Quantitative β-galactosidase assay suitable for high-throughput applications in the yeast two-hybrid system

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Measurement of β-galactosidase (β-gal) activity is an important step in every yeast two-hybrid assay, yet many commonly used methods have distinct disadvantages, such as being only qualitative, time-consuming, and cumbersome when processing large numbers of samples. To overcome these drawbacks, we have implemented a novel technique, termed pellet X-gal assay, that allows simultaneous quantitative measurements from large numbers of samples with a minimum of hands-on time. The method was tested using five different, previously described protein-protein interactions and compared to two standard methods, the colony filter lift and the liquid ONPG assay. Our assay allows accurate quantitative measurements of protein-protein interactions and covers a greater dynamic range than the classic ONPG assay. The novel assay is robust and requires very little handling, making it suitable for applications in which several hundreds of individual protein interaction pairs need to be measured simultaneously.

INTRODUCTION

A major challenge in proteomics today is the identification and characterization of unknown gene products identified in genome sequencing studies (1,2). One powerful strategy aimed at solving this problem is the identification of interacting partners of a given protein using the yeast two-hybrid system (3). In the yeast two-hybrid system, the interaction between two proteins is measured by the reconstitution of a functional transcription factor and the concurrent activation of reporter genes (4). Most yeast two-hybrid systems take advantage of the *Escherichia coli lacZ* gene encoding β-galactosidase (β-gal) as a colorimetric reporter gene, since it is very robust and a variety of qualitative and quantitative assays are available (5). Over the years, a wide range of different assays have been developed to measure β-gal activity both qualitatively and quantitatively. The two most frequently used assays to monitor β-gal activity in the context of two-hybrid studies are based on O-nitrophenyl β-D-galactopyranoside (ONPG) cleavage in a liquid assay (6) and on the evaluation of blue coloration in a colony filter lift assay using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as a substrate (7). Despite their widespread use, both assays have several drawbacks. The colony filter lift assay is a qualitative assay in which the individual coloration of colonies is assessed visually and may differ substantially between colonies, depending on the expression levels of the fusion proteins under investigation and on the efficiency of transfer of the colonies onto the filters (5). In contrast, ONPG-based assays yield a quantitative assessment of β-gal activity (8). A recently described improved method even allows simultaneous processing of large numbers of samples by taking advantage of a microplate reader (9). However, ONPG is a less sensitive substrate than X-gal, and ONPG-based assays often require multiple dilutions of the initial samples to adjust all signals to within the linear range, increasing hands-on time.

More sensitive substrates, such as chlorophenol red-β-D-galactopyranoside (CPRG), chemiluminescent (Tropix® Galacto™-*Star*; Applied Biosystems, Foster City, CA, USA), or fluorescent (resorufin-β-D-gal) substrates are significantly more expensive than either X-gal or ONPG, and consequently, these substrates are not widely used in yeast two-hybrid screens. Commercially available kits, such as the yeast β-gal assay kit marketed by Pierce Biotechnology (Rockford, IL, USA), offer certain advantages, such as efficient lysis of yeast and ease of handling, but they rely on proprietary reagents and are also expensive. In a recent review on the use of β-gal in the yeast two-hybrid system, it was noted that despite the existence of improved methods, 75% of all β-gal assays are still done using either the filter lift or ONPG methods (5).

In designing an improved β-gal assay, it was our aim to achieve quantitative measurements of β-gal levels on large numbers of samples with minimal hands-on time, applying standard laboratory equipment, and avoiding the use of expensive or proprietary reagents. We find the pellet X-gal (PXG) assay to be especially useful in the context of the yeast two-hybrid system, where large numbers of clones have to be quickly screened for β-gal activity.

MATERIALS AND METHODS

Plasmids and Yeast Strains

Bait proteins were expressed as fusions to LexA in the yeast two-hybrid vectors pBTM116 (10) or pLexA-dir (Dualsystems Biotech, Zurich, Switzerland). Preys were expressed as fusions to the activation domain of GAL4 in pACT2 (BD Biosciences Clontech, Palo Alto, CA, USA). The following baits were used: the Bloom helicase (BLM) (amino acids 770–1417 of human bloom helicase), p53 (amino acids 72–390 of human p53), lamin C (amino acids 66–230 of human lamin C), and full-length human DRAL/FHL-2. The following preys were used: large T antigen (amino acids 84–708 of simian virus 40 large T antigen), is2 (amino acids 25391–25874 of human cardiac titin), full-length human myofibrillar creatine kinase (MM-CK), full-length human hMLH1, and truncated human hMLH1 (amino acids 198–756). The *Saccharomyces cerevisiae* strain L40 [*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*] (10) was cotransformed with bait and prey plasmids using the lithium acetate method (11) and selected for histidine prototrophy on minimal medium, containing 2% glucose, 6.7% yeast nitrogen base (BD

Diagnostic Systems, Sparks, MD, USA), complete amino acid mixture lacking histidine, leucine, and tryptophan (Qbiogene, Carlsbad, CA, USA), and 2% bacto agar (BD Biosciences, Franklin Lakes, NJ, USA). Yeast transformants were grown for 3 days at 30°C.

