

# $\beta$ -Galactosidase activity assay using far-red-shifted fluorescent substrate DDAOG

Haibiao Gong<sup>1\*</sup>, Bin Zhang<sup>2</sup>, Garrick Little<sup>1</sup>, Joy Kovar<sup>1</sup>, Huaxian Chen<sup>1</sup>, Wen Xie<sup>2</sup>, Amy Schutz-Geschwender<sup>1</sup>, and D. Michael Olive<sup>1</sup>

<sup>1</sup> LI-COR<sup>®</sup> Biosciences, Lincoln, NE 68504. <sup>2</sup> Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15261, USA

\*Correspondence: LI-COR Biosciences,  
Lincoln, NE 68504, USA;  
Tel: 402-467-0682;  
Fax: 402-467-0825;  
Email: herbert.gong@licor.com

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## ABSTRACT

$\beta$ -galactosidase ( $\beta$ -gal) is commonly used as a reporter gene in biological research, and a wide variety of substrates have been developed to assay its activity. One substrate, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)  $\beta$ -D-galactopyranoside (DDAOG) can be cleaved by  $\beta$ -gal to produce 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO). On excitation, DDAO generates a far-red shifted fluorescent signal. Using this substrate, we developed a  $\beta$ -gal activity assay method. The DDAO signal was stable for at least 18 h. The signal intensity was linearly related to both the enzyme amount and substrate concentration. An optimized buffer for  $\beta$ -gal/DDAOG assay was also formulated. When compared with the colorimetric substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), the signal-to-background ratio of the DDAOG method was approximately 12-fold higher. The  $\beta$ -gal/DDAOG assay method was also tested in transiently transfected cells employing both a pharmacologically and genetically inducible gene expression systems. The ability to detect signal induction is comparable to a similar assay using luciferase as the signal generating moiety. The  $\beta$ -gal/DDAOG assay method should provide a fluorescent reporter assay system for the wide variety of  $\beta$ -gal systems currently in use.

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*Fluorescence*  
*DDAOG*  
*Reporter gene*

## INTRODUCTION

$\beta$ -galactosidase ( $\beta$ -gal<sup>1</sup>, EC 3.2.1.23), the product of the *lacZ* gene of *Escherichia coli*, is the most commonly used reporter gene applied to the study of genetics, cell and molecular biology [1]. The applications of the  $\beta$ -gal reporter gene include measuring promoter activity in transiently or stably transfected cells [2, 3], determining protein interaction and translocation using  $\beta$ -gal enzyme fragment complementation [4], and monitoring tumor growth in animal models [5, 6].

The  $\beta$ -gal enzyme is composed of 1024 amino acids. In *E. coli*, the biologically active enzyme exists as a tetramer of four identical subunits [7, 8].  $\beta$ -gal catalyzes the hydrolysis of  $\beta$ -galactosides at an optimal pH of 7.2. The natural reaction catalyzed by  $\beta$ -gal is to hydrolyze the disaccharide lactose to galactose and glucose. Although it has fairly strict specificity at the galactosyl position, it is adept at hydrolyzing  $\beta$ -D-galactopyranosides with a wide variety of aglycons with divergent chemical composition. As a result, the  $\beta$ -gal enzyme can recognize and hydrolyze a wide range of substrates. These include the colorimetric substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) and chlorophenol red  $\beta$ -D-galactopyranoside (CPRG) for cell based assays [5, 9], and 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal) for histological analysis. More sensitive  $\beta$ -gal assay methods based on the fluorogenic substrate fluorescein di- $\beta$ -D-galactopyranoside (FDG) [10], or the chemiluminometric substrate Galacton [11] have also been developed.

In the current study, we developed a sensitive  $\beta$ -gal activity assay method utilizing a fluorescent substrate. This  $\beta$ -gal substrate, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)  $\beta$ -D-galactopyranoside (DDAOG), can be cleaved by  $\beta$ -gal enzyme to produce 7-hydroxy-9H(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO). The excitation and emission maxima of DDAO are 646 and 659 nm, respectively, and are shifted dramatically from those of DDAOG (excitation/emission = 465/608 nm) [6, 12]. The DDAO fluorescent signal can be detected by imaging in a spectral region that yields high signal-to-noise ratio due to the lower autofluorescent background.

Although previous attempts have reported the use of DDAOG as a substrate to assay  $\beta$ -gal activity in mouse tissues [6, 12], no work has been done to systemically study this method in cell culture systems. To address this issue, we analyzed in detail the factors that affect the signal intensity when DDAOG was employed as a substrate to assay  $\beta$ -gal activity in cultured cells. In addition, we compared the sensitivity of this method with the most widely used ONPG method.

