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Research Article

A *Saccharomyces cerevisiae* cell-based quantitative β-galactosidase assay compatible with robotic handling and high-throughput screening

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Abstract

Reporter-gene assays that employ the *Escherichia coli lacZ* gene are ubiquitously employed in biological research. However, we were not able to readily identify a quantitative method that worked reliably with yeast (*Saccharomyces cerevisiae*) cells and that was compatible with high-throughput screening and robotic liquid handling tools. We have therefore adapted a commercially available assay employing a 6-*O*-β-galactopyranosyl–luciferin substrate to provide the required sensitivity with minimal sample handling times. Our assay uses only one-tenth of the reagents suggested by the reagent manufacturer (Promega) for equivalent assays with mammalian cell cultures and produces rapid, sensitive and reproducible analysis with as little as 1 μl yeast cell culture and with <100 cells. We demonstrate that the assay is compatible with yeast strains generated by the systematic yeast deletion project and functions equally well with genomically integrated or plasmid-encoded *lacZ* reporters and with cells grown in complex or defined media. The high-sensitivity, miniaturized format reduced sample handling required will make this assay useful for a wide range of applications.

Keywords: **β-galactosidase; high-throughput screening; *lacZ* reporter assay luciferase**

Introduction

Reporter assays that employ the *Escherichia coli lacZ* gene are ubiquitous in research. However, for a variety reasons, including the relative insensitivity of traditional substrates and interference with colorimetric detection, other reporters (e.g. luciferase or green fluorescent protein) have become increasingly popular tools (Bovee *et al*., 2004). Luminescent or fluorescent β-galactosidase substrates are now available and offer a route to increase the sensitivity of detection and range of applications for monitoring gene expression with a range of strains containing *lacZ* fusions (Oender *et al*., 2006; Serebriiskii and Golemis, 2000). At the same time, advances in microplate technology have allowed both assay miniaturization and the possibility to save time, reagents and increase dramatically the number of samples analysed (Berg *et al*., 2000; Brouchon-Macari *et al*., 2003). We were interested in using a reporter assay that would be compatible with the robotically controlled liquid-handling systems used in high-throughput compound library screens, and which therefore required minimal sample handling and no centrifugation steps. After assessing previously developed methods, we were able to make improvements that have resulted in a reproducible, cost- and time-efficient assay that is suitable for high-throughput analyses. Our optimized procedure uses as little as 1 μl

cell culture and can detect light from <100 cells. Reagent volumes/costs are minimized and a reproducible output signal is produced that is stable for at least 8 h.

Materials and methods

Yeast strains and plasmids

Strain genotypes are given in Table 1. GP4213 and the isogenic GP4198 were described previously (Richardson *et al*., 2004). The *HIS4–lacZ* fusion in these strains is a fusion of the *HIS4* promoter region (−670 from the mRNA start site to +34 within the coding region), so that the 10th codon of *HIS4* is fused in-frame to the 8th codon of *lacZ*, and this construct was integrated at *ura3-52* by homologous recombination, as described previously (Lucchini *et al*., 1984). BY4743 (Brachmann *et al*., 1998) was obtained from EUROSCARF and was transformed with p180 [*GCN4–lacZ CEN4 ARS1 URA3*] (Hinnebusch, 1985), using a modified lithium acetate method (Gietz and Woods, 2006). The *GCN4–lacZ* fusion plasmid contains the *GCN4* promoter, 591 nucleotide mRNA leader and the N-terminal 55 codons of *GCN4* fused in-frame to *lacZ*.

Assay protocol

Cell growth

Yeast cells were grown either in flasks using standard techniques and then aliquoted into multiwell plates for assay, or grown directly in 96- (200 µl) or 384-well (50 µl) clear flat-bottomed assay plates sealed with a breathable membrane (BreatheEZ membrane, Sigma). Assay plates were typically incubated at 30 °C without shaking, and were not stacked directly to enable air circulation space. The assay is compatible with cells grown in either complex yeast extract peptone dextrose- (YPD) or synthetic dextrose (SD)-based growth media (Adams *et al*., 1998). For large-scale screening projects where a single yeast strain was used, it was possible to generate a series of frozen aliquots (15% v/v glycerol, −80 °C) of the starting cells, so that all cell growth times were optimized and reproducible. When large numbers of individual strains were analysed, the method developed by Gietz and Woods to inoculate cells into microplates efficiently was used (Gietz and Woods, 2006).