β-Gal Assays

PXG assay. For each interaction pair, several colonies were picked from the selection plates and inoculated into snap-cap tubes containing 5 mL of selective medium. Cultures were grown from an absorbance $(A)_{546} < 0.1$ to an A_{546} of 0.8–1. One absorbance unit of yeast was pelleted by centrifugation at 2000× *g* for 5 min. The supernatant was discarded, and cell lysis was performed by two freeze-thaw cycles (3 min in liquid nitrogen, 3 min in a 37°C water bath). Pellets were subsequently resuspended in 20 µL water, transferred to a transparent flat bottom 96-well microplate, mixed with 100 µL phosphate-buffered saline (PBS) buffer, pH 7.4, containing 500 µg/mL X-gal (Applichem, Darmstadt, Germany), 0.5% (w/v) agarose, and 0.05% (v/v) β-mercaptoethanol and incubated at room temperature. The β-gal enzymatic activity was monitored by recording the color development at different time points using a flatbed scanner. Images were quantified using National Institutes of Health (NIH) Image and results were displayed using Microsoft® Excel®.

ONPG assay. The ONPG assay was essentially performed as described previously (6). One absorbance unit of yeast cells per sample was used, except for p53-large T and hMLH1-BLM, where only 0.05 A was used due to the strong β-gal activity found with these interaction pairs. Cells were pelleted in Eppendorf® tubes and resuspended in 500 µL Z-buffer (6). Ten microliters chloroform and 15 µL 0.2% sodium dodecyl sulfate (SDS) were added, and the tubes were vortex mixed briefly. After a 5-min incubation at 28°C, 100 µL ONPG solution (6) were added. The reaction was stopped after 10 min for p53-large T and after 25 min for all other samples by adding 250 µL chilled 1 M Na_2CO_3. Absorbance was determined at 420 nm. Results were normalized against cell density and incubation time.

Colony filter lift assay (7). Colonies were transferred from selective plates onto Whatman 3 MM paper (Whatman, Kent, UK) and immersed in liquid nitrogen for 5 min. The filters were thawed at room temperature and overlaid with X-gal buffer [PBS buffer, pH 7.4, 0.5% agarose, 0.01% (w/v) X-gal]. The filters were incubated overnight at room temperature. To ensure that filter-lifts from different transformants could be compared directly, in each case, care was taken to transfer equal amounts of yeast from the plate to the filter.

RESULTS AND DISCUSSION

Quantification of Interactions Using the PXG Assay

Initially, the PXG assay was tested

using several well-characterized protein interaction pairs: (*i*) the tumor suppressor p53 and the simian virus 40 large T antigen (12,13); (*ii*) the BLM and either the full-length human mismatch repair protein hMLH1 or a truncated variant of hMLH1 (ΔhMLH1) (14,15); (*iii*) the four-and-a-half LIM-only protein DRAL/FHL-2 and the muscle isoform of creatine kinase (MM-CK) (16); and (*iv*) the interaction between DRAL/FHL-2 and the is2 region of M-band titin (is2) (16). Measurements were carried out on yeast transformants grown in liquid selective media as described in Materials and Methods. Yeast pellets were mixed directly with X-gal substrate in PBS buffer, and color development was recorded using a flatbed scanner.

β-Gal activity was highest for the interaction between the tumor suppressor p53 and large T antigen, with samples developing a strong blue color within 5 min (Figure 1, A and B). This is in accordance with in vitro measurements on p53 and large T antigen [association constant (K_A) of 2×10^8 M^{-1}] (17). As previously observed, BLM and the repair protein hMLH1 also showed strong β-gal activity, whereas the truncated version of hMLH1 (ΔhMLH1) displayed considerably lower β-gal activity. DRAL/FHL-2 and the is2 region of titin and DRAL/FHL-2 and MM-CK were ranked as intermediate and low affinity interactions, respectively. No significant β-gal activity was observed in the negative controls co-expressing large T antigen and lamin C or empty vectors only.

Based on the results of the PXG assay, the strength of interactions can be ranked as follows: p53-large T > BLM-hMLH1 >> DRAL/FHL-2-is2 > BLM-ΔhMLH1 > DRAL/FHL-2-MM-CK (Figure 1B). Thus, the PXG assay is able to detect an entire spectrum of interactions and allows the quantitative assessment of β-gal activity.

