Technical Bulletin

β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer

INSTRUCTIONS FOR USE OF PRODUCT E2000.

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I. Description

β-Galactosidase is a commonly used reporter molecule. The β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer[a] (Cat.# E2000) is a convenient method for assaying β-galactosidase activity in lysates prepared from cells transfected with β-galactosidase reporter vectors such as the pSV-β-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-β-D-galactopyranoside). Samples are incubated for 30 minutes, during which time the β-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 and Storage Conditions

Product	Cat.#
β-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 5X Buffer
- 10ml Assay 2X Buffer
- 100u β-Galactosidase
- 35ml Sodium Carbonate, 1M
- 1 Protocol

Storage: Store at –20°C. The Reporter Lysis 5X Buffer may be stored at room temperature. The Sodium Carbonate may be stored at room temperature, 4°C or –20°C with no change in stability.

Unit Definition: One unit of β-Galactosidase hydrolyzes 1 micromole of o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol and galactose per minute at pH 7.5 and 37°C.

III. Cell Lysate Preparation

The Reporter Lysis Buffer (RLB) allows β-galactosidase, luciferase and chloramphenicol acetyltransferase (CAT) assays to be performed from the same lysate prepared from cells cotransfected with vectors carrying these genes. In the cell lines that we have tested (BALB/3T3 and HeLa cells), β-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 are typically prepared 48 hours post-transfection. We also recommend preparing lysates from cells that have not been transfected with a β-galactosidase gene. This negative control allows a correction for endogenous levels of cellular β-galactosidase or its isozymes.

III.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 (Mg^{2+}- and 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.
3. Add a sufficient volume of 1X RLB to cover the cells (400µl for a 60mm culture dish, 900µl 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.

III.B. Preparation of Lysate from Adherent Cells Using Cell Culture Lysis Reagent

1X 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 β-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.

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

III.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^{2+}- and Ca^{2+}-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. Remove all of the PBS 1X buffer 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 150µl of 0.25M Tris-HCl (pH 8.0).

(!) Longer centrifugation times can result in compacted cell pellets that are difficult to resuspend.

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, 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.

IV. β-Galactosidase Assays

IV.A. Standard Assay

Preparation of a standard curve is optional. If a standard curve is desired, 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 in 1X Reporter Lysis Buffer (100µl of lysate plus 50µl of 1X Reporter Lysis Buffer) is a good starting dilution, but up to 150µl 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 β-galactosidase gene.
3. Pipet 150µl of the appropriately diluted (or undiluted) cell lysates into labeled tubes.
4. Add 150µl 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.
7. Stop the reactions by adding 500µl of 1M Sodium Carbonate. Mix by vortexing briefly.

! Read the absorbance immediately after addition of 1M Sodium Carbonate (3).

8. Read the absorbance at 420nm.

IV.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, 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. Mix 30µl of lysate with 20µl of 1X Reporter Lysis Buffer as a good starting dilution, but up to 50µl 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 β-galactosidase gene.
3. Pipet 50µl of the appropriately diluted (or undiluted) cell lysates into labeled wells of a 96-well plate.
4. Add 50µl of Assay 2X Buffer to each well of the 96-well plate.

IV.B. 96-Well Plate Assay (continued)

5. Mix all samples by pipetting the well contents. Place a cover on the plate.
6. Incubate the plate at 37°C for 30 minutes or until a faint yellow color has developed. Color development continues for approximately 3 hours.
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.

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

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, 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, 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

V.A. Preparation of Standard Curve for Standard Assays

If a standard curve is desired, use standards containing between 0 and 6.0×10^{-3} 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 vortex. Add 10µl of this 1:100 dilution to 990µl of 1X Reporter Lysis Buffer, and vortex to make a 1:10,000 stock solution. Using this stock, prepare 150µl of each β-Galactosidase standard per tube as described below.

Prepare fresh enzyme dilutions each time the standard curve is performed.

β-Galactosidase Standard (milliunits)	Volume of 1:10,000 Stock	Volume of 1X Reporter Lysis Buffer
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.

