

# $\beta$ -Galactosidase Enzyme Assay System with Reporter Lysis Buffer

Technical Bulletin No. 097

INSTRUCTIONS FOR USE OF PRODUCT E2000. *PLEASE DISCARD PREVIOUS VERSIONS.*  
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**I. Description**

$\beta$ -Galactosidase is a commonly used reporter molecule. The  $\beta$ -Galactosidase Enzyme Assay System with Reporter Lysis Buffer<sup>(a)</sup> (Cat.# E2000) is a convenient method for assaying  $\beta$ -galactosidase activity in lysates prepared from cells transfected with  $\beta$ -galactosidase reporter vectors such as Promega's pSV- $\beta$ -Galactosidase Control Vector (Cat.# E1081).

The standard assay is performed by adding a diluted sample to an equal volume of Assay 2X Buffer that contains the substrate ONPG (*o*-nitrophenyl- $\beta$ -D-galactopyranoside). Samples are incubated for 30 minutes, during which time the  $\beta$ -Galactosidase hydrolyzes the colorless substrate to *o*-nitrophenol, which is yellow. The reaction is terminated by addition of sodium carbonate, and the absorbance is read at 420nm with a spectrophotometer (1).

## II. Product Components

Product	Cat.#
$\beta$ -Galactosidase Enzyme Assay System with Reporter Lysis Buffer	E2000

This system contains sufficient reagents for 65 standard assays or 200 assays in a 96-well plate format. Includes:

- 30ml Reporter Lysis Buffer, 5X
- 10ml Assay 2X Buffer
- 100u  $\beta$ -Galactosidase
- 35ml Sodium Carbonate, 1M
- 1 Protocol

**Storage:** Store at  $-20^{\circ}\text{C}$ . The 5X Reporter Lysis Buffer may be stored at room temperature. **Note:** The Sodium Carbonate may be stored at room temperature,  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  with no change in stability.

**Unit Definition:** One unit of  $\beta$ -Galactosidase hydrolyzes 1 micromole of *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) to *o*-nitrophenol and galactose per minute at pH 7.5 and  $37^{\circ}\text{C}$ .

## III. Cell Lysate Preparation

The Reporter Lysis Buffer (RLB) allows  $\beta$ -galactosidase, luciferase and chloramphenicol acetyltransferase (CAT) assays to be performed from the same cell lysate cotransfected with vectors carrying these genes. In the cell lines we have tested (BALB/3T3 and HeLa cells),  $\beta$ -galactosidase activity in the Reporter Lysis Buffer is significantly higher than the activity in lysates prepared with the freeze-thaw method (Section III.C; 2). In general, we recommend using RLB to prepare the lysates (Section III.A); however, alternative procedures are provided in Sections III.B and III.C for cases where this is incompatible with the experimental goals of the researcher.

For transient expression assays, cell lysates typically are prepared 48 hours post-transfection. We also recommend preparing lysates from cells that have not been transfected with a  $\beta$ -galactosidase gene. This negative control allows a correction for endogenous levels of cellular  $\beta$ -galactosidase or its isozymes.

### A. Preparation of Lysate from Adherent Cells Using Reporter Lysis Buffer

#### Materials to Be Supplied by the User

(Solution composition is provided in Section VIII.A.)

- PBS 1X buffer ( $\text{Mg}^{2+}$ - and  $\text{Ca}^{2+}$ -free)
  1. Add 4 volumes of water to 1 volume of 5X RLB to produce a 1X stock solution.
  2. Remove the growth medium from the cells to be assayed. Wash the cells twice with PBS 1X buffer, being careful not to dislodge any of the cells. Remove as much of the final wash as possible using a pipet tip.
  3. Add a sufficient volume of 1X RLB to cover the cells (400 $\mu\text{l}$  for a 60mm culture dish, 900 $\mu\text{l}$  for a 100mm dish). Rock the dish slowly several times to ensure complete coverage of the cells.
  4. Incubate at room temperature for 15 minutes, slowly rocking the dish several times during the incubation.

5. Scrape all areas of the plate surface, then tilt the dish and thoroughly scrape the cell lysate to the lower edge of the plate. Using a pipet, transfer the cell lysate to a microcentrifuge tube and place the samples on ice.
6. Vortex the tube for 10–15 seconds, then centrifuge at top speed in a microcentrifuge for 2 minutes at 4°C. Transfer the supernatant to a fresh tube.
7. The lysates may be assayed directly or stored at –70°C for at least 2 months.

