“One plate/three-reporter” assay format for the detection and validation of yeast two-hybrid interactions

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We describe a novel assay format for the Gal4-based yeast two-hybrid-system, in which the readout from three different reporter genes is measured sequentially in a single microplate. Activation of the URA3, MEL1, and lacZ reporters in response to a protein-protein interaction is monitored by measuring sequentially: (i) growth in medium lacking uracil, (ii) \( \alpha \)-galactosidase activity, and (iii) \( \beta \)-galactosidase. The data thus generated permit elimination of many false positive signals and provide a preliminary measurement of reporter activation-strength that may be confirmed by further analysis. The assay procedure is inexpensive and requires few liquid-handling steps. It is appropriate for automated high-throughput interaction mating assays, validation of putative interactor strains and hybrid-protein self-activator tests.

INTRODUCTION

Knowledge of the protein-protein interactions occurring within a cell provides insight into gene function and nominates proteins for pharmaceutical modulation. The yeast two-hybrid (Y2H) system (1) has been used to chart the complete interactome of several organisms (2–6). However, for organisms with large genomes, novel techniques that enhance the veracity and throughput of the Y2H system are required to uncover protein-protein interactions comprehensively. The accuracy of Y2H analysis is enhanced by monitoring multiple different reporter genes engineered into a host cell. The most commonly used reporters include genes required for prototrophic cell growth in the absence of a particular amino acid or base, and the bacterial lacZ gene encoding \( \beta \)-galactosidase (reviewed in Reference 7). In addition, some strains of *Saccharomyces cerevisiae* contain the MEL1 gene (8) that is expressed under the control of the Gal4 transcription factor and codes for a secreted \( \alpha \)-galactosidase enzyme (9,10). Thus, MEL1 provides an endogenous reporter gene compatible with the Gal4-based Y2H system (8). Specific assays for the separate analysis of \( \alpha \)-galactosidase and \( \beta \)-galactosidase activity have been described (10–13), and lacZ has been used as a reporter in large-scale automated screens (14).

To maximize the amount and quality of data generated by high-throughput Y2H screens, we have developed an assay format (the sequential triple-reporter assay) in which the readout from the URA3, MEL1, and lacZ reporter genes is measured sequentially in a single microplate.

MATERIALS AND METHODS

Growth Medium

Yeast cells were grown in liquid yeast extract, peptone, dextrose (YPD) medium or selective dropout broth (DOB) medium with supplements (all from Qiobiogene, Carlsbad, CA, USA) or on rich YPD or selective complete minimal (CM) agar plates (Teknova, Halfmoon Bay, CA). Mating reactions were performed in liquid medium containing 2X YPD.

Yeast Strains and Plasmids

The haploid Y2H host strains YULH (MATa) and N106r (MAT\( \alpha \)) have been described (4). Both strains contain the MEL1 and lacZ reporters driven by the *MEL1* UAS, while YULH alone contains the GAL1::URA3 reporter. Haploid strains, containing fusions of Gal4\( _{AD} \) or Gal4\( _{AD} \) to full-length *Drosophila* cDNAs were mated to produce CGY diploid control strains. The cDNAs were chosen based on consistent function; they reproducibly promoted strong, medium, and weak reporter activation, respectively, in the Y2H system, even after rescue from and re-introduction into a yeast host strain as described in the text. Bait genes were fused to GAL4\( _{BD} \) in vector pDBGal4CAM (Stratagene, La Jolla, CA, USA) while preys were fused to GAL4\( _{AD} \) in vector pACT2 (BD Biosciences Clontech, Palo Alto, CA, USA).

The hybrid proteins encoded in diploid strains CGY-1D and CGY-3D participate apparently in a strong and weak interaction, respectively, while those encoded in diploid strains CGY-2D and CGY-23D participate apparently in a medium-strength interaction. The hybrid proteins encoded in strain CGY-4D do not interact, while a chromosomal mutation in this strain confers uracil prototrophy independent of a Y2H interaction.