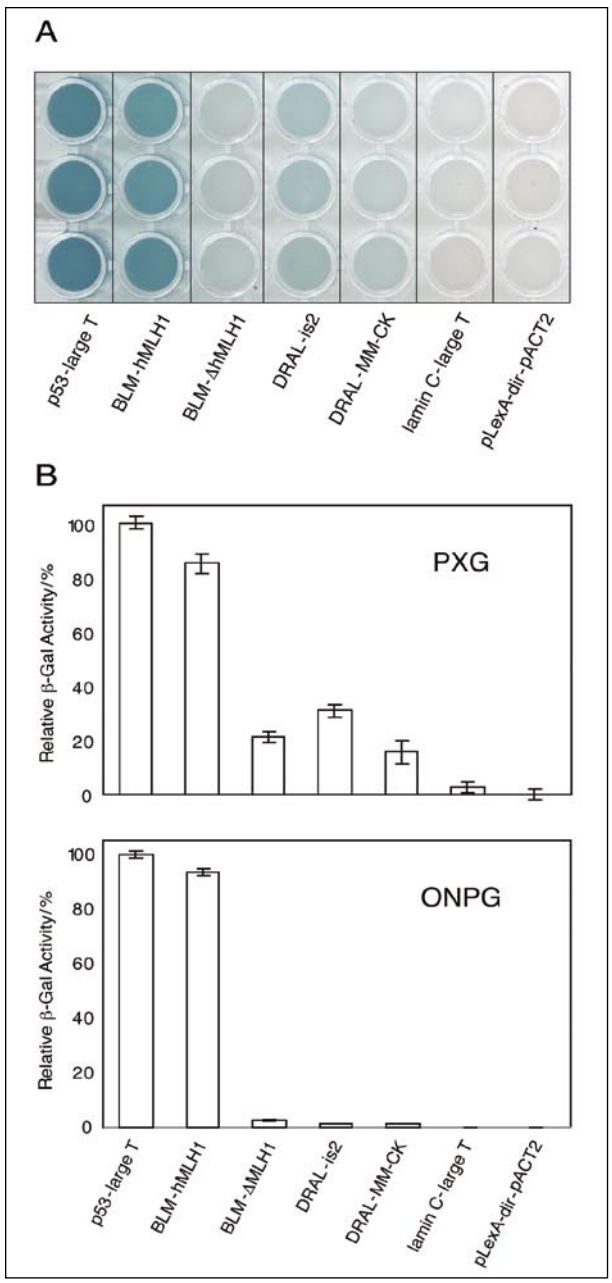

Figure 1. Quantitative β-gal measurements of various protein interaction pairs using the PXG and ONPG assays. (A) Scanned image of a PXG assay in a 96-well microtiter plate after 30 min of incubation. Three independent transformants were assayed per protein interaction pair. (B) Relative β-gal activity in the PXG and ONPG assays. The p53-large T interaction was set arbitrarily to 100% for both assays. Error bars represent the standard deviation from three independent experiments in both assays. Note the increased sensitivity of the PXG assay for weak interactions when compared to the ONPG assay. β-gal, β-galactosidase; PXG, pellet X-gal; ONPG, O-nitrophenyl β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Comparison of the PXG Assay with the ONPG Assay

To ensure that the PXG assay yielded quantitative measurements of inter-

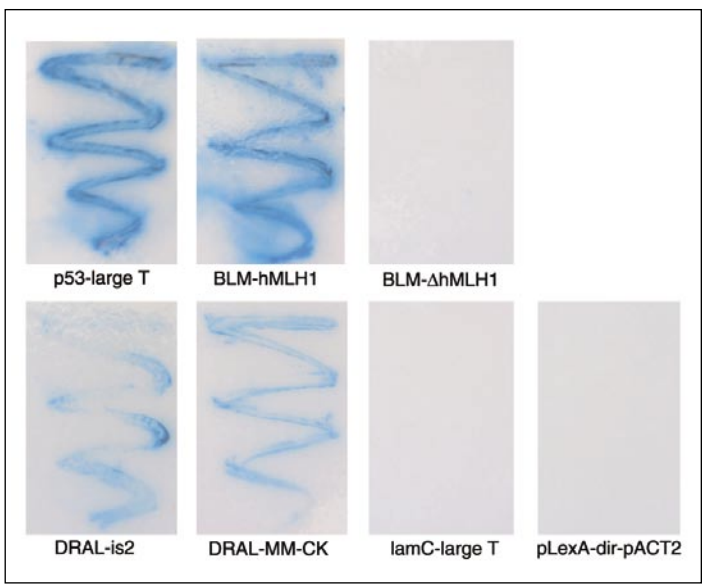

Figure 2. Qualitative β-gal measurements of various protein interaction pairs using the colony filter lift assay. Images of filter lifts were made after overnight incubation. The strength of individual interactions can be roughly estimated and correspond to PXG measurements (see Figure 1B). The interaction between BLM and ΔhMLH1 is too weak to be detected efficiently; only a few faintly blue spots are visible, but the majority of the yeast on the filter remains unstained. BLM, the Bloom helicase; ΔhMLH1, a truncated variant of the full-length human mismatch repair protein hMLH1.