The advantages of this method include the following: i) the assay has higher sensitivity compared to conventional  $\beta$ -gal activity assays using ONPG as substrate; ii) the product DDAO is highly stable, offering a wider signal detection window; and iii) the assay method is simple and efficient. The cell lysis and  $\beta$ -gal activity assay can be performed in a single buffer optimized for high sensitivity.

## MATERIALS AND METHODS

### Chemicals

DDAO and DDAOG were purchased from Invitrogen (Carlsbad, CA, USA) and dissolved in dimethyl sulfoxide (DMSO) as stock solution. ONPG, phorbol 12-myristate 13-acetate (TPA), and purified  $\beta$ -gal enzyme were purchased from Sigma (St. Louis, MO, USA). ONPG and TPA were dissolved in DMSO.  $\beta$ -gal enzyme was reconstituted in 50 mM Tris-HCl (pH7.3) supplemented with 1 mM MgCl<sub>2</sub>.

### Cell culture

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (complete DMEM). For  $\beta$ -gal activity assay in 9L/lacZ cells, except as otherwise noted, cells were seeded at  $1.5 \times 10^4$  cells per well in 96-well plates and cultured overnight before cell lysis and  $\beta$ -gal activity assay.

### $\beta$ -gal activity assay using DDAOG as substrate

Cell lysis and  $\beta$ -gal activity assay were performed in the same buffer. We used the M-PER mammalian cell protein extraction reagent from Pierce (Rockford, IL, USA) for our initial experiments. After experimental determination of optimal buffer conditions for the DDAO signal intensity, cell lysis efficiency, and  $\beta$ -gal enzymatic activity, we formulated a buffer for cell lysis and  $\beta$ -gal reaction using DDAOG as substrate. The buffer consisted of 25 mM bicine supplemented with 0.25% Triton X-100<sup>®</sup>, 20 mM NaCl and 1 mM MgCl<sub>2</sub>. DDAOG was diluted in the lysis buffer to a final concentration of 10  $\mu$ M except as otherwise noted.

Cells cultured in 96-well plate were rinsed with phosphate-buffered saline (PBS, pH 7.4) once before adding 50  $\mu$ l of lysis and enzyme reaction buffer with DDAOG to each well. After shaking at room temperature for 5 min, the plate was incubated at 37 °C for 30 min. The plate was then scanned on an Odyssey<sup>®</sup> Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) using the 700 nm-channel (excitation/emission at 685/705 nm) to measure DDAO fluorescence signal. For those experiments that required stop solution, the stop solution Na<sub>2</sub>CO<sub>3</sub> was added to a final concentration of 0.33 M.

### Plasmid constructs

pCMV $\beta$  and p $\beta$ gal-control vector were obtained from Clontech (Mountain View, CA, USA). AP1-gal construct was created by inserting 10 copies of synthetic AP1 binding consensus sequence [13] into the multiple cloning site of p $\beta$ gal-control vector. The structure of the AP1-gal construct was confirmed by sequencing. pFR- $\beta$ gal, pFR-Luc, pFA2-cJun, pFC2-dbd and pFC-MEKK plasmids were purchased from Stratagene (La Jolla, CA, USA).

### Cell transient transfection

The transfection procedure was modified from the previously described method [14]. In brief, HEK293 cells were

seeded in 96-well plate at a density of  $1.5 \times 10^4$  per well in complete DMEM medium. After overnight culture, cells were transfected with plasmids in serum-free DMEM using a Lipofectamine 2000-mediated method (Invitrogen). After 4 h of incubation, the transfection mixture was replaced with complete DMEM medium and cells were cultured for another 24 h before luciferase or β-gal activity assay. When activation of AP1 was necessary, cells were treated with TPA for 24 h before luciferase or β-gal activity assay. The luciferase activity was determined using luciferin as substrate. The signal produced by luciferin reaction was measured on a Wallac Victor<sup>2</sup> plate reader (PerkinElmer, Waltham, MA, USA).

### Determination of DDAO spectrum

The spectrum of DDAO and absorbance at specific wavelengths were determined using an Agilent 8453 Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

## RESULTS

### The signal intensity produced by DDAO cleavage is dependent on the substrate concentration and β-gal enzyme amount

To determine the optimal DDAO concentration for β-gal activity assay in cell culture, we measured the signal intensity after incubation of different concentrations of DDAO with 9L/lacZ, a cell line constitutively expressing β-gal enzyme [6]. We noticed that incubation of DDAO with 9L/lacZ whole cells for 30 min produced a very weak signal, possibly due to the limited access of DDAO to the intracellular β-gal enzyme (Fig. 1A). Signal intensity increased with the increase of incubation time (data not shown). However, release of β-gal enzyme by lysing the cells enhanced the signal dramatically (Fig. 1A). In the range of 1 to 20 μM final concentration, a linear relation was observed between the DDAO cleavage signal and DDAO concentration, with an  $R^2 = 0.9984$  (Fig. 1A). We selected a concentration of 10 μM for subsequent studies.