Absorbance monitoring

Quantitative β-galactosidase assays are usually normalized to either total cell protein or cellular absorbance value (Adams *et al*., 1998; Serebriiskii and Golemis, 2000). Here absorbance at 595 nm was used (A_{595}). Following growth, cells were resuspended using a microplate shaker (1000 r.p.m., manual handling) or by repeated pipetting (robotic handling), and absorbance was measured at A_{595}. As absorbance readers saturate at higher absorbance values when measuring particulate suspensions, such as yeast cells, a correction was applied that must be determined empirically for each microplate reader used. Medium blank-corrected absorbance readings (A_{obs}) of serial dilutions of yeast cells were plotted against expected values and a third-order polynomial curve was fitted to the data ($A_{cor} = A_{obs} + x(A_{obs})^2 + y(A_{obs})^3$, where x and y are constants), as described previously (Warringer and Blomberg, 2003).

Light measurement

Light measurement used the Beta-Glo assay reagents (Promega). Reagents were mixed, aliquoted and stored frozen at −80 °C. Mixed reagent was brought to room temperature prior to use. Cell culture (1–5 µl) was transferred to white 96- or 384-well plates containing Z-buffer [82 mM Na_2HPO_4, 9 mM NaH_2PO_4, pH 7.4, 0.1% SDS,

Table 1. Yeast strain genotypes

Name	Genotype	Reference
BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*	Brachmann *et al.* (1998)
GP4198[a]	*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6Δ gcn2Δ::hisG* pAV1744[*gcd6-R284H CEN6 LEU2*]	Richardson *et al.* (2004)
GP4213	*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6Δ gcn2Δ::hisG* pAV1265[*GCD6 CEN6 LEU2*]	Richardson *et al.* (2004)

[a] GP4198 and GP4213 are isogenic strains.

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5 mM DTT, 1× protease inhibitor–EDTA cocktail (Roche)]. Final cell + Z buffer volumes varied in the range 10–50 µl. Plate contents were mixed either by repeated pipetting (robotic handling) or were covered with plate seal and mixed for 1 min at full speed, using a microplate shaker (manual handling), then incubated for 20 min at room temperature (21°C). As a positive control, purified β-galactosidase [Sigma G5635, 1×10^{-12} g in (25% v/v glycerol, 100 mg/ml BSA +0.1 M Na-phosphate, pH 7.4, stored at −20 °C)] was mixed with Z buffer. An equal volume (10–50 µl) premixed Beta-Glo firefly luciferase substrate (Promega) was added to each well and mixed, as described above. Reactions were incubated at room temperature and read in a luminescence plate reader. For single plate assays, multiple readings were taken over an extended time course (Figure 1). For multi-plate assays, a single read time was used, once the light had reached maximum (60–120 min). Relative light units (RLUs) were calculated: $RLU = [(\text{sample light units} - \text{blank light units})/A_{\text{cor}}]$.

Microplate readers

A Hidex Chameleon plate reader was used for absorbance and luminescence assays performed with manual pipetting. A Perkin-Elmer Envision 2102 multilabel reader was used for robotic automated plate-handling experiments.

Colorimetric β-galactosidase assay

For comparison, some assays were performed using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) and cell extracts using standard methods described previously (Adams *et al*., 1998; Dever, 1997).

Results and discussion

To develop a multi-well plate assay for quantitative assessment of β-galactosidase activity that could be used manually, with multichannel pipettes, or robotically, with automated plate filling and pipetting equipment, we first evaluated a method that used the standard chromogenic substrate Xgal, which had been developed and used previously (Brouchon-Macari *et al*., 2003). However, in our hands this method required centrifugation of multi-well plates and aspiration of culture media prior to cell lysis, and then centrifugation to remove cell debris prior to reading absorbance values. These steps introduced unacceptable variations in replicate samples (data not shown) and were not compatible with high-throughput studies. We therefore assessed the use of a commercially-available β-galactosidase substrate that, when cleaved, releases D-luciferin as a firefly luciferase substrate. Similar assays are marketed by Applied Biosystems (Galacto-Lightplus) and Promega (Beta-Glo). The Beta-Glo assay was developed for use with mammalian cell cultures, but has been used with yeast (Hook *et al*., 2005). This assay uses a single reagent combining a modified β-galactosidase substrate (6-*O*-β-galactopyranosyl–luciferin) that is cleaved to release D-luciferin. D-luciferin with