### **B. Preparation of Lysate from Adherent Cells Using Luciferase Cell Culture Lysis Reagent**


1X Luciferase Cell Culture Lysis Reagent (CCLR, Cat.# E1531) may be used in place of 1X RLB to lyse cells and to dilute cell lysates in the  $\beta$ -Galactosidase Enzyme Assay as described in Section III.A, **provided that** a different stop buffer is used. We recommend the use of 1M Tris base as a stop buffer when samples are in 1X CCLR, because addition of the 1M Sodium Carbonate (provided with the system) causes a precipitate. Please note that samples in 1X CCLR may also be used in luciferase but not in CAT enzyme assays.


### **C. Preparation of Lysate from Adherent Cells Using the Freeze-Thaw Method**

#### **Materials to Be Supplied by the User**

(Solution compositions are provided in Section VIII.A.)

- PBS 1X buffer (Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free)
  - TEN buffer
  - 0.25M Tris-HCl (pH 8.0)
1. Wash the cells with PBS 1X buffer twice, being careful not to dislodge the cells. Aspirate the PBS 1X buffer well after the final wash.
  2. Add 1ml of TEN buffer per 60mm or 100mm dish and incubate the cells for 5 minutes at room temperature.
  3. Scrape the cells and transfer them to a microcentrifuge tube.
  4. Centrifuge the cells at top speed in a microcentrifuge for 1 minute at 4°C. Remove the supernatant and resuspend the pellet by vortexing it vigorously in 150 $\mu$ l of 0.25M Tris-HCl (pH 8.0).
  5. Subject the lysate to 3 rapid freeze-thaw cycles by placing the lysate on dry ice or in a dry ice/ethanol bath until frozen and then thawing it in a 37°C water bath. Vortex the lysate vigorously after each thaw step.
  6. Centrifuge the lysate at top speed for 2 minutes in a microcentrifuge at 4°C. Transfer the supernatant to a fresh tube.
  7. The lysates may be assayed directly or stored at –70°C for at least 2 months.

 **1X Cell Culture Lysis Reagent** can be substituted for 1X RLB but requires a different stop buffer.

 **Longer** centrifugation times can result in compacted cell pellets that are difficult to resuspend.

## IV. $\beta$ -Galactosidase Assays

### A. Standard Assay

Preparation of a standard curve is optional. If a standard curve is desired, please see Section V.

1. Thaw the system components and mix each component well before use. Place the Assay 2X Buffer on ice. See Note 1.
2. It may be necessary to dilute the cell lysates in 1X Reporter Lysis Buffer. A 2:1 dilution of lysate to 1X Reporter Lysis Buffer (100 $\mu$ l of lysate plus 50 $\mu$ l of 1X Reporter Lysis Buffer) is a good starting dilution, but up to 150 $\mu$ l of cell lysate can be used per reaction. As a negative control, prepare the same dilution of a cell lysate made from cells that have not been transfected with the  $\beta$ -galactosidase gene.
3. Pipet 150 $\mu$ l of the appropriately diluted (or undiluted) cell lysates into labeled tubes.
4. Add 150 $\mu$ l of Assay 2X Buffer to each of the tubes.
5. Mix all samples by vortexing briefly.
6. Incubate the reactions at 37°C for 30 minutes or until a faint yellow color has developed. Color development continues for approximately 3 hours. If enzyme activity is low, samples may be incubated overnight if the reaction tubes are tightly capped.
7. Stop the reactions by adding 500 $\mu$ l of 1M Sodium Carbonate. Mix by vortexing briefly.
8. Read the absorbance at 420nm.

### B. 96-Well Plate Assay

This protocol is useful for testing numerous samples. The modified assay is performed directly in 96-well plates, and the absorbance of each sample is read using a plate reader. Preparation of a standard curve is optional. If a standard curve is desired, please see Section V.

1. Thaw the system components and mix each component well. Place the Assay 2X Buffer on ice. See Note 1.
2. It may be necessary to dilute the cell lysates in 1X Reporter Lysis Buffer. Mix 30 $\mu$ l of lysate with 20 $\mu$ l of 1X Reporter Lysis Buffer as a starting dilution, but up to 50 $\mu$ l of cell lysate can be used per reaction. As a negative control, prepare the same dilution of a cell lysate made from cells that have not been transfected with the  $\beta$ -galactosidase gene.
3. Pipet 50 $\mu$ l of the appropriately diluted (or undiluted) cell lysates into labeled wells of a 96-well plate.
4. Add 50 $\mu$ l of Assay 2X Buffer to each well of the 96-well plate.
5. Mix all samples by pipetting the well contents. Place a cover on the plate.
6. Incubate the plate at 37°C (an incubator works well) for 30 minutes or until a faint yellow color has developed. Color development continues for approximately 3 hours. **Due to the small sample volume, we do not recommend incubating the reactions overnight.**



***It is important***

to read the absorbance immediately after addition of 1M Sodium Carbonate (3).