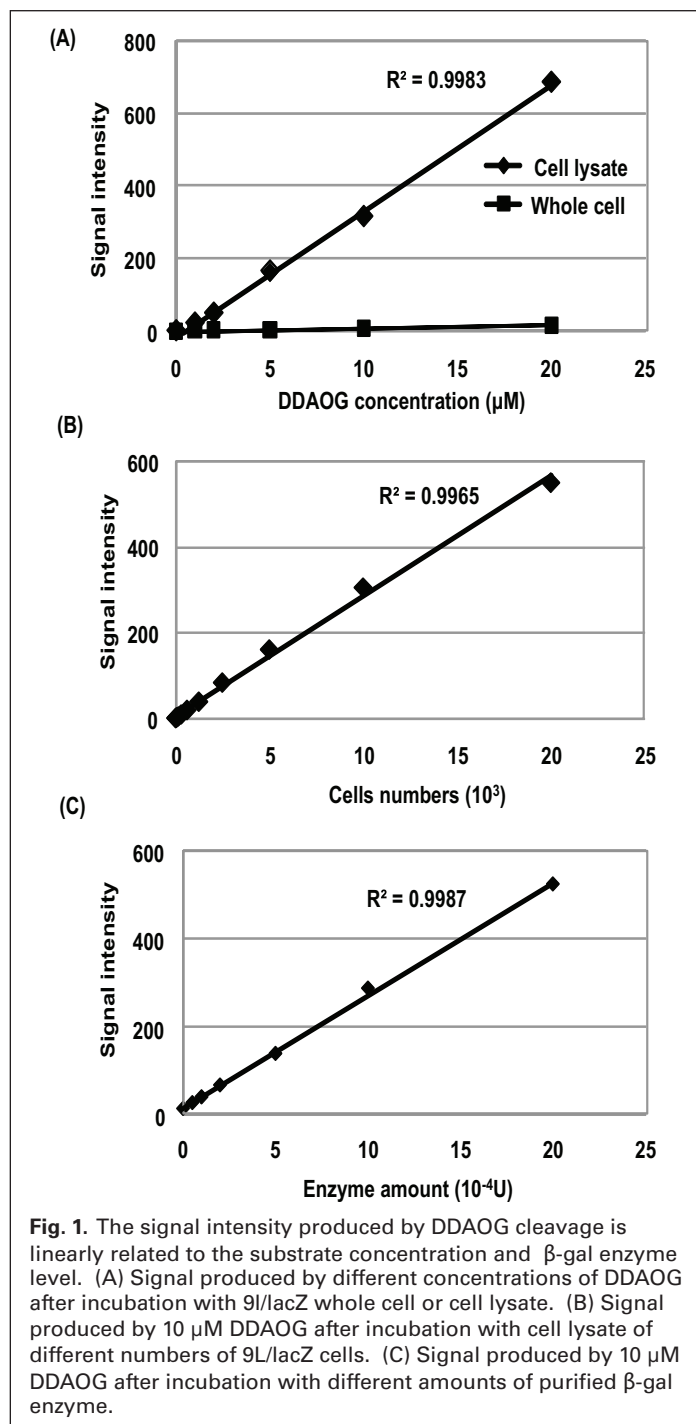
To determine the relationship of β-gal enzyme to the number of cells, different numbers of 9L/lacZ cells were seeded in a 96-well plate. After overnight culture, 10 μM DDAO was added to measure the β-gal activity produced by these 9L/lacZ cells. Fig. 1B shows clearly that the signal intensity of DDAO cleavage increased linearly with the increase in cell numbers. Although the cell numbers shown here were counted when plating, and the actual numbers at the time of assay are unknown, it is likely that the cell number at assay time is proportional to the cell numbers at the seeding time in the range shown in Fig. 1B.

That linear correlation between DDAO cleavage and enzyme concentration was confirmed using the purified β-gal enzyme assay. The signal intensity was proportional to the amount of β-gal enzyme added (Fig. 1C). The correlation is linear over the range of enzyme used (Fig. 1C).

### The DDAO signal is stable

The stability of DDAO signal was determined by incubating DDAO in assay buffer for various time periods at 37°C. The signal intensity of DDAO was measured with the Odyssey instrument after incubation. There was a slight decrease after 30 min of incubation (Fig. 2A). Although the exact reason for the initial decrease is unknown, it may be associated with DDAO molecule aggregation or pH change, as discussed below. No significant change was observed after a longer time of incubation (Fig. 2A).

