

CHAPTER 17

Use of *E. coli lacZ* (β -Galactosidase) as a Reporter Gene

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1. Introduction

Our understanding of the molecular mechanisms that govern gene expression has been facilitated by the ability to introduce recombinant DNA molecules into heterologous cellular systems both in vitro and in vivo. One approach to defining DNA sequences important in the regulation of gene expression is to place controlling elements (e.g., promoter/enhancer sequences) upstream of a DNA coding sequence, introduce these constructs into transgenic animals or cells in culture, and analyze the levels of gene product produced by the introduced construct. Ideally, such a *reporter gene* should encode a product that is stable, and innocuous to the cell or organism in which it is being expressed, and should be readily detectable, even when present in small quantities.

Extensive genetic and biochemical characterization of the *Escherichia coli* (*E. coli*) *lacZ* gene, which encodes the glycoside hydrolase, β -D-galactosidase (β -gal) (EC 3.2.1.23), makes it an ideal choice for use as a reporter gene, and *lacZ* has been used in a variety of systems, including bacteria and yeast (1); cultured cells from mammals (2-8), avians (9), and insects (10); nematodes (11); adult mice (12); developing insect larvae (13-15); and

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murine embryos (16-19). The 3081-bp sequence of the bacterial *lacZ* gene has been determined (20). The first 27 amino acids of the protein can be replaced without affecting enzyme activity (1), allowing translational fusions to be made between either *N*-terminal eukaryotic translation initiation signals or heterologous gene sequences encoding extensive protein domains and the truncated β -gal gene product. Commercially available antibodies to β -gal allow immunochemical localization and detection of β -gal (and β -gal fusion proteins) in fixed cells and cell extracts. Finally, there are a number of chromogenic and fluorogenic substrates and biochemical assays that enable rapid and sensitive detection of β -gal (as few as 5 mol of β -gal in a single viable cell).

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simplified?

Four assays for biochemical detection of β -gal are presented in detail here. The choice of the particular assay system is dependent on experimental need and instrumentation available. The first two assays are used to measure β -gal activity in cell extracts (Table 1). A third assay is presented for histochemical localization of β -gal activity in fixed cells or tissue sections by either light microscope visualization or transmission electron microscopy. Finally, we detail the previously published FACS-gal assay (6, 7) which can quantitate β -gal expression with great sensitivity in viable cells on a cell-by-cell basis. FACS-gal quantitation of *lacZ* expression in single viable cells can be performed in concert with all the standard features of the fluorescence-activated cell sorter (FACS): cloning, sorting, analysis of the distribution of gene expression within a population of cells, and multiparameter analysis.

1.1. Spectrophotometric Assay Using ONPG

A simple and rapid assay to detect β -gal activity relies on the conversion of *O*-nitrophenol- β -D-galactoside (ONPG), a galactoside analog, to yield galactose and the chromophore *O*-nitrophenol (21). *O*-nitrophenol can be detected by measuring its absorbance at 420 nm. Using a spectrophotometer, the assay is sensitive and can be used when performing a quantitative determination of β -gal activity in cell extracts.

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1.2. Fluorometric Assay Using MUG

The most simple and rapid assay to detect β -gal activity in cell extracts relies on the conversion of 4-methyl-umbelliferyl- β -D-galactoside (MUG), a nonfluorescent galactoside analog, to the highly fluorescent molecule methylumbelliferone. The MUG product is measured using a fluorometer (excitation set at 350 nm, and fluorescence emission read at 450 nm). The assay is extremely sensitive and is useful for quantitative measurements of

Table 1
 β-Galactosidase Assays

Substrate	K_m^a	Instrument	Sensitivity ^b
Cell Extracts:			
MUG	170 μM	Fluorometer	10 ⁶
ONPG	110 μM	Spectrophotometer	10 ⁹
Intact Individual Cells:			
X-Gal		Microscope	10 ⁵
FDG	17 μM	FACS	1-10

^aBinding constant; references: MUG, M. Roederer, unpublished data; ONPG, (32); FDG, (34).

