mating-type tester strains for the ability of the selected cells to form diploids (a property of haploid cells).

6.4. Use of Pretransformed AD Libraries

It is now possible to obtain yeast cells that have been previously transformed with an AD library of interest. This circumvents the low-efficiency transformation of yeast (low as compared to *E. coli*) and substitutes a mating procedure to introduce the bait plasmid. In this procedure, an excess of yeast cells of one mating type containing the bait plasmid is allowed to mate with cells of the opposite mating type containing the AD plasmid. This occurs in solution for 20–24 h at 30°C in YPD supplemented with adenine (if necessary) with gentle aeration (30–50 rpm). This prevents the yeast from settling to the bottom of the flask, whereas rapid shaking reduces mating efficiency. The cells are then collected and plated to conditions that allow growth of only the diploid cells. Titration of the number of cells screened and the mating efficiency are necessary in this approach. Another valuable application of this protocol is screening of the same library with multiple baits, thus avoiding numerous large-scale transformations.

3

Media Formulations for Various Two-Hybrid Systems

Michael Saghbini, Denise Hoekstra, and Jim Gautsch

1. Introduction

The two-hybrid system, which was originally developed by Fields ***(1)***, represents the only successfully tested tool to study protein-protein interaction in a living cell. *Succhoromyces cerevisiae*, the species of choice for this system, is a model eukaryotic organism with a stable, well-defined, and easy-to-manipulate genetic system. It is available in many different strains with stable nutritional marker mutations permitting rescue with vectors carrying the appropriate wild-type markers.

S. cerevisiae is essentially processed like bacteria. Culturing is simple, economical, rapid, and nonhazardous. This yeast grows nonselectively on rich medium (yeast extract, peptone, dextrose [YPD]) and selectively (i.e., with selected vectors) on synthetic defined (SD) minimal medium containing the appropriate dropout nutritional supplement. Supplements enhance the growth of yeast cells by providing amino acids and nucleotide precursors, thereby bypassing the need for *de novo* synthesis ***(2,3)***.

Media for *S. cerevisiae* are readily available from commercial sources as individual components, powder mixes, or prepoured plates. The composition of rich medium is well defined, yet certain deletion mutant strains grow differently on YPD obtained from different sources. The components of minimal medium are also well defined with the exception of the nutrient supplements. With the advent of improved versions of the original two-hybrid system, different nutrient supplements have emerged, sometimes with minor variations ***(4–7)***. Making the various supplement mixes required for a successful screen, as most two-hybrid system suppliers recommend, is impractical and time-

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Table 1
YPD Formulation

Final concentration	Amount per liter (g)
1% Yeast extract	10
2% Peptone	20
2% Dextrose (D-glucose)	20
2% agar	20 (only solid medium)

consuming. As a matter of convenience, we have organized dropout nutrient supplements by closest match to readily available dropout mixtures (BIO101).

In an attempt to facilitate assignment of minimal medium required for a particular task in a two-hybrid screen, the genotypes of reporter strains are tabulated in an easy-to-read format. Media requirements are presented for the three major two-hybrid systems: system I ***(4)***, system II ***(5)***, and system III ***(6)***. Media requirements are also presented for the following specialized systems: the split and reverse hybrid systems ***(8,9)***, the trihybrid system with an inducible third party expression ***(10)***, the trihybrid system for detecting RNA-protein interactions ***(11)***, the trihybrid system for detecting protein-protein interactions dependent on posttranslational modifications ***(12)***, the SOS-based recruiting system ***(13)***, the one-hybrid system ***(14–16)***, and the mammalian two-hybrid system ***(17,18)***. In all cases, reference to commercially available two-hybrid systems is made when applicable.

2. Yeast Rich Medium (YPD)

2.1. Composition

YPD, also called YEPD, is the most commonly used rich medium for growing *S. cerevisiae* when special conditions are not required. It provides an excess of amino acids, nucleotide precursors, vitamins, and essential metabolites needed for optimal cell growth (**Table 1**). Yeast cells divide every ~90 min when grown in YPD during the exponential phase of the growth cycle ***(2,3)***. YPD can be prepared as a broth or a solid medium with the addition of agar to a final concentration of 2%. Sterile YPD broth can be stored at room temperature for several months. YPD plates can be stored in sealed plastic bags for more than 3 mo at room temperature or 4°C.

2.2. Source of YPD Makes a Difference in Growth Rate of Certain Mutant Yeast Strains

YPD has a defined composition yet there are many different sources for the individual components that define this medium. **Figure 1** shows serial dilution

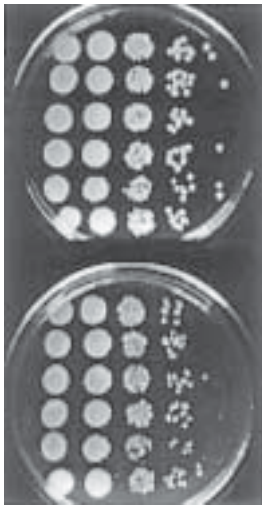

Fig. 1. Growth on YPD obtained from different sources. Several deletion mutant yeast strains are spotted at four increasing dilutions on YPD plates made from ingredients obtained form different sources (top plate vs bottom plate). Photograph is taken after 2 d of growth at 30°C.

of several mutant yeast strains grown on YPD obtained from different sources (top plate vs bottom plate). The slower growth rate observed at the highest dilution (bottom plate far right vs top plate) is an indication that not all YPD rich media are equivalent.

2.3. The Addition of Adenine to YPD Enhances Growth of Certain Yeast Two-Hybrid Strains

Yeast reporter strains with a mutation in the *ADE2* gene (*ade2*) accumulate a red pigment and appear as red colonies ***(19)***. The accumulation of this pigment slows growth ***(20)***. The addition of adenine slows the accumulation of red pigment, leading to enhanced growth rate. **Table 2** lists the various yeast two-hybrid strains that are grown on YPD containing adenine, YPAD. Different amounts of adenine are sometimes used than the 0.003% recommended by Sherman ***(2)***.

