Highly sensitive microplate β-galactosidase assay for yeast two-hybrid systems

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BioTechniques 35:446-448 (September 2003)

The yeast two-hybrid system is a powerful tool to monitor protein-protein interactions. It relies on the expression of hybrid proteins in the yeast nucleus and the activation of reporter genes when a hybrid transcription factor (e.g., Gal4) is reconstituted via two interacting proteins (1). The method has been used at the genome level in organisms ranging from viruses to plants to assess protein-protein interaction networks using prototrophy markers as reporter genes (2–4). A second reporter gene, *LacZ*, offers the possibility of quantitative measurements of β-galactosidase activity by a colorimetric method (5). A β-galactosidase test in bacteria has been transposed to microplates (6). In the case of yeast, the conditions for cell lysis in microplates have been improved (7,8), but for truly high-throughput two-hybrid tests (4,9,10), the β-galactosidase reporter activity is still assayed in test tubes. In this report, we have successfully adapted yeast culture, permeabilization, and β-galactosidase assay conditions to microtiter format, and the optimization of all these steps led us to increase the sensitivity of the enzyme assay by at least 50-fold compared to classical methods. Direct kinetic recording instead of end point measurements allows for the processing of high- and low-activity samples in the same plate with good reproducibility. The colorimetric assay costs approximately $200/1000 plates, which is about 200-fold less expensive than a commercially available β-galactosidase assay kit. The method can be used for the various types of yeast two-hybrid systems currently in use.

To set up the method, we used a two-hybrid system in which the DNA-binding domain was the LexA protein from *Escherichia coli* that has been previously described (11) in L40 strain. We then extended the method to the other LexA-based system (12) in EGY48 strain with reporter plasmid pSH18:34 and to a system in which the DNA-binding domain was the DNA-binding domain of the yeast transcription factor Gal4 itself (BD Biosciences Clontech, Erembodegem, Belgium) in Y190 strain. The two-hybrid systems and yeast strains have been previously described (13). Yeast cells expressing interacting pairs were grown in glucose medium (galactose in the case of Reference 12) with appropriate selection. With the classical approach, to assess the reporter gene *HIS3* (or *LEU2*), yeast patches were replicated on agar medium that lacked the appropriate amino acids, and growth was visualized after 48 h (Figure 1A). The reference method for the β-galactosidase assay was a filter test, using X-gal as a substrate (5), in which a blue coloration developed in 1–10 h (Figure 1B). To adapt the test to microplates, we modified several steps. Yeast culture was made in microplate format instead of culture tubes. Culture medium (0.5 mL), consisting of 2% glucose, 6.7 g/L yeast nitrogen base without amino acids, supplemented with an amino acid mixture lacking the appropriate amino acids for selection (Qbiogene S.A., Illkirch, France), was inoculated with a toothpick from a patch on agar plates, and yeast cells were cultured in deep-well, conical-bottom microplates (Eppendorf, Le Peck, France) in a thermoblock shaker with a microplate holder. We found the Thermomixer Comfort model (Eppendorf) provided the vigorous shaking (900 rpm) needed to prevent the yeast

Table 1. Protocol for the Assay of β-Galactosidase Activity in Microplates

Yeast Culture

1. To inoculate, transfer from a solid culture plate to 0.5 mL medium with appropriate amino acid selection.
2. Culture for 20 h with shaking in a deep-well, conical-bottom microplate.
3. Dilute 50 µL culture in 1 mL medium.
4. Culture for 48 h at 30°C.

β-Galactosidase Assay

1. Transfer 200 µL culture into a U-bottom microplate.
2. Quantify yeast cells by absorbance at 590 nm[a].
3. Centrifuge the microplate at 1500× *g*.
4. Discard supernatant fractions.
5. Add 100 µL 100 mM sodium phosphate[b] buffer, pH 7.5, containing 0.1% sodium dodecyl sulfate.
6. Shake at 800 rpm for 10 min at 30°C.
7. Add 2.5 mM o-nitrophenyl-β-D-galactopyranoside (ONPG) in 25 µL.
8. Measure absorbance at 414 nm in an iEMS microplate reader for 10 min at 30°C to obtain kinetic recording.
9. Determine β-galactosidase activity from linear part of ONPG hydrolysis curve[c].

[a] $1.0 \times A_{590} = 6 \times 10^6$ yeast cells.

[b] Consisting of 82 mM Na_2HPO_4 and 12 mM NaH_2PO_4.