^bSensitivity is expressed as the minimum number of enzyme molecules required to obtain a measurable signal; thus, smaller numbers indicate greater sensitivity.

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β-gal levels in cell populations. Only a few thousand cells are required for accurate determinations. The assay is particularly useful for screening many populations by using conventional microtiter dish fluorescence readers, such as a Fluoroskan (Flow Laboratories). It has the widest dynamic range of the β-gal assays, allowing quantitation of activity in cell extracts having an average of 5 mol-10⁶ mol of β-gal/cell.

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 assay?

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1.3. Histochemical Assay Using X-Gal

Detection of β-gal activity *in situ* within cells in culture or in organs from transgenic animals can be performed using a histochemical stain (22) (see Figs. 1 and 2). This technique has been used to analyze cell lineages and patterns of gene expression within various tissues of eukaryotes during their development, e.g., rat nervous system (23), *Drosophila* embryos (13-15), murine embryos (16-18), and nematodes (11), and in cell-culture systems to study aspects of viral gene expression (5,6,9,24-26). In conjunction with plasmids that express β-gal in mammalian cells (27), this stain can also be used to assist in the identification of optimum conditions for DNA transfection or electroporation (see Fig. 1). The substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), is hydrolyzed by β-galactosidases to generate galactose and soluble indoxyl molecules, which in turn are converted to insoluble indigo. The deep blue color generated by the hydrolysis of X-gal by β-gal facilitates cellular localization of the β-gal activity.

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Cells or tissues to be stained are first washed in phosphate-buffered saline (PBS) and fixed using an appropriate fixative (e.g., gluteraldehyde/paraformaldehyde). The components of the stain are

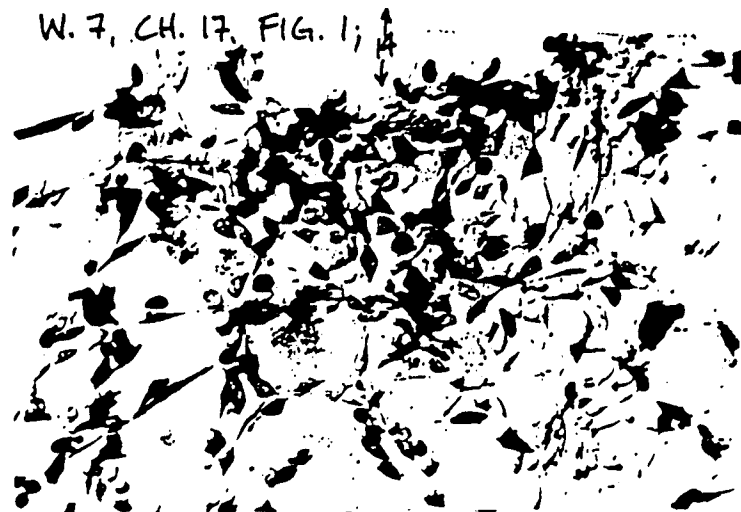


Fig. 1. GP +env AM12 cells expressing *E. coli* β -gal detected by X-gal histochemical stain. GP +env AM12 cells, a Moloney murine leukemia retroviral packaging cell line derived from NIH 3T3 cells, were electroporated with pCMV β (27), a vector that expresses *E. coli* β -gal under control of the human cytomegalovirus immediate early promoter. Forty-eight hours postelectroporation, cells were fixed and stained for β -gal activity with the X-gal stain as described in the text. Cells expressing β -gal at different levels can be seen as staining different shades of blue (gray). Cells not expressing β -gal, or expressing at a level below the sensitivity of the stain, appear clear. This illustrates one of the uses of this technique to assist in optimizing conditions for electroporation of different cell lines (see Chapter 5). Magnification 400 \times .