2.4. Uses of YPD/YPAD in Two-Hybrid Screens

The rich medium YPD/YPAD is used for propagation of reporter strains and for growth whenever selection is not required. It can be used with positive selection agents such as Zeocin™. This antibiotic is used to select recombinant yeast, bacteria, and insect and mammalian cells carrying the ble gene product

Table 2
Two-Hybrid Strains Grown on YPAD

Strain	Reference	Recommended amount of adenine added to YPD
L40	***5***	0.01% Adenine
cdc25-2	***13***	0.004% Adenine sulfate
YRG-2	***21***	0.004% Adenine sulfate
S-260	***22***	0.003% Adenine sulfate
PJ69-2A	***7***	0.003% Adenine sulfate

(***[23]***; Invitrogen). Rich medium is also used for recovery purposes prior to plating cells from a large-scale transformation or a mating assay on selective medium ***(4,5)***. Mating assays facilitate the processing of putative positive yeast clones obtained from two-hybrid screens.

3. Yeast SD Medium

3.1 Definition

SD medium for yeast is also known as complete minimal and synthetic complete. It consists of a defined mixture of salts, vitamins, and a nitrogen source collectively known as yeast nitrogen base (YNB; **ref. *24***; **Table 3**) to which a carbon source, usually dextrose, is added along with nutrient supplements consisting of various amino acids and nucleotide precursors (**Table 4**). SD medium supports the vigorous growth of virtually all strains of *S. cerevisiae* with a doubling time of ~140 min during the exponential phase of growth ***(2,3)***. SD lacking nutrients is known as omission or dropout medium. It is used to select for yeast containing vectors with specific nutritional markers. For example, dropout medium lacking tryptophan and leucine (SD–trp–leu) selects for the presence of plasmids carrying the TRP and LEU markers. Dropout medium containing agar is referred to as SDA.

3.2. Different Carbon Sources Used in SD Medium

Yeast cells can grow on a variety of carbon sources. The most commonly used carbon/energy source is D-glucose (dextrose). Other fermentable carbon sources also can be used such as galactose, maltose, fructose, and raffinose. They are added to a final concentration of 2% to either rich or minimal medium ***(2,3)***. Glucose-free galactose (<0.01% glucose) is particularly useful to induce transcription of genes fused to Gal1/Gal10 promoters. The incorporation of this inducible expression cassette into the library vector of yeast two-hybrid

Table 3
YNB Formulation

Nitrogen Source	
Ammonium sulfate	5000 mg/L
Salts	
Potassium phosphate, monobasic	1000 mg/L
Magnesium sulfate	500 mg/L
Sodium chloride	100 mg/L
Calcium chloride	100 mg/L
Vitamins	
Biotin	0.002 mg/L
Pantothenate, calcium	0.4 mg/L
Folic acid	0.002 mg/L
Inositol	2 mg/L
Niacin	0.4 mg/L
PABA	0.2 mg/L
Pyridoxine, Hcl	0.4 mg/L
Riboflavin	0.2 mg/L
Thiamine, Hcl	0.4 mg/L
Trace Elements	
Boric acid	0.5 mg/L
Copper sulfate	0.04 mg/L
Potassium iodide	0.1 mg/L
Ferric chloride	0.2 mg/L
Magnesium sulfate	0.4 mg/L
Sodium molybdate	0.2 mg/L
Zinc sulfate	0.4 mg/L
Total	6.7 g/L

systems allows the detection of interacting proteins that are potentially toxic to yeast cells ***(6)***. Usually 1% raffinose is added to minimal medium containing 2% galactose to give the cells a better growth advantage without affecting the induction process. In addition, nonfermentable carbon sources can be used such as glycerol, ethanol, and acetate: 3% glycerol, 3% ethanol, 2% glycerol + 2% ethanol, and 2% potassium acetate ***(2,3)***.

3.3. Nutritional Supplements

The addition of a mixture of amino acids containing uracil and adenine to YNB containing a carbon source completes the SD growth medium. Dropout nutrient supplements can be made by mixing the various components, as per

Table 4
Nutritional Supplements for Various Yeast Two-Hybrid Systems

Nutritional supplements[c]	CSM		HSM		BSM	
	(mg/L)	Ref.	(mg/L)	Ref.	(mg/L)	Ref.
Two-hybrid, trihybrid, and split-hybrid yeast reporter strains[a]	SFY526	***(25)***				
	CG1945	***(26)***				
	HF7c	***(26)***				
	Y190	***(27)***				
	Y187	***(27)***			S-260	***(22)***
	AH109[d]				YRG-2	***(21)***
	CY770	***(28)***			cdc25-2	***(13)***
	Y153	***(29)***			EGY48	***(32)***
	AMR70	***(5)***			EGY191	***(32)***
	GGY::171	***(30)***			EGY194	
	YI 584, YI596	***(8)***				
	Y1671	***(8)***	L40-coat	***(11)***		
	pJ69-4A	***(7)***	L40-ura	***(11)***	MaV95, 96, 97	
	YPB2	***(31)***	L40	***(4)***	103, and 203	***(9)***
One-hybrid yeast reporter strains[b]	W303	***(33)***				
	GGY1	***(14)***				
	YM4271	***(16)***				
	yWAM2	***(15)***				
	YM954, YM955	***(34)***				
Aspartic acid	80		50		100	
Serine			50		375	
Glutamic acid					100	
Histidine	20		50		20	
Arginine	50		100		20	
Threonine	100		100		200	
Proline			50			
Cysteine			100			
Tyrosine	50		50		30	
Valine	140		50		150	
Methionine	20		50		20	
Lysine	50		100		30	
Isoleucine	50		50		30	
Leucine	100		100		60	
Phenylalanine	50		50		50	
Tryptophan	50		100		40	

Table 4 *(continued)*

Nutritional supplements[c]	CSM (mg/L)	CSM Ref.	HSM (mg/L)	HSM Ref.	BSM (mg/L)	BSM Ref.
Adenine		10		100		40
Uracil		20		100		20
Total medium (mg/L)		790		1250		1285

[a]The genotype for these strains is shown in **Table 5**.
[b]The genotype for these strains is shown in **Table 6**.
[c]All listed strains can use the CSM supplement mixture with no effect on growth rate. CSM, BSM, and HSM are available from Qbiogene in any dropout combination.
[d]Available from Clontech.
CSM, complete supplement mixture; BSM, Brent supplement mixture; HSM, Hollenberg supplement mixture.

formulation, and grinding to a fine powder with a ball mill or a mortar and pestle; they can also be obtained commercially.

