Odyssey®

Infrared Imaging System

Protocol

Near-Infrared Fluorescent β-galactosidase Reporter Gene Assays Using DDAOG

Published June 2009. The most recent version of this Protocol is posted at http://biosupport.licor.com/support.

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INTRODUCTION

The *lacZ* reporter gene from *E.coli* is widely used in genetics and cellular and molecular biology [1]. Its gene product, β-galactosidase (β-gal), has been used to monitor promoter activity in transiently or stably transfected cells [2, 3], analyze protein interactions and translocation using β-gal enzyme fragment complementation [4], and monitor tumor growth in animal models [5, 6].

This protocol describes a sensitive β-gal activity assay that uses the DDAOG substrate, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside, to generate a near-infrared fluorescent signal that can be detected with the Odyssey® Infrared Imaging System [7]. A detailed examination of this assay was published in *Analytical Biochemistry* in 2009 [7]. DDAOG is cleaved by the β-gal enzyme to produce 7-hydroxy-9H(I,3-dichloro-9,9-dimethylacridin-2-one) (DDAO). The excitation/emission maxima of DDAO are 646 nm/659 nm, respectively – a dramatic shift from those of uncleaved DDAOG (ex/em: 465 nm/608 nm) [6]. The DDAO fluorescent signal can be detected in the Odyssey 700 nm channel without interference from uncleaved DDAOG.

The assay has several advantages over conventional β-gal assays:

1. Higher sensitivity than assays using ONPG as substrate, due to its improved signal-to-noise ratio.
2. The DDAO fluorescent signal is very stable, so timing of detection is less critical.
3. Cell lysis and the subsequent β-gal activity assay can be performed in a single buffer, making the assay more simple and efficient. Both pH and detergent concentration are important for obtaining optimal signal [7].

REAGENTS REQUIRED

DDAOG (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside)

Invitrogen (Carlsbad, CA) D-6488

Lysis and Reaction Buffer

Sterilize buffer after preparation by passing through a 0.2 μM filter; store at room temperature

Bicine	25 mM
Triton X-100	0.25%
NaCl	20 mM
$MgCl_2$	1 mM

Adjust pH to 7.8

Stop Buffer

$NaCO_3$ 1 M

DDAOG Stock Solution

Dissolve DDAOG in DMSO to a final concentration of 10 mM. Store at -20°C and protect from light.

DDAOG Reaction Solution

Dilute the DDAOG stock solution 1:1000 in Lysis and Reaction Buffer to obtain a final concentration of 10 μM. Prepare fresh solution immediately before use.

If β-gal expression is very low, you may wish to use a higher DDAOG concentration (for example, 50 μM).

Recommended microplates

Proper selection of microplates can significantly affect the results of your analysis, as each plate has its own characteristics (including well depth, plate autofluorescence, and well-to-well signal crossover). The bottom of the plates must be clear to allow light to pass through.

- The following 96-well plates are recommended by LI-COR Biosciences:
  - Nunc™ (Part Number 167008, 161093)
  - Falcon™ (Part Number 353075, 353948)

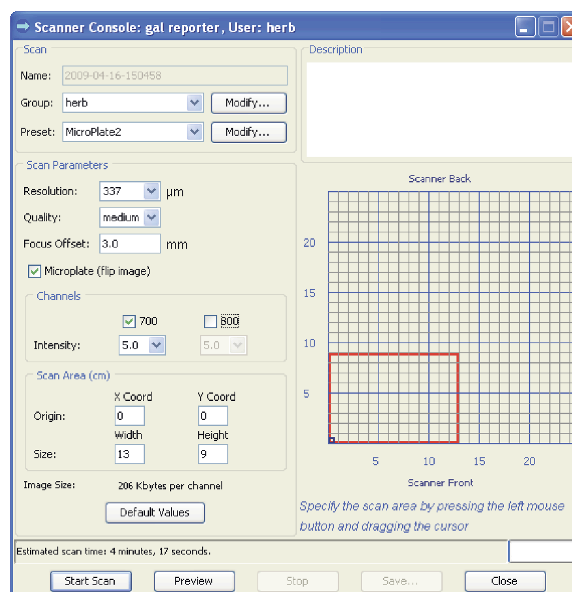
- Do not use plates with white walls, because the white surface increases light scatter and will create significant background noise.
- If you use plates other than those recommended above, the optimal focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets. Use the same intensity settings for each scan. After reviewing the collected scans, use the focus offset with the highest signal-to-noise as your focus offset for experiments.

PROTOCOL

Assay

1. Remove cell culture medium.
2. Optional: wash cells once with PBS.
   Note: If the cells are attached loosely to the plate, this wash step may cause cell loss and should be omitted.
3. Add 50 µl DDAOG Reaction Solution to each well of the 96-well plate.
   Note: if using a different size of well or reaction vessel, you may need to adjust the reaction solution volume.
4. Shake for 5 min at room temperature.
5. Incubate for 20-60 min at 37 °C (the optimal incubation time for your experiment can be determined by imaging the plate every 15 min).
6. *Optional:* Add 25 µl stop buffer to each well (final concentration 0.33 M).
   Should you add stop buffer? Things to consider:
   - *While you are scanning one region of the plate, the reaction will continue to progress in other wells. To most accurately measure the enzymatic activity in ALL wells at a given time point, you must add stop buffer.*
   - *If necessary, you can stop the reaction with stop buffer and save the plate at room temperature to be imaged later. Protect from light during storage.*
   - *When determining the optimal incubation time for your reactions, you must let the reaction proceed and cannot use stop buffer.*
   - *Addition of stop buffer may decrease signal intensity, because it dilutes the reaction solution in the well.*
7. Image plate with Odyssey® Infrared Imaging System.