The high stability of the DDAO signal suggested that the signal produced by DDAO cleavage is also stable. The signal intensity after incubation of DDAO with 9L/lacZ



**Fig. 1.** The signal intensity produced by DDAO cleavage is linearly related to the substrate concentration and β-gal enzyme level. (A) Signal produced by different concentrations of DDAO after incubation with 9L/lacZ whole cell or cell lysate. (B) Signal produced by 10 μM DDAO after incubation with cell lysate of different numbers of 9L/lacZ cells. (C) Signal produced by 10 μM DDAO after incubation with different amounts of purified β-gal enzyme.

lysate was monitored over time. Due to the continuous accumulation of DDAO generated by  $\beta$ -gal action, the signal intensity increased when the mixture was incubated for a longer time (Fig. 2B). However, when the  $\beta$ -gal catalyzed enzymatic reaction was terminated by a stop buffer ( $\text{Na}_2\text{CO}_3$ , final concentration 0.33 M), the signal was stabilized. No significant change was observed during the incubation time period (Fig. 2B).

#### **The DDAO signal is dependent on pH and detergent concentration in the buffer**

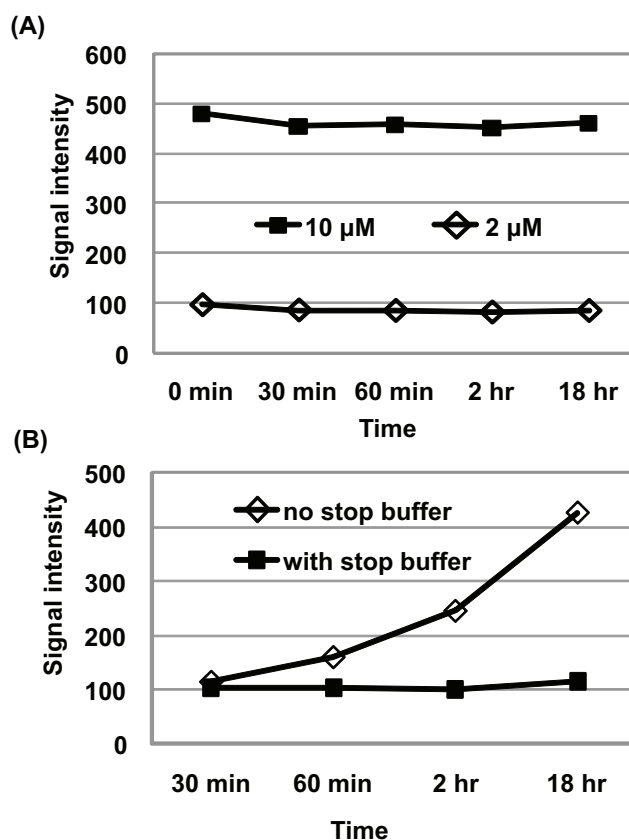
Because the  $\beta$ -gal enzymatic activity is reflected by the DDAO signal intensity, it was of interest to investigate the factors that affect DDAO signal. First, we examined the effect of buffer pH on DDAO signal. The absorbance and excitation signal of DDAO solution with different pH values was measured. Pure water was used as the solvent for DDAO dilution to exclude the effect of buffer component. The pH of 10  $\mu\text{M}$  DDAO in water was approximately 5.8. The absorbance and emission signal reached a maximum at pH 7.6 (Fig. 3).

Our preliminary data indicated that detergent could enhance the DDAO signal in the buffer. We investigated the detergent effect in detail using bicine buffer (25 mM bicine containing 1 mM  $\text{MgCl}_2$ , 20 mM NaCl, pH 7.8) as the basic buffer. Triton X-100 was used as detergent and was adjusted to different concentrations. DDAO absorbance and excitation signal in bicine buffer without Triton X-100 is comparable to that in water at pH 7.8 (data not shown). The absorbance and excitation signal increased with the increase of detergent concentration, reaching a maximum level when 0.25% of Triton X-100 was used (Fig. 4). The peak of DDAO absorbance was shifted slightly to a higher wavelength (from 646 to 651 nm) when detergent was added (data not shown). The addition of Triton X-100 to 0.25% increased the emission signal by 4.3-fold (from 89 to 471).