***Do not*** incubate

reactions overnight.

7. Stop the reaction by adding 150µl of 1M Sodium Carbonate. Mix by pipetting the contents of each well. Avoid producing bubbles, which may interfere with absorbance readings; if present, bubbles may be removed by piercing with a fine gauge needle.
8. Read the absorbance of the samples at 420nm in a plate reader (see Note 5).

**Notes:**

1. If crystals are present in the 1M Sodium Carbonate, warm the solution to 37°C to dissolve the crystals and then leave it at room temperature. If a precipitate is present in the Assay 2X Buffer, warm briefly in a 37°C water bath to dissolve and then place the solution on ice.
2. The 96-well plate assay is configured for a plate that has a maximum well volume of approximately 300µl. For plates with different maximum well volumes, the reaction may be scaled up or down proportionally.
3. Plate readers generally perform best using plates that have flat-bottomed, optically clear wells.
4. The coatings applied to some 96-well plates may inhibit the β-galactosidase reaction. To test for this, perform identical reactions in a 96-well plate and in microcentrifuge tubes. Stop the reactions, pipet the tube reactions into the plate wells and read all samples in a plate reader. The absorbance values should be the same for both types of samples.
5. Some plate readers are limited in the number of wavelengths at which they can read. Although the peak absorbance of the reaction product is near 420nm, other wavelengths close to 420nm may be used to monitor the reaction. The greatest sensitivity is obtained with wavelengths of 410–430nm.


**V. Standard Curves**


**A. Preparation of Standard Curve for Standard Assays**

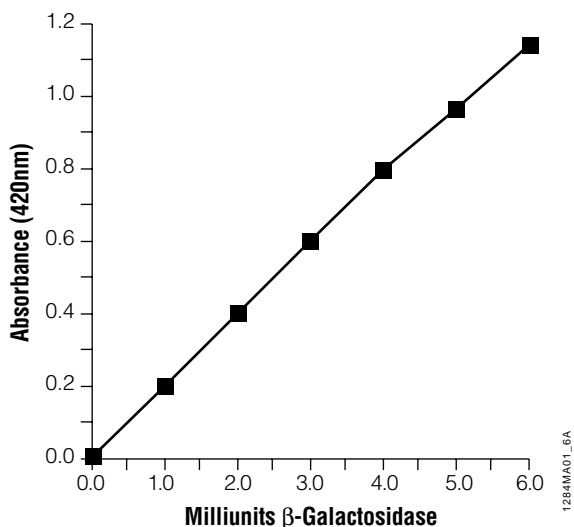
If a standard curve is desired, use standards between 0 and 6.0 x 10<sup>-3</sup> units of β-Galactosidase. Prepare the following dilution series in 1X Reporter Lysis Buffer immediately before use. Add 10µl of 1u/µl β-Galactosidase to 990µl of 1X Reporter Lysis Buffer and mix. Then add 10µl of this 1:100 dilution to 990µl of 1X Reporter Lysis Buffer and mix it to make a 1:10,000 stock solution. Using this stock, prepare 150µl of each β-Galactosidase standard per tube as described below.

<b>β-Galactosidase Standard (milliunits)</b>	<b>Volume of 1:10,000 Stock</b>	<b>Volume of 1X Reporter Lysis Buffer</b>
0	0µl	150µl
1.0	10µl	140µl
2.0	20µl	130µl
3.0	30µl	120µl
4.0	40µl	110µl
5.0	50µl	100µl
6.0	60µl	90µl

1. Follow the protocol described in Section IV.A, Steps 4–8.
2. Plot the absorbance at 420nm versus concentration of β-Galactosidase standards. An example of a standard curve prepared by the standard assay method is shown in Figure 1.

 **It is important** to read the absorbance immediately after addition of 1M Sodium Carbonate (3).

 **If a** standard curve is used, prepare fresh enzyme dilutions each time the assay is performed.



**Figure 1. A sample standard curve for the standard assay.** This standard curve was prepared as described in Section V.A. Samples were incubated for 30 minutes at 37°C.