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1. Sodium phosphate to buffer the pH of the system and to provide sodium ions, which activate the β -gal enzyme;
2. Magnesium ions, a cofactor for the enzyme;
3. Potassium ferrocyanide and potassium ferricyanide, which together act as an oxidation catalyst to increase the rate of conversion of the soluble (and therefore diffusible) indoxyl molecules to the insoluble indigo form, thereby enhancing localization of the blue color; and
4. X-gal, the chromogenic indicator.

The stain is prepared and used to cover the fixed cells of whole or sectioned tissues. Following an incubation period, the indigo dye can be seen by eye or low-power microscopy. Further, the insoluble indigo dye precipitated in this manner is electron-dense, facilitating fine analysis of subcellular localization of β -gal fusion proteins by electron microscopy (see Notes). Although this technique for detection of β -gal activity is not as sensitive as an immunocytological method (5) or FACS-gal (6), it has the advantage relative to the ONPG and MUG methods that it can detect a single cell expressing β -gal within a population of nonexpressing cells.

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Callouts...



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Fig. 2. Histochemical-based detection of *E. coli* β -gal in muscle of a transgenic mouse using X-gal stain. Leg muscle from an F2 transgenic mouse (G. R. MacGregor and P. A. Overbeek, unpublished observations), the transgene of which is composed of the Rous (avian) sarcoma virus long terminal repeat juxtaposed to the *E. coli* β -gal gene with RNA processing signals derived from SV40, fixed and stained for β -gal activity using X-gal as described in the text. Following staining, the tissue was embedded in plastic, sectioned, and counterstained with hematoxylin and eosin. Note the heterogeneity of β -gal expression as detected by the X-gal stain. It is probable that additional myofibers contain β -gal activity at levels below the sensitivity of detection afforded by this technique. Magnification (A) 40 \times , (B) 400 \times .

1.4. Analysis of β -Gal Expression in Viable Cells Using FACS-Gal

Of the four assays described here, the analysis of β -gal expression in cultured cells using fluorescein di- β -D-galactoside (FDG) and a FACS is the most powerful (6, 7). The assay relies on the hydrolysis of FDG by β -gal inside viable cells and the subsequent detection of the product, fluorescein, with the laser excitation and detection systems of the FACS. Cells to be analyzed are loaded with FDG by brief hypotonic shock at 37°C for 1 min. At the end of 1 min, the cells are returned to isotonic conditions by dilution into ice-

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cold media. Although fluorescein passes through the cell membrane 200 times faster at 37°C than at 5°C, the V_{max} of β -gal is reduced only about 10-fold by this temperature change. Thus, by lowering the temperature following loading of cells with FDG, β -gal activity proceeds without leakage of the fluorescein or FDG substrate from cells, thereby permitting discrimination between β -gal-positive and -negative subpopulations of cells (see Fig. 3). However, if the cells are allowed to warm to above 15°C, the fluorescein generated in β -gal-positive cells will leak through the cell membrane and be taken up by other cells, including β -gal⁻ cells, yielding a population that stains homogeneously positive for β -gal expression when analyzed by FACS.

The FACS-gal assay is unique in that cells can be analyzed, sorted, and cloned using the FACS on the basis of their level of β -gal activity and are viable throughout the process. Furthermore, it is both quantitative and extremely sensitive—under standard conditions, as few as 5 mol in a single cell can be detected. FACS-gal is more sensitive than immunocytochemical methods or the X-gal histochemical stain—for example, approx 1000 mol are necessary for a cell to be stained homogeneously blue by X-gal (Table 1). The fluorescence observed (fluorescein produced) is monotonically related to the number of β -gal tetramers present in the cell; calculations (MR, GPN, SF, and LAH) indicate that fluorescence = (number of β -gal tetramers)^{1.8} × constant × time. There exist many applications for this technique, including analysis of DNA transcription rates, studies of cellular epigenetic effects, transgene studies in immune lineage cells correlated to expression of cell-surface differentiation markers, and isolation of control elements for gene expression (e.g., using an “enhancer trap” system).