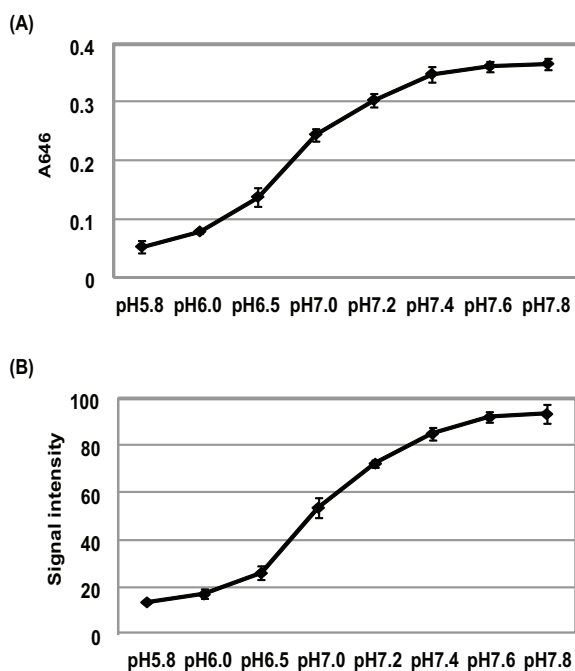
We also investigated the detergent effect on the signal intensity of DDAOG cleavage after incubation with 9L/lacZ cells. In this case, the detergent exerts multiple effects on cell lysis,  $\beta$ -gal enzymatic activity, and signal intensity of the product DDAO. As expected, the signal increased in the presence of higher concentrations of Triton X-100. The highest level was achieved when 0.275% Triton X-100 was used (Fig. 4C). The signal decreased sharply with further increases in the concentration of Triton X-100, possibly due to the detrimental effect of detergent on  $\beta$ -gal enzymatic activity (Fig. 4C). Based on these results, we selected 0.25% Triton X-100 for the lysis and  $\beta$ -gal reaction buffer.

#### **The signal-to-background ratio of DDAOG method is higher compared with that of the conventional ONPG method**

An important issue of the  $\beta$ -gal enzymatic activity assay is the background noise from endogenous enzyme contained in cells. We investigated the background signal in several cell lines, including HEK293, A431, HeLa, HepG2, MDA-MB231, and NIH3T3. The background signals of these cell



**Fig. 2.** The stability of DDAO signal. (A) The time course of signal produced by 2  $\mu\text{M}$  or 10  $\mu\text{M}$  DDAO diluted in  $\beta$ -gal assay buffer. (B) The time course of signal produced by DDAOG cleavage after incubation with 9L/lacZ cell lysate with or without addition of stop buffer.



**Fig. 3.** Effect of pH on the DDAO absorbance and DDAO emission signal. (A) Quantification of DDAO absorbance at 646 nm. DDAO was diluted in water to a final concentration of 10  $\mu\text{M}$ . NaOH was added to adjust pH of DDAO solution. (B) Emission signal measured on Odyssey of the same DDAO solutions in (A).

lines ranged from 6 to 11 relative fluorescence intensity units (RFU). The 9L/lacZ signal was 339 RFU, approximately 42 times higher than the average signal of cell lines without lacZ expression (Fig. 5A). As a comparison, the signal was about 3.5 times higher than background using a commercial kit and ONPG as the enzyme substrate (Fig. 5B). These results seem to indicate that the sensitivity using DDAOG as the enzyme substrate is considerably

higher than that of an assay using ONPG. The signal-to-background ratio of the DDAOG method is approximately 12-fold higher than that of the ONPG method.

### Reporter gene assay in cells after transient transfection

It is well-established that AP1 binding element mediates TPA-induced gene transcription [13, 15]. We used this system to measure drug-induced gene expression using the β-gal/DDAOG activity assay. AP1-gal plasmid was transiently transfected into HEK293 cells. TPA was added to stimulate AP1-controlled β-gal expression. As shown in Fig. 6A, the β-gal activity assayed using DDAOG in cells treated with TPA was significantly higher than that in control (DMSO treated) cells.

We also tested the DDAOG method using the PathDetec® *trans*-Reporting System (Stratagene). In this system, the reporter gene coding sequence, such as β-gal or Luciferase, was put under the control of the yeast GAL4 binding element. The activation domain of c-Jun is linked to the GAL4 DNA binding domain (DBD) to form a fusion protein. On activation of c-Jun, GAL4 DBD of the fusion protein binds to the GAL4 binding element and activates the expression of the reporter gene under the control of GAL4. MEKK was used to activate the c-Jun fusion protein in this study. When β-gal was used as the reporter gene, the DDAOG cleavage signal in MEKK activated cells was approximately 2.5-fold that of control cells (Fig. 6B). The fold induction was similar to the result obtained when β-gal expression vector was replaced with luciferase expression vector.