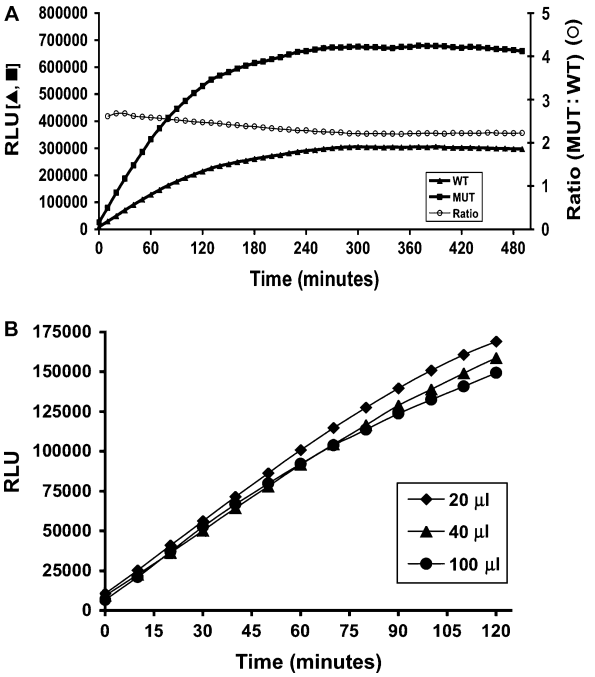

Figure 1. Time courses of modified Beta-Glo assays measuring *HIS4–lacZ* activity. (A). Extended time course comparison of isogenic wild-type (▲; GP4213) and mutant (■; GP4198) activity with data recorded every 10 min over 8 h. Relative light units (RLU) calculation is described in Materials and methods. The ratio (○) remains constant over the entire course. (B) Reducing the assay volume has no negative impact on the initial kinetics of the reaction. Assays done with 1 µl cell culture ($n = 12$), CV ≤ 10% for each reading after 10 min incubation

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ATP is the substrate for firefly luciferase (Hannah *et al*., 2003). When used as recommended by the manufacturer, this kit was highly sensitive but did not generate a stable or reproducible light-output signal with our combination of cells and *lacZ* reporters (data not shown). Rather than abandoning the assay, we experimented with a range of parameters and have now developed a robust assay.

As yeast cell lysis releases cellular proteases, we hypothesized that protease activity could be interfering with the assay and that including protease inhibitors might enhance and stabilize the light output. In addition, to improve cell permeabilization/lysis, we diluted our cell cultures in a neutral buffer containing SDS. The resulting assay protocol (see Materials and methods) is extremely simple, sensitive and reproducible and gives a light output that is stable for >8 h (see e.g. Figure 1A). Briefly, cell culture (1–5 µl) is mixed with a modified Z-buffer containing SDS for a short time to promote cell lysis/permeabilization, and then as little as 10 µl Beta-Glo reagent is added and light output recorded in a microplate reading luminometer. As such small culture volumes are required for each assay, we have found that multiple readings can be taken from a single micro-culture grown in either 96- or 384-well format plates. To normalize light units to cell culture density, we used a previously developed method to compensate for saturation of absorbance readings read in microplate readers (see Materials and methods).

To evaluate our assay and compare it to the traditional chromogenic substrate (Xgal assays), we compared the activity of a wild-type and an isogenic missense mutation in a gene (*GCD6*) that indirectly increases *HIS4–lacZ* reporter gene activity by derepressing a transcription factor important for *HIS4* expression (Pavitt, 2005). We found that the expression level in the mutant was consistently 2–2.3-fold greater than wild-type expression at all time points (Figure 1A, labelled Mut). This compared well with data previously obtained with an Xgal substrate (Table 2, rows 3–4; Richardson

Table 2. Summary comparison of β-galactosidase data

				Beta-Glo assays[d]						Xgal assays		
				Vol in rxn (µl)			Units					
	lacZ fusion[a]	Strain[b]	Growth medium[c]	Cell cult	Z buff	Beta-Glo	RLU	%CV	*n*	Units	%CV	*n*
	Robotic assays[e]											
1	*HIS4*	GP4213	YPD	5	20	25	4.6×10^7	10	16			
2	*HIS4*	GP4198	YPD	5	20	25	9.2×10^7	9	16			
	Manual assays[e]											
3	*HIS4*	GP4213	YPD	1	19	20	1.29×10^5	9	12	271[f]	2	3
4	*HIS4*	GP4198	YPD	1	19	20	3.33×10^5	16	12	575[f]	3	3
5	*HIS4*	GP4213	YPD	1	49	50	1.18×10^5	10	12			
6	*HIS4*	GP4213	YPD	1	19	20	1.22×10^5	6	12			
7	*HIS4*	GP4213	YPD	1	9	10	1.33×10^5	4	12			
8	*HIS4*	GP4213	YPD	2	8	10	2.74×10^5	10	12			
9	*HIS4*	GP4213	YPD	2	18	20	2.41×10^5	6	12			
10	*HIS4*	GP4213	YPD	3	17	20	3.02×10^5	6	12			
11	*HIS4*	GP4213	YPD	5	15	20	4.12×10^5	6	12			
12	*GCN4*	BY4743	SD	3	17	20	1.27×10^5	16	20	53	10	6
13	*GCN4*	BY4743	SD+5MT	3	17	20	2.45×10^5	16	20	134	12	6