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2. Materials

2.1. ONPG Analysis

1. PM-2 buffer (9): 33 mM NaH₂PO₄, 66 mM Na₂HPO₄, 0.1 mM MnCl₂, 2 mM MgSO₄, 40mM β -mercaptoethanol (stock is 14.2 M), pH 7.3. Filter the solution through a 0.45- μ m disposable filter. It is stable at 4°C for up to 1 mo.
2. O-Nitrophenyl- β -D-galactopyranoside (ONPG, Sigma cat. no. N-1127). Dissolve at 4mg/mL in PM-2 buffer. Warm at 37°C, with vortexing, to form solution. Make fresh for each assay.
3. 1M Na₂CO₃.
4. Protein assay kit (BioRad cat. no. 500-0006 or equivalent).
5. Bovine serum albumin (50 mg/mL solution from BRL, catalogue no. 5561UA, or equivalent).

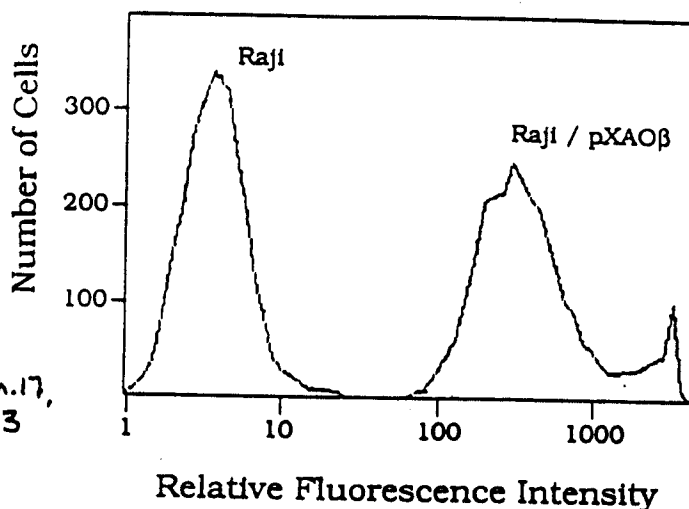
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β -Galactosidase Reporter Gene

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Fig. 3

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Fig. 3. Detection of human B lymphoblastoid cells expressing *E. coli* β -gal using FACS-FDG. Human Raji B lymphoblastoid cells were electroporated with an Epstein-Barr virus origin of replication episomal cDNA expression vector (G418 resistant) expressing the *E. coli* β -gal gene (pXAO β , G. R. MacGregor, unpublished results) and a population of stable transfectants generated by selection with G418. This population was analyzed for expression of β -gal using the FACS-FDG method and chloroquine treatment as described in the text. The figure shows the result of an experiment in which equal numbers of parental Raji cells and the polyclonal Raji/pXAO β population were mixed and analyzed for β -gal expression using FACS-FDG. Unelectroporated Raji cells can be distinguished with ease from the polyclonal population of Raji cells expressing the β -gal gene. Note the heterogeneity of expression revealed within the population by this method.

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6. Disposable plastic cuvetts (1 mL) with 10-mm light path.
7. Phosphate-buffered saline (PBS): 15 mM Na Phosphate, pH7.3; 150 mM NaCl.

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Na Phosphate
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2.2. MUG Analysis

1. Z-buffer: 60 mM Na₂PO₄·7H₂O; 40 mM NaH₂PO₄·H₂O; 10 mM KCl; 1 mM MgSO₄·7H₂O. Adjust pH to 7.0 with NaOH or HCl.
2. 1% TX-100: 1 mL Triton X-100 detergent in 100 mL (final vol) H₂O.
3. MUG stock: 3 mM methylumbelliferyl- β -D-galactoside (Sigma cat. no. M-1633) in Z-buffer. MUG at this concentration is not soluble at room temperature. To prepare the stock solution, add the appropriate amount of solid to Z-buffer and heat to boiling to form solution (should

take 5-10 min). Aliquot into 1-mL tubes, and freeze until use. Just before starting the reaction, dissolve the stock by putting it in boiling water (it should be completely in solution in 1-2 min at 80°C). Do not let the stock sit at room temperature for >10-15 min before using, since it may precipitate. If it does, simply reheat.