Sequential Triple-Reporter Assay

Diploid colonies (of strains CGY-1D to CGY-4D) were inoculated into selective liquid medium (100 \( \mu \)L; lacking tryptophan, leucine, and uracil) in a flat-bottom 384-well microplate (Bioone, Greiner Lake Mary, FL, USA) and incubated at 30°C for 3 days in a sealed container. To test activation of the URA3 reporter gene, the cells were resuspended, diluted 100-fold in selective medium (lacking tryptophan, leucine, and uracil), inoculated into a flat-bottom 384-well plate (100 \( \mu \)L culture volume) and incubated at 30°C for 3 days in a sealed container. (Under these conditions the final cell density achieved by cells containing weak or strong two-hybrid interactions was similar, and background growth by cells containing noninteracting proteins was below the level of detection of \( A_660 \) measurement.) The cells were resuspended, and an aliquot of each culture (45 \( \mu \)L) was transferred to a second 384-well plate containing glucose (50% v/v), mixed and archived at
-80°C. As an option at this stage, the $A_{660}$ of the cultures remaining in the original (assay) plate were measured to quantify cell growth (see alternative procedure below). To measure $\alpha$-galactosidase activity encoded by MEL1, 20 µL Buffer ZLX [3.75× Z-buffer (1×: 16.1 g/L Na$_2$HPO$_4$ · 7H$_2$O, 5.5 g/L NaH$_2$PO$_4$ · H$_2$O, 0.75 g/L KCl, 0.246 g/L MgSO$_4$ · 7H$_2$O, pH 7.0), 23.4 mM X-a-Gal (p-nitrophenyl $\alpha$-d-galactopyranoside; Sigma, St. Louis, MO, USA), and 188 U/mL lyticase (Fluka, Buchs, Switzerland)] were added to the cultures, mixed, and incubated at room temperature for 60–180 min. Color development (at A$_{410}$ or $\Delta$A$_{410}$/A$_{660}$) was measured using a PowerWave X™ Select platereader with KC4 software (BioTek Instruments, Winooski, VT, USA). The $\Delta$A$_{410}$/A$_{660}$ reading provides a measurement of $\alpha$-galactosidase activity correlated to the amount of biomass present and eliminates the necessity for an independent A$_{660}$ measurement to correct for growth, while similar results were obtained by both approaches. To subsequently measure $\beta$-galactosidase activity encoded by the lacZ reporter gene, 25 µL of Buffer CPRG-N [0.5 mg/mL CPRG (chloro phenol red-$\beta$-d-galactopyranoside; Roche Molecular Biochemicals, Indianapolis, IN, USA) and 0.8% IGPAL CA-630 (Sigma)] was added to each well, mixed, and incubated at room temperature for 60 min (or longer, up to an overnight incubation) prior to measurement of color development (A$_{578}$ or $\Delta$A$_{578}$/A$_{660}$). For both the $\alpha$-galactosidase and $\beta$-galactosidase assay, we found that the rate of color development could be adjusted conveniently by varying the concentration of substrate and/or lyticase (thereafter held constant in every assay of a particular screen) depending on time and cost requirements. Data was processed in Microsoft® Excel® by subtracting the background signal generated by negative control strain CGY-4D from the signals obtained from positive interactor strains (strains containing plasmids encoding two interacting proteins).

**Interaction-Mating**

Bait and prey plasmids were recovered from the diploid control strains CGY-1D, CGY-23D, CGY-3D, and CGY-4D (RPM® yeast plasmid isolation kit; QBiogene) and re-introduced into strain YULH and N106r, respectively, by standard procedures (15). Diploid cells of strain CGY-1D, CGY-23D, CGY-3D, and CGY-4D were regenerated by interaction mating as described previously (14) with the following modifications. The relevant haploid transformant cells were grown in selective medium (3 mL) by incubation at 30°C overnight with agitation.
Proteomic Technologies

Figure 1. Sequential (A) α-galactosidase and (B) β-galactosidase assay for the detection of protein-protein interactions using the Gal4-based Y2H system. Diploid colonies (CGY-1D to CGY-4D) were inoculated into selective liquid medium and assayed as described in Materials and Methods. Incubation time for the α-galactosidase and β-galactosidase assay was 90 and 120 min, respectively. Data for each strain are the average (± SD) of assays performed in triplicate and corrected for background signals produced in the absence of a Y2H interaction (strain CGY-4D).

Figure 2. Sequential detection of reporter gene activity in diploid cells generated by interaction mating. Mating and selective outgrowth of diploid cells was performed as described in Materials and Methods. The diploid cells tested by sequential (A) α-galactosidase and (B) β-galactosidase assays were generated by interaction mating (open bars) or by growing diploid cells directly without mating (closed bars). In each case, the number of diploid cells assayed for α-galactosidase and β-galactosidase activity was approximately 5.5 × 10^5 per well. Incubation time for the α-galactosidase and β-galactosidase assay was 180 and 60 min, respectively. Data are presented as in Figure 1.