[a] Gene to which β-galactosidase activity relates (*HIS4* fusion integrated at *URA3; GCN4* fusion carried on centromeric plasmid).
[b] GP4213 and BY4743 are wild-type haploid and diploid strains, respectively; GP4198 is isogenic to GP4213 and contains a mutant *gcd6* allele that indirectly activates *HIS4* transcription.
[c] 2 mM 5MT added for 6 h prior to cell harvest where indicated.
[d] The volume of reagent combinations used in each assay and the relative light units (RLU) determined as described in Materials and methods, with the coefficient of variation (%CV) and number of replicates (*n*).
[e] Robotic assays used fully automated liquid and plate handling and 384-well format plates. Manual assays used multichannel pipettes and 96- or 384-well format plates.
[f] Derived from previously published data (Richardson *et al*., 2004).

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et al., 2004). We also found that reducing the total assay volume to as little as 20 µl (1 µl cell culture +9 µl buffer +10 µl Beta-Glo reagent) had no negative impact on the reaction kinetics (Figure 1B; Table 2, rows 5–7), thus making a considerable cost saving in reagents. Importantly, including different volumes of cell culture in the assays showed that the assays were proportional to the number of cells assayed (Table 2, rows 7–11).

To assess the sensitivity of the assay, eleven 200 µl microcultures were each five-fold serially diluted seven times and 2 µl of each was assayed in parallel. In addition, 2 µl of each dilution was spotted onto YPD medium to determine the mean cell numbers present in each assay. Figure 2 shows that the light output was proportional over at least three orders of magnitude, and that <100 cells generate a signal above background with our plate reader. These studies demonstrate that our assay is highly sensitive.

A variation of this method was developed to use in high-throughput screening of chemical compounds, using automated liquid plate-fillers, pipetting equipment, etc., and a robotic plate carrying arm to transport 384-well assay plates between equipment. The method developed worked well (Table 2, rows 1–2) and has been used to screen a chemical library. The results can be found on the Pubchem BioAssay database (**http://pubchem.ncbi.nlm.nih.gov/**) under Assay Identification No. 688.

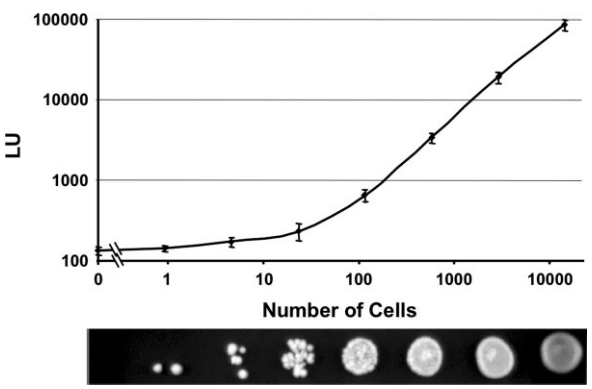

Figure 2. Assay is proportional between 100 and 10 000 cells. Upper panel shows a double-log scale plot of light units (LU) recorded against mean number of cells assayed from five-fold serially diluted cultures. Error bars show SD ($n = 11$). Mean cell numbers were calculated from spots of the serially diluted cultures grown on solid YPD medium. One set only is shown in the lower panel

Finally, we also show that our methods are applicable for use with the 'BY' strains used by the systematic gene deletion project (Winzeler *et al*., 1999) and plasmid-encoded *lacZ* fusions. We transformed the diploid BY4743 with the *URA3* plasmid p180, which encodes an in-frame fusion with codon 53 of the *GCN4* gene (Hinnebusch, 1985). *GCN4* expression is regulated by amino acid availability. Assays were performed on cells grown in 96-well plates containing minimal supplemented SD medium, and to one half of the cultures 5-methyl tryptophan (5MT), an inhibitor of tryptophan synthesis, was added after 2 h and growth continued for 6 h. In parallel, and for direct comparison, assays using Xgal were performed as described (Dever, 1997). As shown in Table 2 (rows 12–13), both the traditional Xgal and Beta-Glo assays gave equivalent results in terms of relative units; however, the Beta-Glo assays require considerably less time to complete, as no cell extracts are prepared.

In conclusion, the modifications we have introduced to a commercially available β-galactosidase assay should make this assay widely applicable in many high-throughput and functional analysis projects, including two-hybrid applications. The reductions in both sample and reagent volumes, and in sample-handling time needed to complete the assays, will reduce significantly the costs and increase screening capacity when undertaking large-scale transcriptional analyses or two-hybrid screening projects. Alternatively, this assay can be used manually to allow a higher number of sample replicates to be performed for smaller-scale experiments more easily than for other published protocols.

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