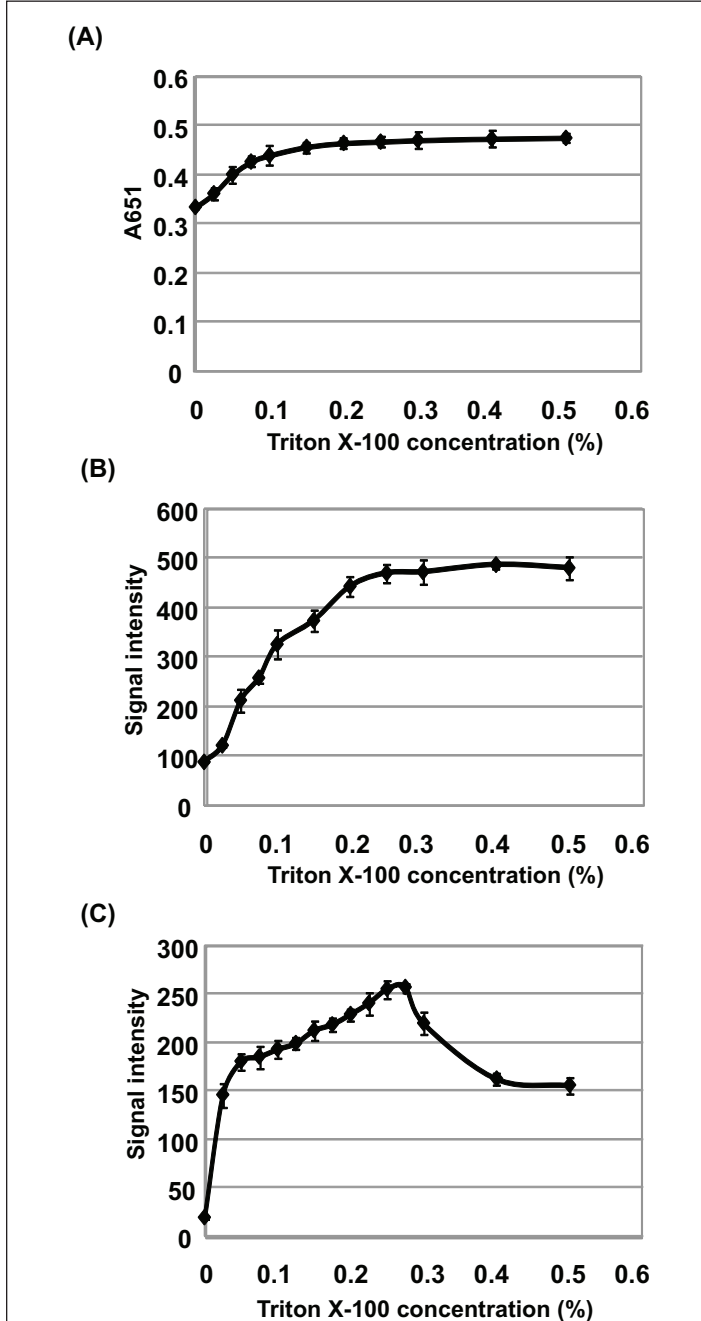


Fig. 4. Effect of detergent concentration on the DDAO absorbance, DDAO emission signal, and DDAOG cleavage after incubation with 9L/lacZ cells. (A) Quantification of DDAO absorbance at 651 nm. DDAO was diluted in bicine buffer (pH 7.8) to a final concentration of 10 μM. Triton X-100 was added to different concentrations. (B) Emission signal measured on Odyssey of the same DDAO solutions in (A). (C) DDAOG was diluted to a final concentration of 10 μM in the same buffers as in (A). DDAOG solutions were added to 9L/lacZ cells and incubated for 30 min before measuring the signal intensity on Odyssey.