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4. STOP buffer: 300 mM Glycine; 15 mM EDTA; adjust pH to 11.2 with NaOH. The pH of this solution is critical: make sure that a 1:2 dilution of STOP with Z-buffer yields a pH of 10.5 or greater.

2.3. X-Gal-Based Histochemical Assay

1. Stock solutions of 1M Na₂HPO₄, 1M NaH₂PO₄, and 1M MgCl₂: Prepare in double-distilled (dd) or Millipore milliQ-grade H₂O and stored at room temperature.
2. Stock solutions of 50 mM potassium ferricyanide (K₃Fe [CN]₆) and 50 mM potassium ferrocyanide (K₄Fe [CN]₆): Prepare in ddH₂O and store in foil-wrapped glassware (in the dark) at 4°C, where they are stable for at least 3 mo.
3. X-Gal stock: Dissolve in N,N-dimethyl formamide at 20 mg/mL and store in a glass container (not polycarbonate or polystyrene) in the dark at -20°C.

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0.05%
formaldehyde

4. 4% paraformaldehyde (Wear a mask and gloves when handling paraformaldehyde): In a fume hood, dissolve 8 g of powder in 150 mL of 0.1M sodium phosphate, pH 7.3 (66 mM Na₂HPO₄ or 33 mM NaH₂PO₄), stirring and heating to 60°C. Add 10N NaOH at a rate of 1 drop/min until the solution clears. Bring the volume to 200 mL with 0.1M sodium phosphate, pH 7.3. Store at 4°C for up to 1 mo.

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5. Gluteraldehyde (Fischer) is purchased as a 25% solution.
6. To prepare the working fixative (2% paraformaldehyde/0.2% gluteraldehyde), combine 50mL of 4% paraformaldehyde with 49.2 mL of 0.1M sodium phosphate, pH 7.3, and 0.8 mL of 25% gluteraldehyde. This can be stored at 4°C for up to 1 wk.

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7. X-Gal stain: 100 mM sodium phosphate, pH 7.3 (66 mM Na₂HPO₄, 33 mM NaH₂PO₄), 1.3 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, and 1mg/mL X-Gal. Filter through a 0.45-µm disposable filtration unit prior to use.

8. Phosphate-buffered saline (PBS): 15 mM sodium phosphate, pH 7.3; 150 mM NaCl.

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section 2.1,
#1.

1) Pour media off cells
 2) 5 minutes in PBS @ 0.05% formaldehyde on ice
 3) 2 washes w/ PBS.
 4) Add ferr/ferr cyanide w/ 1mg/ml X-gal.
 240µL 50mg/mL into 12mL ferriferous cyanide
 500x glutaraldehyde
 8hrs to overnight in incubator
 view w/out filter
 DMSO @ 50mg/ml