### B. Preparation of Standard Curve for 96-Well Plate Assays

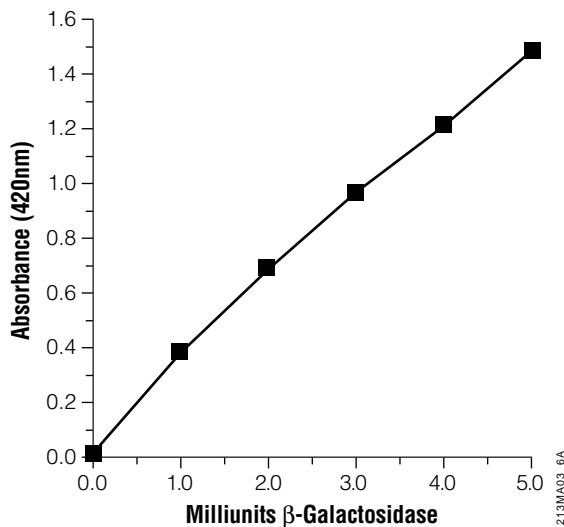
If a standard curve is desired, use standards between 0 and  $5.0 \times 10^{-3}$  units of  $\beta$ -Galactosidase. Prepare the following dilution series in 1X Reporter Lysis Buffer immediately before use. Add 10 $\mu$ l of 1u/ $\mu$ l  $\beta$ -Galactosidase to 990 $\mu$ l of 1X Reporter Lysis Buffer and vortex. Then add 10 $\mu$ l of this 1:100 dilution to 990 $\mu$ l of 1X Reporter Lysis Buffer and vortex to make a 1:10,000 stock solution. Using this stock, prepare 50 $\mu$ l of each  $\beta$ -Galactosidase standard per well as described below.

$\beta$ -Galactosidase Standard (milliunits)	Volume of 1:10,000 Stock	Volume of 1X Reporter Lysis Buffer
0	0 $\mu$ l	50 $\mu$ l
1.0	10 $\mu$ l	40 $\mu$ l
2.0	20 $\mu$ l	30 $\mu$ l
3.0	30 $\mu$ l	20 $\mu$ l
4.0	40 $\mu$ l	10 $\mu$ l
5.0	50 $\mu$ l	0 $\mu$ l

1. Follow the protocol described in Section IV.B, Steps 4–8.
2. Plot the absorbance at 420nm versus concentration of  $\beta$ -Galactosidase standards. An example of a standard curve prepared by the 96-well plate method is shown in Figure 2.

### VI. In situ Staining of Cells for $\beta$ -Galactosidase Activity

Cells transfected with the pSV- $\beta$ -Galactosidase Control Vector and expressing  $\beta$ -galactosidase can be visualized by microscopy (4). The cells appear blue following fixation and incubation with the substrate X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside). For comparison, it is important to include control cells that have not been transfected with a  $\beta$ -galactosidase vector in order to visualize the level of background activity due to endogenous  $\beta$ -galactosidase or its isozymes. The following protocol is for use with a 60mm culture dish.



**Figure 2. A sample of the standard curve for the 96-well assay.** This standard curve was prepared as described in Section V.B. Samples were incubated for 30 minutes at 37°C.

### A. Staining Protocol

#### Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.A.)

- PBS 1X buffer (Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free)
- glutaraldehyde solution
- X-Gal solution

1. Wash the cells twice with PBS 1X buffer. Remove all of the final wash buffer.
2. Fix the cells by adding 2ml of glutaraldehyde solution and incubating for 15 minutes.
3. Remove the glutaraldehyde solution and rinse gently 3 times with PBS 1X buffer. It is important to remove residual glutaraldehyde, which could inhibit  $\beta$ -galactosidase activity.
4. Add 1ml of X-Gal solution per plate of cells. Incubate the cells at 37°C between 1–16 hours until the cells are visibly stained. The exact incubation time must be optimized for each set of transfections.
5. Remove the X-Gal solution. Cover the cells with 1X PBS.
6. View the cells with a phase contrast or light microscope. To obtain a permanent record of the results, photograph in situ stained cells on the same day of the experiment.
7. For long-term storage (weeks to months) of in situ stained cells, store the cells under 70% glycerol at 4°C.



**If a** standard curve is used, prepare fresh enzyme dilutions each time the assay is performed.



**Caution:** Glutaraldehyde is a carcinogen. Avoid contact with skin and avoid inhalation. Use in a fume hood and discard waste according to your institution's procedures.