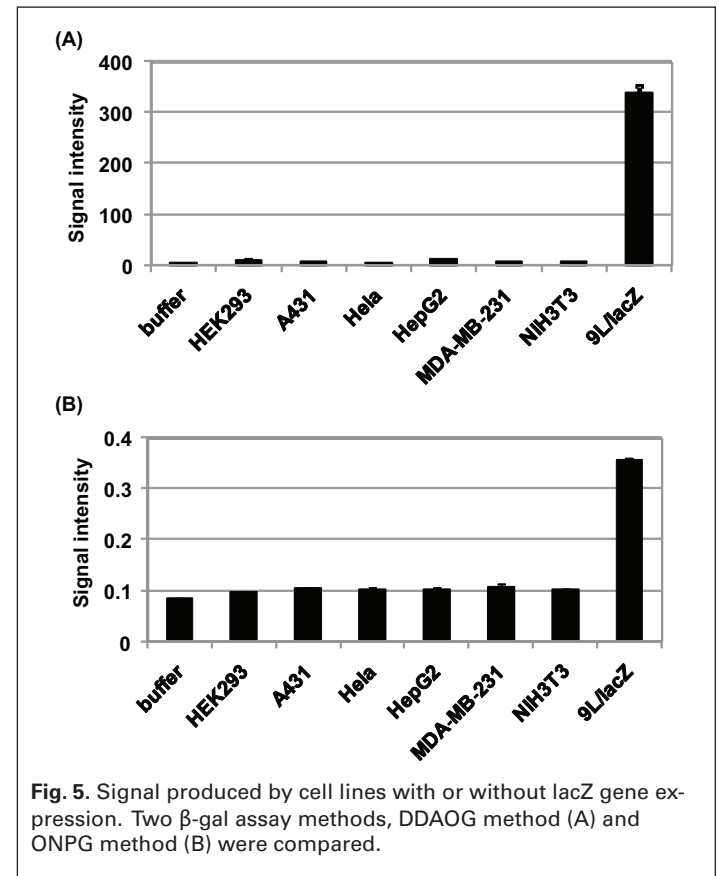


Fig. 5. Signal produced by cell lines with or without lacZ gene expression. Two β-gal assay methods, DDAOG method (A) and ONPG method (B) were compared.

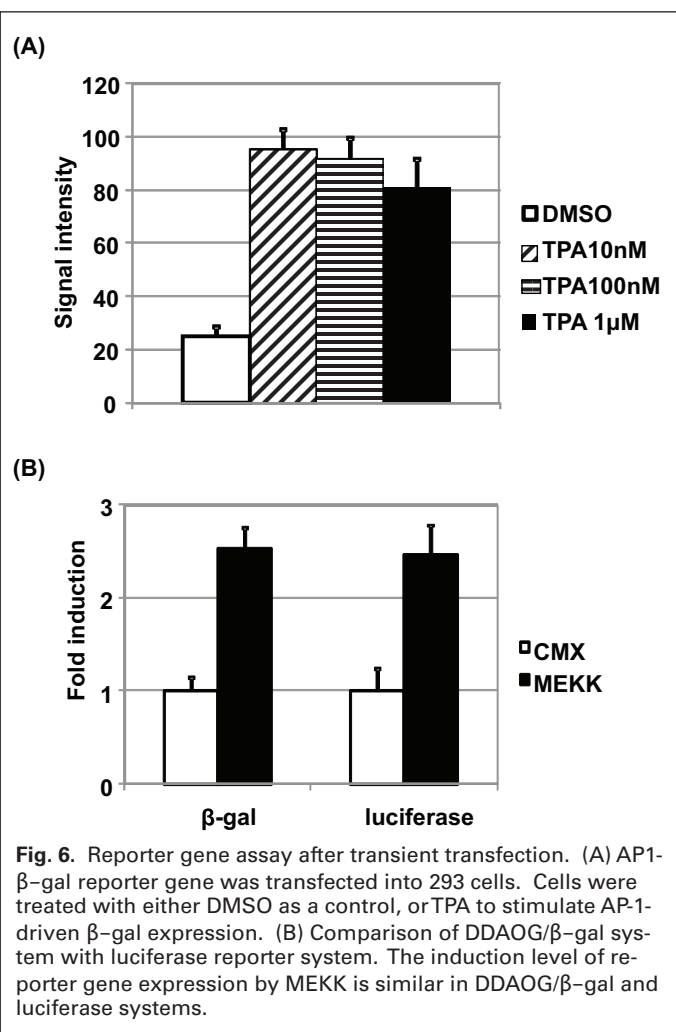
## DISCUSSION

The  $\beta$ -gal reporter gene is widely used in the biological research. Although originally from *E. coli*, this reporter is functional in many other organisms, such as yeast, *Caenorhabditis elegans*, *Drosophila* and mammals. Other advantages of  $\beta$ -gal system include the stability of  $\beta$ -gal enzyme, easy activity assay procedure, and availability of a broad range of substrates [1]. These advantages allow detection of  $\beta$ -gal enzyme using various methods, including microtiter plate assays and flow cytometry for cells, immunohistochemical assay for tissue sections, and potentially *in vivo* animal imaging. Given the superior sensitivity of fluorescence, we attempted to use the fluorescent substrate DDAOG to assay  $\beta$ -gal activity.

There is a 50-nm red shift of emission peak when DDAOG is cleaved to DDAO by  $\beta$ -gal, enabling specific detection of DDAO with minimal background from DDAOG [6, 16]. The signal produced by DDAOG cleavage is linearly related to the substrate DDAOG concentration and enzyme amount in the range that we tested. This feature is highly desirable for the enzymatic activity assay. We selected 10  $\mu$ M DDAOG for  $\beta$ -gal activity assay of 9L/lacZ stable cell line, and 293 cells transiently transfected with  $\beta$ -gal reporter genes. The concentration of 10  $\mu$ M DDAOG is very low compared to the ONPG concentration used for  $\beta$ -gal activity assay, which was in the range of 1 to 10 mM [3, 17]. To further increase sensitivity, the DDAOG concentration can be increased to 50  $\mu$ M (Gong *et al.*, unpublished data).