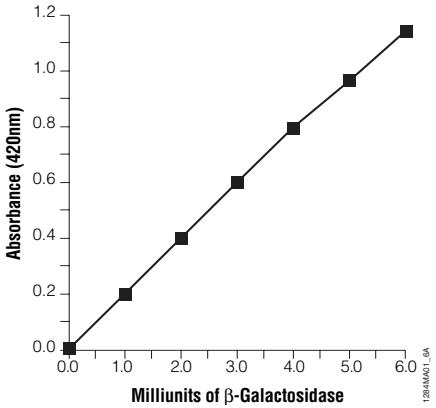

Figure 1. A sample standard curve for the standard assay. This standard curve was prepared as described in Section V.A. Samples were incubated for 30 minutes at 37°C. Numerous factors can affect the absorbance readings, so your standard curve may differ from this example. For this reason, generate a standard curve for each set of β-galactosidase assays performed.

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

If a standard curve is desired, use standards containing between 0 and 5.0×10^{-3} 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 vortex. Add 10µl of this 1:100 dilution to 990µl of 1X Reporter Lysis Buffer, and vortex to make a 1:10,000 stock solution. Using this stock, prepare 50µl of each β-Galactosidase standard per well as described below.

Prepare fresh enzyme dilutions each time the standard curve is performed.

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

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

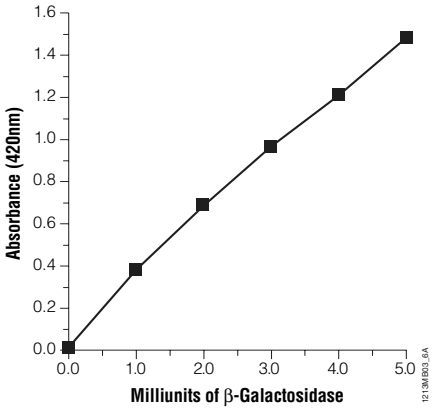

Figure 2. A sample standard curve for the 96-well assay. This standard curve was prepared as described in Section V.B. Samples were incubated for 30 minutes at 37°C. Numerous factors can affect the absorbance readings, so your standard curve may differ from this example. For this reason, generate a standard curve for each set of β-galactosidase assays performed.

VI. In situ Staining of Cells for β-Galactosidase Activity

Cells transfected with the pSV-β-Galactosidase Control Vector and expressing β-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-β-D-galactopyranoside). For comparison, it is important to include control cells that have not been transfected with a β-galactosidase vector to visualize the level of background activity due to endogenous β-galactosidase or its isozymes. The following protocol is for use with a 60mm culture dish.

Materials to Be Supplied by the User
(Solution compositions are provided in Section VIII.A.)

- PBS 1X buffer (Mg^{2+}- and Ca^{2+}-free)
- glutaraldehyde solution
- X-Gal solution

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.

1. Wash the cells twice with PBS 1X buffer. Remove all of the final wash buffer.
2. Fix cells by adding 2ml of glutaraldehyde solution and incubating for 15 minutes.

VI. In situ Staining of Cells for β-Galactosidase Activity (continued)

3. Remove the glutaraldehyde solution and rinse gently 3 times with PBS 1X buffer. It is important to remove residual glutaraldehyde, which could inhibit β-galactosidase activity.
4. Add 1ml of X-Gal solution per plate of cells. Incubate the cells at 37°C for 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 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.

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 β-galactosidase reporter gene co-transfections. *Promega Notes* **41**, 11–4.
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

VIII.A. Composition of Buffers and Solutions

Assay 2X Buffer

200mM	sodium phosphate buffer (pH 7.3)
2mM	$MgCl_2$
100mM	β-mercaptoethanol
1.33mg/ml	ONPG

β-Galactosidase

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

glutaraldehyde solution

0.25%	(v/v) glutaraldehyde

Prepare in PBS 1X buffer.

PBS 1X buffer (Mg^{2+}- and Ca^{2+}-free)

137mM	NaCl
2.7mM	KCl
8.1mM	Na_2HPO_4
1.47mM	KH_2PO_4

The final pH should be 7.4 at 25°C.