Because accurate and homogeneous mixtures are essential for successful and reproducible two-hybrid screens, strict guidelines are needed for commercial manufacturing. The production process should follow good laboratory practice guidelines. Ideally, raw materials are carefully chosen for their ability to promote vigorous growth on yeast minimal medium. Computerized nutrient dropout recipes automatically calculate the amounts of raw materials needed for each dropout mixture while keeping track of inventory and lot number assignments. Bar-coded raw materials are carefully measured on computerized electronic scales to keep track of amounts and components used for each batch. After a careful check of composition by quality assurance personnel, the dropout mixes are individually milled to a fine powder. They are further verified by high-performance liquid chromatography (HPLC) analysis before packaging.

Different supplement mixtures, sometimes with minor variations, have been recommended with the advent of various versions of the original yeast two-hybrid system ***(4–7)***. Making these dropout supplements is a tedious and time-consuming process. **Table 4** matches the nutritional requirement for the various yeast reporter strains to dropout nutrient mixes produced by Qbiogene following the strict guidelines outlined in this subheading.

3.4. HPLC Analysis of Supplements

Nutritional supplements consist of a defined mixture of amino acids and nucleotide precursors (**Table 4**). Thus, it is possible to detect their components by HPLC analysis using commercially available kits. The kit used in our laboratory is the Waters AccQ-Fluor™ Reagent kit (cat. no. 052880). Amino acids are derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and

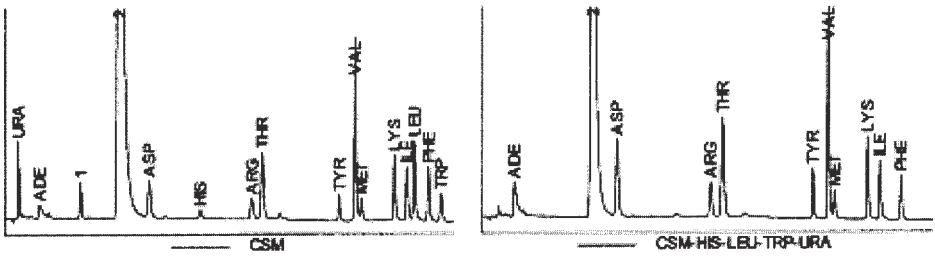

Fig. 2. HPLC analysis of nutrient supplement mixtures. Chromatograms for CSM and CSM–his–leu–trp–ura is shown. This is taken from the certificate of analysis for that quadruple dropout (Qbiogene). 1, underivatized TRP; 2, derivatizing agent.

separated by reverse-phase HPLC on a C18 column. They are then detected using a an ultraviolet detector at 554 nm.

The nucleotide precursors adenine and uracil are normally not detected once the nutrient supplement mixture is derivatized. However, the untreated adenine and uracil can be detected at 554 nm at concentrations used in minimal dropout medium. Injecting a derivatized mixture followed by an underivatized one allows the detection of adenine, uracil, and the rest of the nutrient supplement components on a single chromatogram (**Fig. 2**).

4. Preparation and Storage of Specialized Yeast/Bacterial Minimal Medium Used in Two-Hybrid Screens

4.1. Yeast Minimal Medium Containing 3-Amino-1,2,4-triazole

3-Amino-1,2,4-triazole (3-AT) is used to suppress leaky *HIS* reporter genes by competitively inhibiting the *HIS3* gene product ***(4)***. The amount of 3-AT employed is host strain and bait dependent, varying from a 0 to 100 m*M* final concentration. It must be empirically determined via titration experiments. The lowest possible concentration of 3-AT should be used to minimize the negative effect on transformation efficiency.

4.1.1. Preparation of Medium Containing 3-AT

1. Prepare a 1 *M* 3-AT stock solution in H_2O by heating at 50°C to dissolve all solids if necessary. Sterile filter and store at 4°C for ~1 mo.
2. Add the appropriate amount of 3-AT to autoclaved minimal medium ± agar that has been cooled to ~50°C, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing 3-AT should be poured thick (110–120 mL of medium/15-cm plate) to prevent drying during the screening process as the appearance of putative positive colonies is monitored for up to 10 d following a two-hybrid screen. Plates containing 3-AT are stable for ~1 mo when stored at 4°C in sealed plastic bags.

4.2. Yeast Minimal Medium Containing Cycloheximide

Cycloheximide blocks peptide elongation during translation. It is added to a final concentration of 1–10 µg/mL of medium depending on the yeast reporter strain used; for example, Y190 requires only 2.5 µg/mL ***(4)***. Cycloheximide acts to prevent the growth of cells that contain the wild-type *CYH2* gene. The loss of vectors containing the *CYH2* allele confers a growth advantage to cells that carry the resistant allele chromosomally (cyh^R) when grown on medium containing cyloheximide ***(35)***. Incorporation of the *CYH2* gene into the bait vector permits the selective loss of that vector. This facilitates the introduction of nonspecific baits, by mating, to test the authenticity of interaction of putative positive yeast clones following a successful two-hybrid screen ***(4)***.