action strengths, it was compared with the most frequently used quantitative β-gal assay, the ONPG assay. To enable us to directly compare the results of the PXG and ONPG assays, the same yeast transformants were used in both assays. The PXG assay and the ONPG assay gave similar results for β-gal activity (Figure 1B). Both assays gave comparable results for the strong interactions between p53 and large T antigen and between BLM and full-length hMLH1. Strikingly, the remaining three interactions were ranked as very weak in the ONPG assay (1.5%–2.3% relative β-gal activity), whereas they were classified as intermediate to weak in the PXG assay (16%–31% relative β-gal activity), in accordance with estimates from the colony filter lift assay (Figure 2). A ranking of the weak interactions in the ONPG assay was difficult, as relative β-gal activities were near the threshold of detection (Figure 1B), probably due to the reduced sensitivity of ONPG as a substrate when compared to X-gal. These results suggest that the PXG assay is more suitable than the ONPG assay for quantifying weak interactions and that it covers a greater range of signal intensities. All interactions could be quantified in the same experiment when using the PXG assay, whereas in the ONPG assay, p53-large T and BLM-hMLH1 had to be diluted several times to adjust the signal to the linear range.

Comparison of the PXG Assay with the Colony Filter Lift Assay

Next, we compared the PXG assay with the colony filter lift assay. Here, yeast colonies are directly transferred from the selection plates to filters, lysed, and then overlaid with X-gal substrate (7). The two strongly interacting pairs, p53-large T and BLM-hMLH1, were easily detectable in the colony filter lift assay, as were the intermediate strength pairs DRAL-is2 and DRAL-MM-CK (Figure 2). The weak interaction between BLM and ΔhMLH1 was barely detectable, even after overnight incubation. Only a few spots of faint blue color were visible on the filter (Figure 2, BLM-ΔhMLH1). In contrast, both the PXG assay and the ONPG assay allowed detection of the BLM-ΔhMLH1 interaction and even ranked it as slightly stronger than the interaction between DRAL and MM-CK. The failure of the filter lift assay to detect the interaction between BLM and ΔhMLH1 was reproducible in several independent experiments (data not shown). It highlights a drawback of the colony filter assay, since it is often difficult to reproduce handling steps such as the transfer of yeast from the plate onto the filter and the efficiency of lysis of

the transferred yeast colonies (8). In contrast, the β-gal activity measured in the PXG assay is a mean value of approximately 10^7 cells, and therefore, values are more homogeneous and reproducible than in the colony filter lift assay. It has also been noted that certain bait/prey combinations exhibit differences in toxicity depending on whether they are grown on solid or liquid medium (5,18). This may explain why the interaction between BLM and ΔhMLH1 is detectable in the PXG and ONPG assays, which use yeast grown in liquid medium, whereas it is not detectable in the filter assay, which uses yeast grown on solid medium.

When compared to the colony filter lift, the PXG assay was also capable of discriminating more accurately between protein pairs with different affinities. Visual inspection of the PXG plate shows the DRAL-is2 interaction to be stronger than the DRAL-MM-CK interaction, whereas this distinction cannot be made based on the results of the filter lift assay (compare Figures 1 and 2).

In summary, the PXG assay allows quantitative measurements of protein-protein interactions with very little hands-on time, especially when dealing with a large number of samples that need to be processed in parallel. In contrast, the processing of large numbers of samples is challenging when using substrates such as ONPG or CPRG, in which measurements have to be taken in a spectrophotometer and inaccuracies in handling pose a consistent problem. A recently described assay by Serebriiskii and coworkers offers another solution by using a plate reader to accurately measure large numbers of interactions simultaneously with high accuracy (9).

We believe that the PXG assay has several advantages over β-gal assays described to date: (*i*) it allows for both a quick assessment of the relative strength of different interactions by simple visual inspection and the accurate quantification over a wide range of interaction strengths; (*ii*) it takes advantage of equipment available in every laboratory; and (*iii*) it involves very few handling steps, which is an advantage when simultaneously assaying many different interactions in the context of a yeast two-hybrid screen.

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