[c] β-galactosidase unit: increase in absorbance at 414 nm $\times$ min^{-1} $\times$ $1000/(A_{590})$.

from settling. Aeration was assured via a plastic gas-permeable lid. The culture time was found to greatly affect β-galactosidase activity, and an optimal culture time of 48 h at 30°C was determined. After 20 h, 50 µL of the yeast suspension were diluted in 1 mL fresh medium. For the assay, 0.2 mL of the yeast suspension were transferred to U-bottom plates and quantified by reading the absorbance at 590 nm in a microplate reader (iEMS Labsystem; Thermo Life Science France S.A., Cergy Pontoise, France). Approximately 6×10^6 yeast cells, corresponding to an absorbance of 1.0, were used in each assay. The microplate was centrifuged at 1500× *g* in a CR-4.12 centrifuge (Jouan S.A., St. Herblain, France) with a M4 rotor for 5 min, and the supernatant was discarded. Permeabilization was performed in 100 µL 100 mM sodium phosphate buffer containing sodium dodecyl sulfate (SDS) (instead of 5% chloroform) by shaking for 10 min at 800 rpm at 30°C. Because of possible precipitation, we omitted KCl. We also found that Mg^{2+} and β-mercaptoethanol were unnecessary (data not shown). The SDS solution (10%) was purchased from Invitrogen, Cergy Pontoise, France. Other chemicals and o-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma-Aldrich, Saint Quentin Fallavier, France. The reaction was initiated by the addition of 2.5 mM ONPG in 25 µL,