4.2.1. Preparation of Medium Containing Cycloheximide

1. Prepare a 10 mg/mL cycloheximide stock solution in H_2O and sterile filter. Store at –20°C for later use.
2. Add the appropriate amount of cycloheximide to autoclaved minimal medium ± agar that has been cooled to ~50°C, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing cycloheximide are stable for 2 to 3 mo when stored at 4°C in sealed plastic bags.

4.3. Yeast Minimal Medium Containing 5-Fluoroorotic Acid

5-Fluoroorotic acid (5-FOA) is converted to a toxic product, 5-fluorouracil, by the *URA3* gene product. Yeast cells that contain the *URA3* marker grow on medium lacking uracil and are unable to grow on medium containing 5-FOA. This property is used to select for the loss of vectors carrying the wild-type marker in certain two-hybrid systems ***(11)***. It is also used to design a reverse two-hybrid system that positively selects for dissociation of interacting proteins ***(9)***.

4.3.1. Preparation of Medium Containing 5-FOA

1. Add up to 2 g of 5-FOA to 500 mL of H_2O and dissolve by stirring at 50–60°C for ~1 h (*see* **Note 1**).
2. Add 5 mL of 2.4 mg/mL uracil and sterile filter (*see* **Note 2**).
3. Combine with 500 mL of 2X minimal dropout medium ± agar that has been autoclaved and cooled to ~50°C.
4. Swirl to mix and pour plates if preparing agar-containing medium. Plates containing 5-FOA are stable for 2 to 3 mo when stored at 4°C. The recipe can be scaled to any desired amount.

4.4. Yeast Minimal Medium Containing 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

LacZ is a common reporter gene in yeast two-hybrid strains. Checking for β-galactosidase activity can be accomplished in several ways with different

levels of sensitivity. The least sensitive detection method involves growing colonies on 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) plates. One yeast reporter strain (EGY48) can be used with this detection method ***(6)***. The filter lift assay is much more sensitive than growing cells on X-gal plates, and it is the most commonly used method ***(36)***. The liquid assay can be the most sensitive method if using a chemiluminescent substrate, but it requires a luminometer or scintillation counter (Galacton-Star®; Clontech)

4.4.1. Preparation of X-Gal Medium Plates

1. Add dropout minimal medium powder, enough to make 1 L, to 900 mL of H_2O. Add 20 g of agar, autoclave at 121°C for 15 min, and cool to ~50°C.
2. Add 2 mL of 20 mg/mL X-gal in *N*,*N*-dimethylformamide.
3. Add 100 mL of 1 *M* potassium phosphate, pH 7.0, that has been sterilized by autoclaving, swirl to mix, and pour plates if preparing agar-containing medium. Plates containing X-gal are stable for 2 to 3 mo when stored protected from light at 4°C in sealed plastic bags.

4.5. Yeast Minimal Medium Containing Tetracycline

Tetracycline is used in the split-hybrid system to modulate the tetracycline repressor (TetR) activity if the bait has intrinsic activation capabilities ***(8)***.

4.5.1. Preparation of Medium Containing Tetracycline

1. Prepare a 25 mg/mL tetracycline stock solution in 50% ethanol. Store at –20°C for later use.
2. Add the recommended concentration of tetracycline to autoclaved minimal medium ± agar that has been cooled to ~50°C and pour plates if preparing agar-containing medium. Plates containing tetracycline are stable for ~ 1 mo when stored protected from light at 4°C in sealed plastic bags.

4.6. Bacterial Medium for Selection of Vectors Carrying Yeast Markers

An essential step in two-hybrid screens is determining the authenticity of the interaction between the newly uncovered library proteins and the bait. One strategy requires the isolation of library vectors from putative positive yeast clones to be later reintroduced with the original bait and nonspecific baits after amplification in bacteria. If both bait and library vectors carry the same bacterial antibiotic marker, as is the case with most two-hybrid systems, it is still possible to selectively recover the library vector. This selective recovery is based on the ability of the *TRP* and *LEU* yeast marker, carried on library vectors, to complement bacteria with a *trpC* and *leuB* mutation, respectively.

4.6.1. Plates for Selective Recovery of Library Vectors Carrying TRP Marker: Bacterial Strain KC8 **(6)**

1. Add 11.3 g M9 minimal salts 1 part formulation (cat. no. 3037-012; Qbiogene), 0.74 g of CSM-trp (cat. no. 4510-012), and 20g of agar to 990 mL of H_2O. Autoclave and cool to ~50°C.
2. Add 10 mL of 20% sterile glucose.
3. Add 1 mL of sterile filtered 10 mg/mL thiamine-HCl.
4. Add the appropriate amount of antibiotics. Swirl to mix and pour plates. Plates are stable for several months at 4°C in sealed plastic bags.

4.6.2. Plates for Selective Recovery of Library Vectors Carrying LEU Marker: Bacterial Strains KC8, HB101, RR1, JA226, and C600

Prepare medium as in **Subheading 4.6.1** except use 0.69 g of CSM-leu (cat. no. 4510-512). If using HB101 or RR1, add 4 mL of 10 mg/mL sterile, filtered proline. If using C600, add 4 mL of 10 mg/mL sterile, filtered threonine and 4 mL of 10 mg/mL sterile, filtered proline.

5. Sterilization of Yeast Medium: Filtration vs Autoclaving

Autoclaving for 15 min at 121°C (15 psi) is the classic way to sterilize yeast minimal (SD and SD dropouts) and rich media (YPD) in either solid or liquid format. All standard components—yeast extract, peptone, dextrose, YNB, and nutritional supplements—are added together as per formulation to the appropriate amount of water before autoclaving. Temperature-sensitive compounds such as 3-AT, 5-FOA, cycloheximide, tetracycline, Zeocin, and X-gal are added to autoclaved medium after it has cooled to ~50°C from a sterile, filtered stock solution.