2.4. FACS-Gal Analysis

1. Staining media: PBS (15 mM NaPO₄, pH 7.3; 150 mM NaCl) containing 10 mM HEPES, pH 7.3; 4% fetal calf serum.
2. Fluorescein-di- β -galactoside (FDG) was obtained from Molecular Probes, PO Box 22010, Oregon 97402 (cat. no. F-1179). The FDG powder should be a very pale yellow. A dark yellow color indicates the presence of fluorescent contaminants. FDG powder is unstable and will hydrolyze spontaneously over a period of time even if desiccated and kept at -20°C. Therefore, prepare a stock solution as soon as it is received. As a solution, FDG is stable for many months. The 200 mM stock solution is made by dissolving 5 mg of FDG in 38 μ L of 1:1 H₂O:DMSO. Mixing DMSO and H₂O is exothermic, so it is necessary to cool the mixture before adding FDG. This stock should be yellow in color and will remain liquid at -20°C. To prepare a 2 mM working solution, remove 10 μ L and add to 990 μ L of sterile water in a sterile Eppendorf tube. Since 2 mM is near the maximum solubility for FDG, place the solution in a 37°C water bath for about 10 min to dissolve the FDG completely, ensuring that no precipitate remains. The solution should not be held at 37°C for extended periods of time, since this will enhance the rate of spontaneous hydrolysis of the FDG. This solution can be frozen and thawed as required and should be faint yellow in color. Alternatively, one can prepare many tubes of 2 mM FDG solution in aliquots of 1-2 mL and store them, frozen, until needed.

FDG solutions can be contaminated with minor quantities of fluorescent monogalactoside and/or fluorescein, both of which will contribute to background fluorescence. Although quality control ensures that the FDG prepared by Molecular Probes contains <10 ppm fluorescein-equivalent contaminants (an acceptable level for all FACS-related purposes), it is sometimes desirable to remove any background fluorescent components in the FDG stock solution prior to staining cells. This is achieved by photobleaching these fluorescent molecules by holding the Eppendorf tube containing the 2 mM solution directly in the path of the FACS 488-nm argon laser for 1 min or more (move the tube slowly to avoid melting the plastic). Hold the tube in such a manner that the laser strikes the solution at the meniscus. This will ensure optimal spread of the light throughout the solution. **LASER GOGGLES MUST**

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BE WORN DURING THIS PROCEDURE. After this treatment, the solution can be freeze-thawed without rebleaching.

3. Propidium iodide.
4. Nylon screen (optional): Cut 2-in.-square pieces of nylon screen. For sterile work, wrap these in aluminum foil and autoclave. Nitex monofilament screen cloth, cat. no. HD-3-85, was obtained from Fairmont Fabrics, PO Box 1515, Pacifica, CA 94044.

3. Methods

3.1. ONPG Analysis

1. Disperse cells by treatment with trypsin (if adherent) or pelleted (if suspension) and wash once with PBS.
2. Resuspend pelleted cells in PM-2 buffer by vortexing. As a guide, we routinely resuspend 10^6 cells in 0.5–1 mL of PM-2. Transfer to a 1.5-mL Eppendorf tube.
3. Prepare cell extracts by 5 freeze-thaw cycles using liquid nitrogen (or dry ice/ethanol) and a 37°C water bath (*see* Note 3, section 4.1). Vortex samples briefly between each cycle. Following this treatment, spin the samples at 13,500g for 15 min at 4°C to pellet cellular debris.
4. Transfer the supernatant containing soluble protein to fresh Eppendorf tubes and place on ice.
5. Estimate the soluble protein concentration. A suggested technique is that of Bradford (28, *see also* Volume 3, this series), using a commercially available kit (BioRad). Values to generate a standard curve are obtained using BSA standards.
6. Dilute equal amounts of protein from cell extracts in PM-2 buffer to a final volume of 800 μ L and place in disposable 1-mL plastic cuvetts (*see* Note 1, section 4.1). Prepare two negative controls, one with BSA, the other with extract derived from untransfected cells.
7. Incubate the ONPG solution and the samples in the cuvetts separately at 37°C for 15 min to allow temperature equilibration.
8. Initiate the reaction by the addition of 200 μ L of prewarmed ONPG to each cuvet containing protein extracts. Cap the cuvetts with parafilm and invert several times to ensure thorough mixing of the enzyme and substrate. Note the exact time of addition of ONPG. Perform enzyme incubations at 37°C.
9. Monitor the assays visually until a yellow color becomes apparent. For best results, this should give an absorbance at 420 nm of between 0.1 and 1.0.

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"pelleting"?