The DDAO signal is stable in the assay buffer. The time window for signal detection can be extended to 18 h after enzymatic reaction, and this is considerably longer than that with luminescence-based substrates. The wide time window offers more flexibility for signal detection. We observed that the DDAO signal was dependent on pH and detergent concentration in the buffer. Based on our results, we chose pH 7.8 and 0.25% Triton X-100 for the lysis and  $\beta$ -gal reaction buffer. We also incorporated 1 mM  $MgCl_2$  and 20 mM NaCl based on the previous observation that the combination of  $MgCl_2$  and NaCl in the buffer could promote the  $\beta$ -gal activity [18]. The relatively high pH (7.8) in the lysis and  $\beta$ -gal reaction buffer offers two benefits. First, our results indicated that higher pH favors the DDAO signal intensity. Second, the endogenous  $\beta$ -gal activity in mammalian cells, contributed mainly by the senescence-associated  $\beta$ -gal enzyme and lysosomal enzyme, is minimized under higher pH [12, 19, 20]. Interestingly, the peak of DDAO absorbance was shifted to a higher wavelength (from 646 to 651 nm) when detergent was added. This shift moves the absorbance peak closer to the optimal detection wavelength of the Odyssey imaging system. This shift may account for the dramatic signal enhancement by inclusion of detergent in the buffer. 2-Mercaptoethanol (2-ME) is often included in the  $\beta$ -gal assay buffer to reduce oxidation of the enzyme. However, in our assay, 2-ME dramatically decreased the DDAOG cleavage signal (data not shown). So, 2-ME was not included in this protocol.

When comparing the signal produced by 9L/lacZ cells with the background signal of cell lines without lacZ expression,



**Fig. 6.** Reporter gene assay after transient transfection. (A) AP1- $\beta$ -gal reporter gene was transfected into 293 cells. Cells were treated with either DMSO as a control, or TPA to stimulate AP1-driven  $\beta$ -gal expression. (B) Comparison of DDAOG/ $\beta$ -gal system with luciferase reporter system. The induction level of reporter gene expression by MEKK is similar in DDAOG/ $\beta$ -gal and luciferase systems.

the ratio is about 42 using DDAOG substrate. This ratio is considerably higher than that using the ONPG method. We also compared the signal of HEK293 cells with or without  $\beta$ -gal expression following transient transfection with pCMV $\beta$ -gal and control vectors. A similar difference was observed between the DDAOG method and ONPG methods (Gong *et al.*, unpublished data). The combination of high sensitivity of fluorescence imaging and lack of auto-fluorescent background may contribute to a better performance of the DDAOG method and allow detection of lower levels of enzyme expression.

We tested the DDAOG method in a transient transfection system. When pCMV $\beta$ -gal was transfected into HEK293 cells, the signal intensity was comparable to that of 9L/lacZ cells (Gong *et al.*, unpublished data). Moreover, the DDAOG method was capable of detecting the induced  $\beta$ -gal activity by either drug stimulation (in the case of AP1-gal plasmid) or genetic activation (in the case of the PathDetect system). The exchangeable reporter gene in the PathDetect system offers a unique system to compare the efficiency of different reporter genes. We used this system to compare the luciferase reporter system with the  $\beta$ -gal/DDAOG reporter assay, and a similar fold induction was observed. Further study will be needed to test in other cell lines.

In addition to the cell culture system,  $\beta$ -gal has been employed as a reporter gene in small animals [6, 16, 21]. The near infrared (NIR) has the advantages of increased penetration depth and reduced autofluorescence, and is ideal for optical imaging in small animals [22]. Therefore, the emission of DDAO in the far-red range could potentially make it a substrate for detection of  $\beta$ -gal activity *in vivo* [6]. However, we observed very weak signals with DDAOG in whole cells, probably indicating poor cellular penetration by the substrate (Fig. 1A). The results of Tung and coworkers

[6] may, in fact, be detecting released  $\beta$ -gal following cell necrosis in  $\beta$ -gal-expressing tumors rather than actual measurement of intracellular enzyme activity.

In conclusion, we have developed a  $\beta$ -gal enzymatic activity assay system using the far-red-shifted fluorescent substrate DDAOG. The system has been optimized to include a single lysis and reaction buffer and a simple protocol. The method provides a quantitative, high-sensitivity fluorescence assay alternative to other reporter systems.

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**LI-COR**<sup>®</sup> 4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA  
North America: 800-645-4267 • International: 402-467-0700 • FAX: 402-467-0819  
LI-COR GmbH Germany, Serving Europe and Africa: +49 (0) 6172 17 17 771  
LI-COR Ltd. UK, Serving UK, Ireland, Scandinavia: +44 (0) 1223 422104  
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