Autoclaving agar containing yeast minimal medium too long can lead to soft plates (***[6]***; personal observations). Yeast minimal and rich liquid media can be sterile filtered, resulting in faster preparation, less caramelization of carbohydrates, and faster growth of cells ***(2,3,37)***. To prepare sterile, filtered agar medium, a 2X agar solution (20 g/500 mL of H_2O) is prepared and autoclaved for 15 min at 121°C. After cooling to ~50°C, it is mixed with 500 mL of a 2X sterile, filtered minimal dropout medium. The resulting plates exhibit a firm agar surface, more so than their autoclaved counterpart, and will support healthier growth. **Figure 3** shows a growth advantage for one of two randomly selected putative positive yeast clones grown at increasing dilutions on sterile, filtered (right plate, bottom half) vs autoclaved (left plate, bottom half) SD dropout plates. Prepoured sterile, filtered plates are commercially available for all yeast two-hybrid systems from Qbiogene.

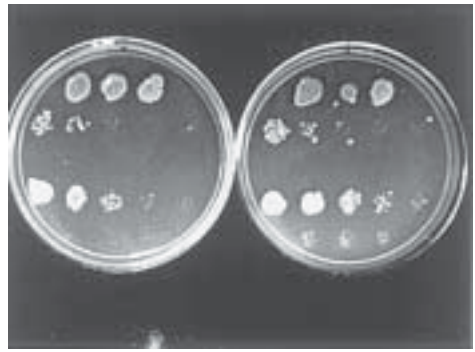

Fig. 3. Growth on sterile, filtered vs autoclaved SD dropout plates. Two randomly selected His^+ and $LacZ^+$ yeast clones from a two-hybrid screen (top half and bottom half of either plate) are plated at increasing dilutions on SD–trp–leu–his + 2 m*M* 3-AT prepared by autoclaving (left plate) and sterile filtration (right plate). The yeast reporter strain used is CG1945. Photograph is taken after 3 d of growth at 30°C.

6. Genotypes for Various Yeast Two-Hybrid Reporter Strains

Reporter strains with the *ade2* mutation exhibit a pink to red colony color in medium containing low adenine ***(19)***. The color may turn darker as the colony ages and the adenine becomes depleted. White colonies will normally form at a rate of 1 to 2% owing to spontaneous mutations that disrupt mitochondrial function. These petite colonies, which grow to a very small colony size (<1 mm in diameter), are to be avoided in two-hybrid screens.

It is generally good practice to verify the genotype of yeast reporter strains as to markers and reporter genes by growing on the appropriate dropout medium. For example, Y190 should display the phenotype Leu^-, Trp^-, Ura^+, and Lys^+ when grown on SD–leu, SD–trp, SD–ura, and SD–lys, respectively. **Tables 5** and **6** show the genotypes for the various yeast two-hybrid and one-hybrid reporter strains, respectively.

7. Media for Various Yeast Two-Hybrid Systems

7.1. Introduction

Different two-hybrid systems have been devised in an attempt to improve on the original system introduced by Fields ***(1)***. Yeast strains with improved reporter genes and more user-friendly bait and library vectors were developed (systems I, II, and III). A two-hybrid system with a cytoplasmic interaction was introduced ***(13)***. Three-hybrid systems were developed with different applications ***(10–12)***. A one-hybrid system to identify DNA-binding proteins ***(14–16)*** and split-hybrid systems that select for dissociation of interacting pro-

teins ***(8,9)*** were also developed. These various systems use different yeast reporter strains and dropout minimal medium to carry out their desired function.

Yeast dropout minimal medium is designated as SD-nutrient. It consists of YNB containing ammonium sulfate, 2% D-glucose, and the appropriate nutrient dropout (**Table 4**) ± agar. SD(gal)-nutrient represents a substitution of dextrose with 2% galactose. SD(gal/raff)-nutrient represents a substitution of dextrose with 2% galactose and 1% raffinose.

7.2. Media for the Three Major Two-Hybrid systems

Tables 7 and **8** give the media and vectors for systems I–III, respectively. **Table 9** lists the commercial two-hybrid systems based on systems I–III.

7.3. Media for SOS Recruiting System

Table 10 provides the media and their functions for the SOS recruiting system.

7.4. Media for Trihybrid Systems

Three different trihybrid systems have been introduced: one with an inducible third-party expression under the control of a methionine regulated promoter ***(10)***, one to identify RNA-binding proteins ***(11)***, and one for the identification of protein-protein interaction dependent on posttranslational modifications ***(12)***.

7.4.1. Media and Their Functions for Trihybrid System with an Inducible Third-Party Expression

Table 11 provides the media and their functions for the trihybrid system with an inducible third-party expression.

7.4.2. Media for Trihybrid System to Identify RNA-Protein Interactions

Table 12 provides the media for identifying RNA-protein interactions in the trihybrid systems. An alternative version of the trihybrid system for the analysis of RNA-protein interactions has been reported ***(39)***. It uses the yeast reporter strain CG-1945. The equivalent of LexA-MS2 fusion protein and MS2-RNAx hybrid RNA are expressed from a single TRP vector. The library is expressed form a LEU vector. Libraries for the these two systems have the same selectable markers yet they cannot be interchangeably used because one is LexA based ***(11)*** and the other is GAL 4 based.

7.4.3. Media for Trihybrid System for Identifying Protein–Protein Interaction Dependent on Posttranslational Modifications

Table 13 provides the media for identifying protein–protein interactions dependent on posttranslational modifications.