TEN buffer

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

X-Gal solution

0.2%	X-Gal (from 2% stock in dimethylformamide)
2mM	$MgCl_2$
5mM	$K_4Fe(CN)_6 \bullet 3H_2O$
5mM	$K_3Fe(CN)_6$

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

VIII.B. Related Products

Product	Size	Cat.#
pSV-β-Galactosidase Control Vector	20µg	E1081
X-Gal	100mg	V3941
Beta-Glo® Assay System	10ml	E4720
	100ml	E4740
	10 × 100ml	E4780
Reporter Lysis 5X Buffer	30ml	E3971
Luciferase Cell Culture Lysis 5X Reagent	30ml	E1531
Luciferase Assay System with Reporter Lysis Buffer	100 assays	E4030
Luciferase Assay System Freezer Pack	1,000 assays	E4530
Luciferase Reporter 1000 Assay System	1,000 assays	E4550
Luciferase Assay Reagent	1,000 assays	E1483
Dual Luciferase® Reporter Assay System	100 assays	E1910
Dual Luciferase® Reporter 1000 Assay System	1,000 assays	E1980

Transfection Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™-50 Reagent	2.1mg	E1811
Tfx™-20 Reagent	4.8mg	E2391
Tfx™ Reagents Transfection Trio	5.4mg	E2400
Transfectam® Reagent for the Transfection of Eukaryotic Cells	1mg	E1231
	0.5mg	E1232
ProFection® Mammalian Transfection System—Calcium Phosphate	40 reactions	E1200
ProFection® Mammalian Transfection System—DEAE-Dextran	40 reactions	E1210

Plasmid Purification Systems

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

pGL4 Luciferase Reporter Vectors

Please visit **www.promega.com** to see a complete listing of our reporter vectors.

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene Promoter	Mammalian Selectable Marker	Cat.#
pGL4.10[*luc2*]	Yes	*luc2*[A]	No	No	No	E6651
pGL4.11[*luc2P*]	Yes	"	hPEST	No	No	E6661
pGL4.12[*luc2CP*]	Yes	"	hCL1-hPEST	No	No	E6671
pGL4.13[*luc2*/SV40]	No	"	No	SV40	No	E6681
pGL4.14[*luc2*/Hygro]	Yes	"	No	No	Hygro	E6691
pGL4.15[*luc2P*/Hygro]	Yes	"	hPEST	No	Hygro	E6701
pGL4.16[*luc2CP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6711
pGL4.17[*luc2*/Neo]	Yes	"	No	No	Neo	E6721
pGL4.18[*luc2P*/Neo]	Yes	"	hPEST	No	Neo	E6731
pGL4.19[*luc2CP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6741
pGL4.20[*luc2*/Puro]	Yes	"	No	No	Puro	E6751
pGL4.21[*luc2P*/Puro]	Yes	"	hPEST	No	Puro	E6761
pGL4.22[*luc2CP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E6771
pGL4.70[*hRluc*]	Yes	*hRluc*[B]	No	No	No	E6881
pGL4.71[*hRlucP*]	Yes	"	hPEST	No	No	E6891
pGL4.72[*hRlucCP*]	Yes	"	hCL1-hPEST	No	No	E6901
pGL4.73[*hRluc*/SV40]	No	"	No	SV40	No	E6911
pGL4.74[*hRluc*/TK]	No	"	No	HSV-TK	No	E6921
pGL4.75[*hRluc*/CMV]	No	"	No	CMV	No	E6931
pGL4.76[*hRluc*/Hygro]	Yes	"	No	No	Hygro	E6941
pGL4.77[*hRlucP*/Hygro]	Yes	"	hPEST	No	Hygro	E6951
pGL4.78[*hRlucCP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6961
pGL4.79[*hRluc*/Neo]	Yes	"	No	No	Neo	E6971
pGL4.80[*hRlucP*/Neo]	Yes	"	hPEST	No	Neo	E6981
pGL4.81[*hRlucCP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6991
pGL4.82[*hRluc*/Puro]	Yes	"	No	No	Puro	E7501
pGL4.83[*hRlucP*/Puro]	Yes	"	hPEST	No	Puro	E7511
pGL4.84[*hRlucCP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E7521

[A]*luc2* = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene.

[a]Certain applications of this product may require licenses from others.

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