Detection with the Odyssey Infrared Imaging System



1. Signal is detected by imaging in the 700 nm channel. Use intensity 5.0 (usually the default setting). It will take about 3 min to image the entire plate.

   Odyssey settings:

   Preset: Microplate
   Resolution: 337 µm (lowest resolution)
   Quality: medium
   Focus offset: 3.0 mm *(Note: you may need to optimize the focus offset for your microplate type)*
   Intensity: 5.0
2. Click check box for "Microplate (flip image)"
3. Under "Channels", uncheck box for "800" (not needed)
   All settings are default except resolution. Using the lowest resolution increases the scan speed and decreases scan time.
4. After each scan, check the plate image. When viewing a color image (red and black), the negative control should be black. A red color in the well indicates a good signal.

   Note: If signal is too low, try imaging the plate again with the scan intensity set to a higher value (for example, 6.0 or 7.0).

   If any wells display white pixels (or cyan blue pixels, if you are viewing a grayscale image), this indicates that the signal from those pixels was too high and it saturated the fluorescence detector. Saturated pixels cannot be accurately quantified.

To eliminate signal saturation:

- *Image the same plate again with the scan intensity set to a lower value (for example, 4.0 or 3.0).*
- *Alternatively, you can repeat the assay with new samples, using a shorter incubation time or lower DDAOG concentration to eliminate signal saturation. However, a DDAOG concentration lower than 5 µM is not recommended.*

Image Analysis

For details about how to apply a quantification grid to the image and analyze the results, please refer to the *Odyssey® Infrared Imaging System User Guide*. Grid placement and alignment are also discussed in the *In-Cell Western Assay Power User Guide* webinar series, at these internet locations:

http://www.licor.com/bio/video/ICW/Section_2/Section_2.jsp
http://www.licor.com/bio/video/ICW/Section_3/Section_3.jsp

GENERAL TIPS

1. Different lots or shipments of microplates may vary in the background fluorescence of the plastic. It is wise to check the background fluorescence levels of a new batch of plates by imaging an empty plate before running the assays.
2. If the β-gal expression is low, use a higher DDAOG concentration (e.g., 50 µM, or a 1:200 dilution of the DDAOG stock solution in Lysis and Reaction Buffer).
3. When the optimum incubation time has been determined, the enzymatic reaction can be stopped with stop buffer if desired (add $NaCO_3$ to a final concentration of 0.33 M).
4. When pipetting, be careful not to introduce bubbles. Bubbles in the wells may refract light during imaging.

EXPERIMENTAL RESULTS

In Figure 1, a β-gal expressing vector (CMV-gal) was used to transfect HEK293 cells grown in a 96-well plate. One day after transfection, the culture medium was removed. β-gal enzymatic activity was assayed using DDAOG as substrate with the above protocol. To demonstrate the effect of DDAOG concentration on signal intensity, different concentrations of DDAOG were used here. In typical experiments, 10 µM DDAOG is recommended. If β-gal expression is low, the concentration of DDAOG can be increased to 50 µM.

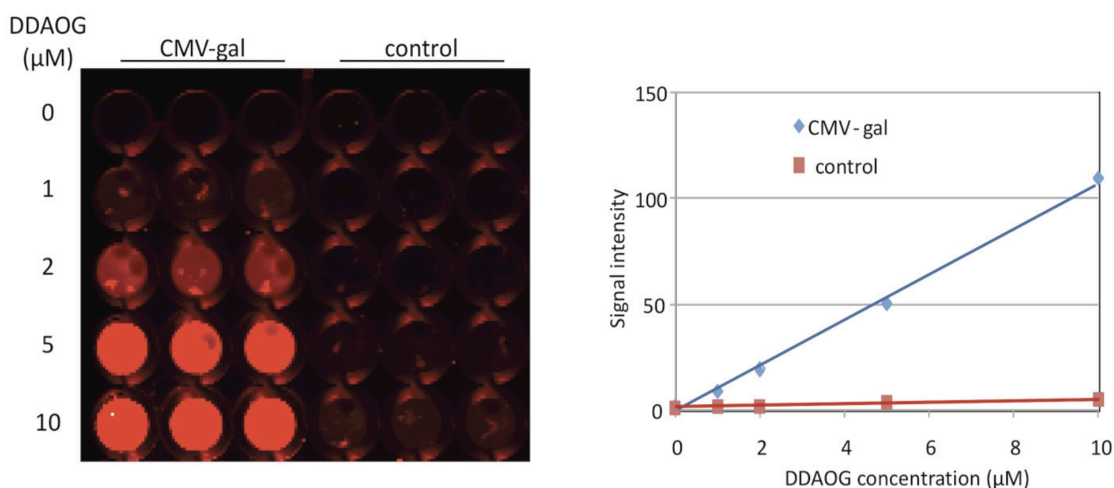


Figure 1. β-gal activity assay using DDAOG as substrate. HEK293 cells were transiently transfected with either a β-gal expressing vector (CMV-gal) or control vector. The signal intensity produced by DDAOG cleavage is linearly related to the substrate concentration. Signal inten-sities obtained using different concentrations of DDAOG (µM) were determined by imaging in the Odyssey 700 nm channel. The original image (A) and quantified data (B) are shown.