Table 5
Genotypes for Yeast Two-Hybrid, Trihybrid, and Split-Hybrid Reporter Strains

Strain[a]	Type	Markers	Other	Reporter
Y190[b] ***(27)***	*a*	*trp, leu, cyh*^{R}	*ade2-101, ura3-52* *gal4, gal80, his3Δ200*	*URA3*: :*GAL1-lacZ* *LYS2*: :*GAL1-HIS3*
Y187 ***(27)***	α	*trp, leu, his*	*ade2-101, met*$^-$ *gal4, gal80, ura3-52*	*URA3*: :*GAL1-lacZ*
L40[b] ***(38)***	*a*	*trp, leu*	*ade2, Lys2-801am* *GAL4, his3Δ200*	*URA3*: :*(Lex A op)*$_8$*-lacZ* *LYS2*: :*(Lex A op)*$_4$*-HIS3*
L40-ura3[b] ***(11)***	*a*	*trp, leu, ura*	*ade2, his3Δ200* *ura3-52*	*ura3*: :*(Lex A op)*$_8$*-lacZ* *LYS2*: :*(Lex A op)*$_4$*-HIS3*
AMR70 ***(38)***	α	*trp, leu, his*	*Lys2-801am*	*URA3*: :*(lexAop)*$_8$*-LacZ*
EGY48 ***(32)***	α	*trp, his, ura*		*(LexA op)*$_6$*-LEU2*
EGY191 ***(32)***	α	*trp, his, ura*		*(LexA op)*$_2$*-LEU2*
cdc25-2 ***(13)***	α	*trp, leu, ura, his*	*ade2-101, lys2-801* *GAL+*	*Cdc25-2*
HF7C ***(26)***	*a*	*trp, leu*	*ade2-101, lys2-801* *Gal4-542, gal80-538* *ura3-52, his3Δ200*	*URA3*: :*(GAL4-17mer)*$_3$*-CYC1-LacZ* *LYS2*: :*GAL1-HIS3*
YI584[b] ***(8)***	*a*/α	*trp, leu*	*ade2/ade2* *hisΔ200/hisΔ200*	*URA3*: :*(LexA op)*$_8$*-TetR* *LYS2*: :*(Tet op)*$_2$*-HIS3*
SFY 526 ***(25)***	*a*	*trp, leu, his*	*ade2-101, lys2-801* *gal4-542, gal80-538* *can*^{1}*, ura3-52*	*URA3*: :*GAL1-LacZ*
AH109[b]	*a*	*trp, leu*	*gal4Δ gal80Δ* *ura3-52, his3Δ200*	*URA3*: :*MEL1-LacZ* *LYS2*: :*GAL1-HIS3* *GAL2-ADE2*

PJ69-4A ***(7)***	*a*	*trp, leu, ura,*	*gal4Δ gal80Δ* *his3-200*	*met2*: :*GAL7-LacZ* *LYS2*: :*GAL1-HIS3* *GAL2-ADE2*
GGY: :*171* ***(30)***	α	*leu, his*	*ade2, met*$^{-}$*, tyr1,* *gal4Δ gal80Δ, ura3-52*	*URA3*: :*GAL1-lacZ*
YPB2^{b} ***(31)***	*a*	*trp, leu,*	*ade2-101, lys2-801, can*^{R} *gal4-542, gal80-538* *ura3-52, his3Δ200*	*URA3*: :*GAL17mer-CYC1-lacZ* *LYS2*: :*GAL1-HIS3*
S-260 ***(22)***	α	*trp, leu, ura*	*ade2-101, can1-100, ho*	*ura3*: :*(Col E1 op)*$_{6}$*-LacZ*
MaV103b ***(9)***	*a*	*leu, trp, cyh2*^{R}	*can1*^{R}*, ade2-101* *ura3-52, his3Δ200* *gal4Δ gal80Δ*	*SPAL10*: :*URA3* *GAL1-LacZ* *LYS2*: :*GAL1-HIS3*

[a]YI596b is similar to YI584b except that the Tet repessor gene is driven by the ADH promoter: URA3: :(LexA op)$_{8}$-ADH-TetR (DeMaggio, personal communication).

YI671^{b} is similar to YI596 except that it is haploid: MAT α (DeMaggio, personal communication).

Y153^{b} ***(29)*** is identical to Y190 except that it is cycloheximide sensitive: *CYH2*.

CY770^{b} ***(28)*** is derived from Y190 by selection on 5-FOA medium to select for loss of URA: : GAL1-LacZ.

YRG-2 ***(21)*** is a derivative of HF7C selected for high-efficiency transformation. It has the same genotype as HF7C.

CG1945 ***(26)*** is a derivative of HF7C selected for cycloheximide resistance: *cyh*^{R}.

MaV203^{b} ***(9)*** is similar to MaV103 except that it is MATα.

MaV95, 96, 97^{b} ***(9)*** are similar to MaV103 with the exception of fewer SPAL sites 5, 7, and 8, respectively.

EGY194 is similar to EGY48 except that it has four lexA operators in the *LEU2* reporter gene: *(LexA op)*$_{4}$*-LEU2*.

[b]The *HIS* reporter gene has different levels of basal expression requiring different amounts of 3-AT to suppress background growth in two-hybrid screens.

Table 6
Genotypes for Yeast One-Hybrid Reporter Strains

Strain	Type	Markers	Other
YM954 ***(34)***	*a*	*trp, leu, ura, his, lys*	*ade2-101, can^R, gal4Δ542, gal80Δ538*
YM955 ***(34)***	*a*	*trp, leu, ura, his, lys*	*ade2-101, can^R, gal4Δ542, gal80Δ538, tyr1-501*
GGY1 ***(14)***	α	*leu, ura, his*	*ade2, tyr, Δgal4, Δgal80*
W303-1a ***(33)***[a]	*a*	*trp, leu, ura, his*	*ade2-1, can1-100*
yWAM2 ***(15)***	α	*trp, leu, his*	*ade2-101, lys2-801, CYH2, Δgal4, Δgal80 URA3::GAL1-LacZ*
YM4271 ***(16)***	*a*	*trp, leu, his, ura, lys*	*ade2-101, ade5, tyr1-501, Δgal4, Δgal80*

[a]*W303-1b* ***(33)*** is similar to *W303-1a* except that it is MAT α.