### VII. References

1. Rosenthal, N. (1987) Identification of regulatory elements of cloned genes with functional assays. *Meth. Enzymol.* **152**, 704–20.
2. Schenborn, E. and Goiffon, V. (1993) A new lysis buffer for luciferase, CAT and  $\beta$ -galactosidase reporter gene co-transfections. *Promega Notes* **41**, 11.

3. MacGregor, G.R. (1991) In: *Methods in Molecular Biology*, Vol. 7: Gene Transfer and Expression Protocols, Murphy, E.J., ed., The Humana Press, Inc., Clifton, NJ, 217.
4. Sanes, J.R., Rubenstein, J.L. and Nicolas, J.F. (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133–42.

## VIII. Appendix

### A. Composition of Buffers and Solutions

#### Assay 2X Buffer

200mM sodium phosphate buffer (pH 7.3)  
 2mM MgCl<sub>2</sub>  
 100mM β-mercaptoethanol  
 1.33mg/ml ONPG

#### TEN buffer

40mM Tris-HCl (pH 7.5)  
 1mM EDTA (pH 8.0)  
 150mM NaCl

#### PBS 1X buffer (Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free)

137mM NaCl  
 2.7mM KCl  
 8.1mM Na<sub>2</sub>HPO<sub>4</sub>  
 1.47mM KH<sub>2</sub>PO<sub>4</sub>

The final pH should be 7.4 at 25°C.

#### glutaraldehyde solution

0.25% (v/v) glutaraldehyde

Prepare in PBS 1X buffer.

#### X-Gal solution

0.2% X-Gal (from 2% stock in DMF)  
 2mM MgCl<sub>2</sub>  
 5mM K<sub>4</sub>Fe(CN)<sub>6</sub> • 3H<sub>2</sub>O  
 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>

Prepare in PBS 1X buffer. Filter the X-Gal solution, which may contain large crystals, through a 0.2µm filter immediately before use.

#### β-Galactosidase

Supplied at 1u/µl in 118mM phosphate buffer, 50% w/v glycerol.

### B. Related Products

Product	Size	Cat.#
pSV-β-Galactosidase Control Vector	20µg	E1081
Reporter Lysis Buffer, 5X	30ml	E3971
Luciferase Cell Culture Lysis Reagent, 5X	30ml	E1531
Luciferase Assay System with Reporter Lysis Buffer <sup>(a,b)</sup>	100 assays	E4030
Luciferase Assay System Freezer Pack <sup>(a,b)</sup>	1,000 assays	E4530
Luciferase Reporter 1000 Assay System <sup>(a,b)</sup>	1,000 assays	E4550
Luciferase Assay Reagent <sup>(a,b)</sup>	1,000 assays	E1483

## Transfection Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent(c)	1.2mg	E2431
Tfx™-50 Reagent(d)	2.1mg	E1811
Tfx™-10 Reagent(d)	9.3mg	E2381
Tfx™-20 Reagent(d)	4.8mg	E2391
Tfx™ Reagents Transfection Trio(d)	5.4mg	E2400
Transfectam® Reagent for the Transfection of Eukaryotic Cells(e)	1mg	E1231
	0.5mg	E1232
ProFection® Mammalian Transfection System, Calcium Phosphate	1 system	E1200
ProFection® Mammalian Transfection System, DEAE-Dextran	1 system	E1210

## Luciferase Reporter Vectors

Product	Size	Cat.#
pGL3-Control Vector(f,g)	20µg	E1741
pGL3-Basic Vector(f,g)	20µg	E1751
pGL3-Promoter Vector(f,g)	20µg	E1761
pGL3-Enhancer Vector(f,g)	20µg	E1771

(a)Certain applications of this product may require licenses from others.

(b)U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and European Pat. No. 0 553 234 have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay. Other patents are pending.

(c)The cationic lipid component of the TransFast™ Transfection Reagent is covered by U.S. Pat. Nos. 5,824,812, 5,869,715 and pending foreign patents.

(d)The cationic lipid component of the Tfx™ Reagents is covered by U.S. Pat. Nos. 5,527,928, 5,744,625 and 5,892,071, Australian Pat. No. 704189 and other pending foreign patents.

(e)Transfectam is a registered trademark of Promega Corporation, which sells the Transfectam® product for research purposes only under a license from Polyplus Transfection. The Transfectam® product was developed by J.P. Behr and J.P. Loeffler and is covered by U.S. Pat. No. 5,171,678 and corresponding foreign patents.

(f)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

(g)U.S. Pat. No. 5,670,356 has been issued to Promega Corporation for a modified luciferase technology.

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