In Figure 2, we examined the relationship between fluorescent signal intensity and amount of enzyme. Signal intensity increased linearly with both cell number (using increasing numbers of 9L/*lacZ* cells that constitutively express the enzyme) and enzyme amount (using a titration of purified β-gal).

The experiment in Figure 3 addressed the background levels observed in cells that do not express β-gal, compared to 9L/*lacZ* cells. Some endogenous β-gal activity may occur in cultured cells, and this background noise can affect the sensitivity of the assay. We assessed the background level in a number of nonexpressing cell lines, including HEK293, A431, HeLa, HepG2, MDA-MB231, and NIH3T3. These cell lines generated background signals in the DDAOG assay that ranged from 6 to 11 relative fluorescence units (RFU). 9L/*lacZ* cells generated a signal of ~340 RFU, an increase of 40-fold compared to the nonexpressing cells (Figure 3A). In contrast, when a commercial ONPG substrate (Sigma, St. Louis, MO) was used, 9L/*lacZ* cells showed only a 3.5-fold signal increase compared to background. This improved signal-to-background level may allow detection of lower levels of enzyme expression.

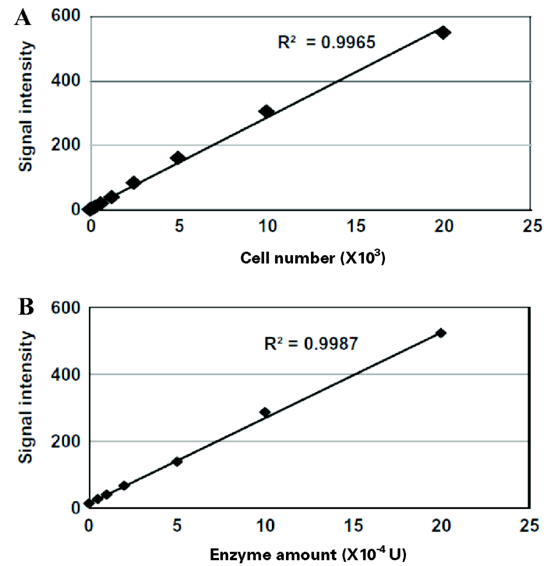


Figure 2. The signal intensity produced by DDAOG cleavage is linearly related to β-gal enzyme level. (A) 9L/*lacZ* cells (ATCC, Manassas, VA), which constitutively express β-galactosidase [6], were cultured and lysed. Graph depicts the signal produced by 10 mM DDAOG after incubation with cell lysate from different numbers of 9L/*lacZ* cells. (B) Signal produced by 10 mM DDAOG after incubation with different amounts of purified β-gal enzyme (Sigma, St. Louis, MO). Plates werer imaged with Odyssey® system.

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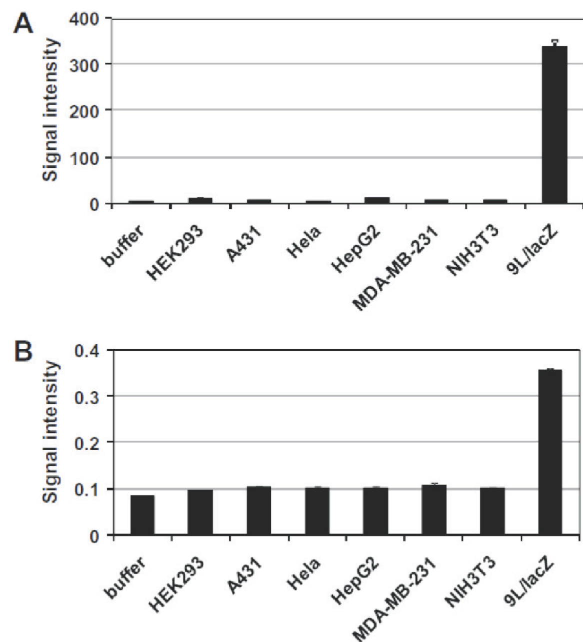

Figure 3. Signal produced by cell lines with or without *lacZ* gene expression. Two β-gal assay methods were compared: (A) DDAOG method and Odyssey imaging, described in this document; (B) conventional colorimetric ONPG method. Using the DDAOG method, the fluorescent signal from 9L/*lacZ* cells was ~42-fold higher than the average background from control cell lines not expressing *lacZ*. With the conventional ONPG method, the signal from 9L/*lacZ* cells was only ~3.5-fold higher than the background from nonexpressing cells.

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