7.5. Media for Split/Reverse Two-Hybrid Systems

Two systems that positively select for dissociation of interacting proteins have been defined. The split-hybrid system (*see* **Table 14)** uses the *HIS3* reporter gene under the control of the Tet R repressor. Interaction between the bait and prey leads to repressor production, which inhibits transcription of the *HIS3* reporter gene, preventing growth on medium lacking histidine. Dissociation restores histidine prototrophy ***(8)***. The reverse two-hybrid system (*see* **Table 15**) uses the URA3 counterselectable marker to positively select for dissociation of interacting protein in the presence of 5-FOA ***(9)***.

7.6. Media for One-Hybrid Systems

Several different versions of the one-hybrid system have been developed that use the following reporter genes: *LacZ* ***(14,33)***, *HIS3* ***(15)***, *HIS3* and *LacZ* ***(16,34,40)***. These reporter genes are under the control of the target DNA fragment to which binding proteins are to be identified. Recombinant reporter genes can either be incorporated into the yeast genome ***(14,16,40)*** or exist on autonomously replicating vectors ***(14,33,34)***. The same libraries used for two-hybrid screens are used for the one-hybrid system if the markers are compatible. Commercially available one-hybrid systems include Matchmaker one-hybrid system (Clontech) (*see* **Table 16**).

Table 7
Media for Systems I–III and Their Functions

System I	System II	System II	Medium function
SD–trp	SD–trp	SD–his	Bait selection
SD–leu	SD–leu	SD–trp	Library selection
SD–trp–leu	SD–trp–leu	SD–his–trp–ura	Bait + library selection
SD–leu+ Cycloheximide	SD–leu or SD–leu–ura+ 10 mg/L Adenine[a]	SD–trp	Bait loss
SD–trp–his ±3–AT	SD–trp–leu–his–ura–lys ±3–AT	SD–ura + X–gal SDgal/raff–ura + X–gal SDgal/raff–ura–his–trp+/–leu	Testing bait for autoactivation of reporter genes[c]
SD–trp–leu–his (±3–AT)	SD–trp–leu–his–ura–lys (±3–AT)	SDgal/raff–his–trp–leu–ura SD–his–trp–leu–ura	Screening bait + library for activation of reporter genes
		SD–ura	*lacZ* reporter vector selection[d]
Y190/Y187	L40[b]/AMR70	EGY48/EGY191	Reporter strains
CSM	CSM/HSM	CSM/BSM	Recommended nutritional supplement (**Table 4**)

[a]Select for segregants that have lost the bait vector carrying the *ADE2* gene by a change in colony color from white to red.

[b]Minimal medium contains added succinate: 10 g of succinic acid + 6 g of NaOH/L of dropout medium.

[c]Autoactivation is ideally carried out in the presence of bait and activation domain vector. Selection for these vectors may not need to be maintained during the testing process, as is the case for the lacZ assay of system III.

[d]The *LacZ* reporter gene is integrated into the yeast genome in systems I and II.

Table 8
Vectors for Systems I–III

	System I	System II	System III
Bait vectors	pAS1/2	pBTM116	pEG202
Library vectors	pACT1/2	pVP16	pJG4–5

Table 9
Commercial Two-Hybrid Systems Based on Systems I–III *(4–6)*

System I	System III
Matchmaker I, II (Clontech) Strains Y153, Y187, CG1945, HF7c, SFY526 HybriZap (Stratagene) Strain YRG2[a] Matchmaker system3[b] (Clontech) Strain AH109	DupLEXA (Origene) Matchmaker LexA (Clontech) Strain EGY48 Hybrid hunter (Invitrogen)[c] Strains EGY48 and L40 Bait selected with Zeocin

[a]Minimal medium contains 1 *M* sorbitol: 182.2 g of sorbitol/L of medium (Stratagene).
[b]Omission of adenine selects for expression of third reporter gene, *ADE2*.
[c]Bait is selected with Zeocin. Medium for L40 strain contains added succinate: 10 g of succinic acid + 6 g of NaOH/L of dropout medium.

Table 10
Media and Their Functions for SOS Recruiting System

Medium	Function
SD–leu	Bait selection/expression
SD–ura	Repressed library selection
SDgal/raff–ura	Expressed library selection
SD–leu–ura	Bait + repressed library slection
Sdgal/raff–leu–ura	Screening bait + library for growth at 36–37°C
Yeast reporter strain: cdc25–2	
Bait vector: expresses bait–hSOS fusion protein	
Library vector: expresses myristoylated fusion protein	
Nutritional supplement: CSM/BSM	

Table 11
Media and Their Functions for Trihybrid System with Inducible Third-Party Expression

Medium	Function
SD–trp + 1 m*M* methionine (met)	Protein 1 and 2 selection[a]
SD–trp–met	Protein 1 and 2 selection/expression
SD–leu	Protein 3 selection/expression
SD–trp–leu + 1 m*M* met	Protein 1 and 3 expression
SD–trp–leu–his + 1 m*M* met	Screening protein 1 and 3 for interaction
SD–trp–leu–met	Protein 1, 2, and 3 expression
SD–trp–leu–his ± 1 m*M* met	Screening all three expressed proteins for interaction
Yeast reporter strains: L40/HF7C	
Bait vectors: pLex9–3H (L40)/ pGBT9–3H/B (HF7C)	
Library vectors: pVP16 (L40)/ pGAD–GH (HF7C)	
Nutritional supplement: CSM/HSM	

[a]1, Suppressed; 2, repressed.