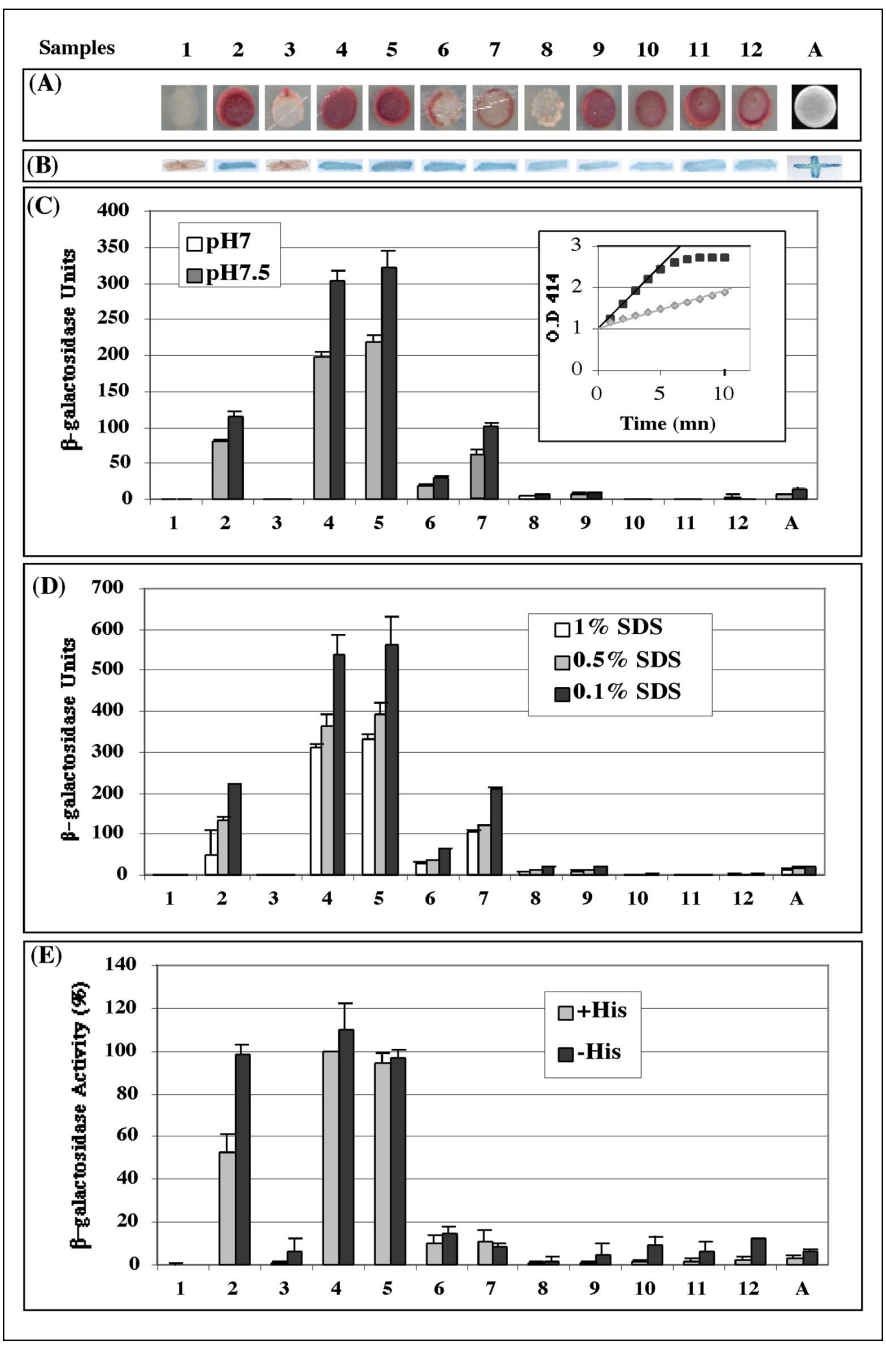

Figure 1. Comparison between qualitative reporter gene expression and our new quantitative β-galactosidase assay. A series of interacting pairs in the LexA-based two-hybrid system (11) (numbered from 2 to 12, 1 being the negative control with one empty vector) were tested under several conditions. Sample A corresponds to Gal4-based two-hybrid system positive control. The β-galactosidase activity is expressed in units (see Table 1). The same samples are used in all panels. (A) Assay of the reporter *HIS3* by growth on solid selective medium lacking histidine (His). (B) Qualitative assay of the reporter gene *LacZ* using X-gal as a substrate. The test was made on filter papers as previously described (5). The blue coloration developed between 1 and 10 h. (C) Comparison of pH 7.0 (gray bars) and pH 7.5 (black bars) for the sodium phosphate buffer in the quantitative β-galactosidase assay. The result of one representative experiment (of five) is shown. All assays were run in triplicate. Insert: direct curves of the kinetics of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis followed at 414 nm. The crude data correspond to sample no. 5, pH 7.5 (black squares), and sample no. 2, pH 7.0 (gray diamonds). (D) Effect of sodium dodecyl sulfate (SDS) concentration used for permeabilization on β-galactosidase activity. The pH of the sodium phosphate buffer was set to 7.5, and concentrations of SDS of 1% (white bars), 0.5% (gray bars), or 0.1% (black bars) were added. Each column in the histogram corresponds to a representative experiment (run in triplicate) from five experiments. (E) Comparison of β-galactosidase activity after growth in selective (-His) or nonselective (+His) medium. The yeast cells were diluted in selective medium (-His, black bars) or nonselective medium (+His, white bars). The optimal assay conditions, sodium phosphate buffer, pH 7.5, and 0.1% SDS, were used. Data were taken from three independent experiments that were normalized to 100% because of slight differences in yeast growth. Results are expressed as the mean ($\bar{x}$), and the error bars represent the standard error of the mean (SEM). The maximal activity corresponds to 745 β-galactosidase units. In the case of the Gal4-based sample A, the maximal activity is 44 β-galactosidase units. The activities for two samples in the LexA/EGY48/pSH18:34 system (12) were 50 and 1463 β-galactosidase units, respectively (data not shown).

and absorbance at 414 nm was recorded for 10 min. The kinetics of ONPG hydrolysis was taken from the linear part of the curve for the determination of β-galactosidase units. The original method (14) was an end point method with an addition of sodium carbonate to stop the reaction and to enhance the yellow coloration of hydrolyzed ONPG. With the modifications to the protocol, the kinetics of ONPG hydrolysis could be directly followed at 414 nm in each well simultaneously. For each well, the linear part of the curve was taken into consideration (see insert to Figure 1C). We checked that the slight turbidity of the reaction medium had no effect on the kinetics and concluded that centrifugation was unnecessary. The key to the method was the improved sensitivity that allowed us to confidently reveal the activity by the quantitative method, even for the weakly positive interacting pairs (see data for sample A, which is representative of the less sensitive Gal4-based two-hybrid system). The figure shows stepwise improvements in sensitivity on the same series of interacting pairs. First, we compared the activities at two standard buffer pH values found in the literature (14,15) and determined that 7.5 was the optimum pH (Figure 1C). Second, at pH 7.5, reducing the concentration of SDS from 1% to 0.1% increased the activity (Figure 1D). Third, at pH 7.5 and with 0.1% SDS, culture in selective medium lacking histidine (His; in systems where *HIS3* is a reporter gene) increased the sensitivity (Figure 1E). Moreover, when the β-galactosidase data are normalized to compensate for growth differences, the reproducibility among three independent experiments is good for samples with high activities as well as for samples with low activities (Figure 1E), with a standard error of less than 10%. Table 1 describes the final protocol.

In conclusion, we have developed a quantitative and highly sensitive two-hybrid test that allows for the simultaneous assay of 96 interactions, even when they are relatively weak. It should be mentioned that quantitative data do not directly reflect affinities because the activation of reporter genes also depends on the level of the expression of the two-hybrid proteins. Our test is inexpensive, compared to commercially available yeast β-galactosidase assay kits, and suited to high-throughput two-hybrid systems currently in use. Moreover, it can be used in high-throughput screening for compounds that affect a specific interaction with minimal manipulation and only one centrifugation step. Another possibility is to use this test to assess the ability of a third partner introduced by transformation or mating procedures (triple hybrid) to enhance or to inhibit a previously characterized two-hybrid interaction.

ACKNOWLEDGMENTS

The authors thank Drs. Rabiet, Willison, and Griffin-Shea for their critical reading of the manuscript.

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Received 28 February 2003; accepted 16 May 2003.

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