Crosses were made by mixing 2 μL of a saturated MATα (bait) culture with 8 μL of the appropriate MATα (prey) culture in 10 μL of medium containing 2× YPD in a V-bottom 96-well plate (DOT Scientific, Burton, MI, USA) followed by incubation at 30°C overnight. The mating reactions were resuspended, diluted 100-fold in selective medium (lacking tryptophan, leucine, and uracil), inoculated into a flat-bottom 384-well plate (100 μL culture volume) and incubated at 30°C for 3 days in a sealed container to permit selective outgrowth of diploid cells. [Dilution of rich medium present in the mating reaction is necessary to prevent nonselective outgrowth of noninteractor cells (14).] The diploid strains CGY-1D, CGY-23D, CGY-3D, and CGY-4D were cultured in an identical manner to that described above except that 10 μL of the saturated diploid culture was added to the 2× YPD-containing medium. Cells were archived and analyzed by sequential α-galactosidase and β-galactosidase assays as described above.

RESULTS AND DISCUSSION

To detect and validate protein-protein interactions using in the Y2H system, we developed a liquid assay that permits readout from three different reporter genes conveniently in a single 384-well microplate. Blue-colored diploid colonies containing putative Y2H interactions were initially selected on agar medium lacking uracil and containing 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal) as described (4). Activation of the URA3 reporter gene was then confirmed by selective growth of diploid cells in liquid medium lacking uracil (not shown). Subsequently α-galactosidase activity expressed from the MEL1 reporter was measured in the presence of Z-buffer (pH 7.0) and lyticase (Figure 1A). Under these conditions, color development (colorless to yellow) occurs in concert with the hydrolysis of X-a-Gal (without the requirement for subsequent pH adjustment), while the concomitant cell lysis increases signal strength via release of intracellular enzyme. Following measurement of color development, detergent and CPRG were added to each well to promote completion of cell lysis and rapid detection of β-galactosidase activity (Figure 1B). We found that the signal ratio between strong, medium-strength, and weak interactor strains was generally similar in the α-galactosidase and β-galactosidase assays. In rare instances where the signal from the α-galactosidase and β-galactosidase assay was not comparable (not shown), interactions were deemed false positives based on the assumption that a true interaction should activate both reporter genes similarly.

For primary detection of protein-protein interactions by Y2H matrix (1 × 1) screening, we next combined the sequential α-galactosidase/β-galactosidase assay format with liquid interaction mating assays. Interaction-mating of MATα and MATα haploid cells, containing bait and prey plasmids respectively, was performed in V-bottom plates. Diploids were inoculated into 100 μL of selective medium following a 100-fold dilution of the mating mixture. A subsequent period of selective outgrowth was included to test for the activation of the URA3 reporter gene and to enrich for diploid cells. Using this approach, the relative strength of the protein-protein interactions detected in different strains correlated well following interaction mating or direct diploid cell growth (Figure 2, A and B; strains 1, 23, and 3). Thus, our assay format provides reliable detection of protein-protein interactions following a 1 × 1 interaction mating assay. Nevertheless, differences in interaction strength were observed in similar strains generated by the different procedures (interaction mating or direct diploid growth) (Figure 2A, strain 1, and Figure 2B, strains 23 and 3). The reason for these
differences is unknown, but they may be due to changes in plasmid copy number during the interaction mating procedure.

In the sequential triple-reporter assay, the readout from three different reporter genes is measured in turn in a single 384-well plate. This format is therefore ideal for automated, high-throughput screening for protein-protein interactions using the Gal4-based Y2H system. Because it is designed for the analysis of small culture volumes (55 µL) in 384-well plates, it is inexpensive (estimated cost per assay, 3.0 cents) and requires only two liquid handling steps. Therefore, it will be useful for the comprehensive mapping of protein-protein interactions occurring in organisms with large genomes. However, as for all Y2H approaches, the interactions detected by high-throughput screening require validation and quantitation by further tests, including measurement of hybrid protein expression level and independent biochemical analysis, such as co-immunoprecipitation experiments. Our approach complements and partially extends a similar assay format described previously (16) by utilizing the URA3 prototrophic reporter in addition to two others in a single microplate assay and by adapting the procedure to accommodate interaction mating assays. While the data generated from three GAL4-dependent reporter genes in the sequential triple-reporter assay increases the veracity of the Y2H technique by reducing false positive results, the elegant dual-bait system described by the previous authors (15,16) is expected to achieve this more effectively at the expense of greater host strain complexity.

We have used our assay format to validate putative “interactor” colonies detected initially on selective agar medium and to detect protein-protein interactions directly in combination with the interaction-mating approach. Moreover, each of the haploid Y2H host strains that we have used contains both the MEL1 and lacZ reporter genes, and thus the sequential αα-galactosidase/β-galactosidase assays we describe can be used to test for self-activating bait or prey plasmids directly in their respective haploid host strains prior to mating.

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COMPETING INTERESTS STATEMENT

The authors declare no conflicts of interests.

REFERENCES


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