Table 12
Media for Identifying RNA-Protein Interactions in Trihybrid System

Medium	Function
SD–trp	LexA–MS2 fusion protein vector selection
SD–ura	MS2–RNAx–hybrid RNA vector selection
SD–leu	Library selection
SD–trp–leu–ura	Selection of all three vectors
SD–trp–leu–ura–his ± 3–AT	Screening for library protein interaction with RNAx
SD–trp–leu + 5-FOA	MS2–RNAx–hybrid RNA vector loss
Yeast strains: L40–ura[a]	
Nutritional supplement: CSM/HSM	

[a]Strain L40 coat can also be used; it has the LexA-MS2 fusion protein vector integrated into its genome. If using this strain, do not omit tryptophan from the medium.

Table 13
Media for Identifying Protein–Protein Interactions Dependent on Posttranslational Modifications

Medium	Function
SD–trp	Repressing bait selection
SD–ura	Repressing library selection
SD–leu	Modifying enzyme vector selection
SDgal–trp–leu	Testing modified bait for activation of reporter gene
SD–leu–trp–ura	Screening library for interaction to modified bait
SDgal–trp–leu–ura	
Yeast reporter strain: S-260	
Nutritional supplemet: CSM/BSM	

Table 14
Media for Splithybrid System

Medium	Function
SD–trp	Bait selection
SD–leu	Prey selection
SD–trp–leu	Bait + prey selection
SD–trp–leu–lys–ura	
SD–trp–leu–lys–ura±his ±tet ±3–AT	Modulation with 3–AT and tetracycline
SD–trp–leu–lys–ura–his ±3–AT ±tet	Positive selection for dissociation
Yeast strains: Y1584, Y1596, Y1671	
Bait vector: pBTM116	
Prey vector: pVP16, PVP16–LacZ	
Nutritional supplement: CSM	

Table 15
Media for Reverse Two-Hybrid System[a]

Medium	Function
SD–leu	Bait selection
SD–trp	Prey selection
SD–leu–trp	Bait + prey selection
SD–leu–trp + 5–FOA	Selecting for disruption of protein interaction

Table 15 *(continued)*

Medium	Function
SD–trp–leu–ura	Selecting for positive interaction (LacZ)
SD–trp–leu–his ± 3–AT	Selecting for positive interaction (HIS3)
Yeast reporter strain: MaV103/203 bait vector: pPC97–CYH2 Prey vector: pMV257 Nutritional supplement: CSM/BSM	

[a]This system is available from Gibco-BRL (ProQuest™ Two-Hybrid System).

Table 16
Media for One-Hybrid Systems

Medium	Function
SD–leu, SD–trp, or SD–ura	DNA–reporter gene selection (A)
SD–trp or SD–leu	Library selection (B)
SD–A–B ± his[a]	A and B selection to screen for DNA-binding proteins
SD(gal/raff)–A–B ± his[a]	A selection and B selection/expression to screen for DNA-binding proteins
Yeast strains: YM954/YM955 ***(33)***, W303–1a ***(32)***, GGY1 ***(12)***, YM4271 ***(14)***, yWAM2/PCY2 derivative ***(13)***, Nutritional supplement: CSM	

[a]A and B represent the nutrient to be dropped out for selection; for example, if the DNA reporter gene construct has the URA marker and the library carries the LEU marker, the following medium is used for a library screen: SD–ura–leu ± his or SD(gal/raff)–leu–ura ± his (histidine is dropped only if using the *HIS3* reporter gene).

7.7. Media for Mammalian Two-Hybrid System.

The mammalian two-hybrid system, like its yeast counterpart, uses the reconstitution of a transcription factor to drive expression of a detectable reporter gene. Reporters used in this system include the chloramphenicol acetyltransferase ***(17)*** and the luciferase ***(41)*** genes. Bait, library, and reporter vectors are introduced into mammalian cells by transient transfection ***(42)***. Different mammalian cell lines have been successfully used in this system ***(17,18,41,43,44)*** (*see* **Table 17**).

Table 17
Cell Lines and Media for Mammalian Two-Hybrid System

Cell line	Medium[a]
293 cells ***(43)***	Low-glucose DMEM with 2 m*M* glutamine + 10% FCS
CHO ***(17)***	MEM alpha + 10% FCS
Hela ***(45)***	MEM + 10% FCS
CV1 ***(18)***	DMEM + 10% FCS
SAOS2 ***(44)***	DMEM + 10% FCS
C3H10T1/2 ***(41)***	DMEM + 10% FCS
Jurkat ***(41)***	RPMI + 2 m*M* glutamine + 1 m*M* Na pyruvate + 10% FBS

[a]The various media listed are commercially available as sterile liquid solutions or powder mixes that must be reconstituted in H_2O and sterilized by filtration (e.g., Gibco-BRL, Fisher, VWR, Thomas, Beckton Dickinson, HyClone). FCS is added shortly before use. The following antibiotics can be added to prevent bacterial contamination: 20–50 µg/mL of gentamicin sulfate or 100 µg/mL of penicillin + 100 µg/mL of streptomycin ***(42)***. A mammalian two-hybrid system is available from Clontech and Stratagene. DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FCS, fetal calf serum.

Mammalian cells are much more finicky than bacteria or yeast and are susceptible to contamination by these organisms. They have a long generation time (12–48 h). They require much stricter sterile techniques for maintenance and propagation. Culturing is usually done in sterile hoods following good sterile techniques. Only high-quality purified water is used to prepare medium. Sterile disposable items are used as much as possible. A special set of glassware is often dedicated for tissue culture purposes, because even a trace amount of common laboratory chemicals can be toxic to mammalian cells. Following these guidelines ensures a healthy population of cells resulting in efficient transfections, which are essential for successful library screens ***(42)***.

8. Notes

1. One gram of 5-FOA (0.1% final concentration) is traditionally used to select for the loss of URA marker ***(3)***. A range of 0.05–0.2% 5-FOA is used in the reverse two-hybrid system ***(9)***.
2. Uracil is ommitted from this medium if one is screening for dissociation of interacting proteins using the reverse two-hybrid system.

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