β -Galactosidase Reporter Gene

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10. Add 500 μ L of 1M Na_2CO_3 to each cuvet. This adjusts the pH of the reaction to approx 11, stopping further enzymatic conversion of ONPG. Note carefully the duration (in minutes and seconds) of each reaction (*see* Note 2, section 4.1).
11. Read the sample absorbance at 420 nm. Calculate β -gal activity as follows: $U = (380 \times A_{420})$ divided by time (in minutes), where 380 is a constant such that 1 U is equivalent to conversion of 1 nmol of ONPG/min at 37°C (9).

3.2. MUG Analysis

1. Resuspend cells to be assayed in Z-buffer. A reasonable starting concentration is about 5×10^5 /mL. Deposit up to 105 μ L of the cell suspension in a well of a microtiter dish (*see* Notes 1-6, section 4.2).
2. Add 15 μ L of 1% TX-100 to each well (final concentration: 0.1%). Let these incubate for 5-10 min to solubilize the cells completely.
3. Add 30 μ L of 3 mM MUG to each well. Time each reaction.
4. Add 75 μ L of STOP to each well (1:2 dilution) after appropriate incubation time (*see* Note 7, section 4.2). The STOP solution not only stops the reaction completely, but also increases the methylumbelliferone fluorescence sixfold.
5. Measure the fluorescence of the reactions (excitation set at 350 nm and fluorescence emission read at 450 nm).

3.3. X-Gal-based Histochemical Assay

3.3.1. Adherent Cells

1. Aspirate media from the monolayers of cells to be assayed, and rinse gently but thoroughly twice with PBS.
2. Overlay the cells with fixative in a tissue-culture dish with fixative and incubate at 4°C for 5 min. (*see* Note 7, section 4.3)
3. Aspirate the fixative and rinse gently twice with room temperature PBS.
4. Aspirate the PBS and overlay the fixed cells with X-gal stain.
5. *See* steps 5-6 of section 3.3.2.

3.3.2. Suspension Cells

1. Spin down the cells (from 10^4 to 10^7). Wash once with 2 mL of PBS. Aspirate PBS.
2. Agitate the tube to dissociate the pellet. Add 1 mL of fixative and incubate at 4°C for 5 min.
3. Spin down the cells and aspirate the fixative. Wash twice with 2 mL PBS. Aspirate the PBS.

4. Resuspend the fixed cells in 2 mL of X-gal stain. Transfer cells to 24-well culture dishes.
5. Replace the tissue-culture-dish lids and incubate in a humidified incubator at 37°C until a blue color develops (this may take anywhere from 30 min to overnight).
6. View the cells under a microscope. Cells expressing β -gal that is detectable by X-gal will appear blue (*see* Figs. 1 and 2).

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3.4. FACS-Gal Analysis

3.4.1. Cell Preparation

1. Populations of cells expressing *E. coli* β -gal are harvested from exponentially growing cultures prior to staining (*see* Note 1, section 4.4). Trypsinize adherent cells or spin down suspension cells.
2. Resuspend the cells in staining media and pipet to obtain a single-cell suspension. When working with FACS, cells must be in a single-cell suspension. If the cells are clumped as a result of inadequate trypsinization, cell death, or other reasons, clumps must be removed prior to running the analysis by passing the cells through a nylon screen.
3. Adjust the cell suspension to 10^7 cells/mL (*see* Note 2, section 4.4).
4. Aliquot 50 μ L of cells into a 4-mL FACS tube (Becton Dickinson cat. no. 2058) and place cells on ice.

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3.4.2. Staining for *E. coli* β -Gal Activity

1. Place the cells in the FACS tube in a 37°C water bath for 10 min.
2. Add to the cells 50 μ L of 2 mM FDG in H₂O prewarmed to 37°C and mix rapidly and thoroughly (important). This part of the reaction can be scaled up or down without cause for concern. However, it is important that an equal volume of cells and 2 mM FDG in H₂O be used to generate the hypotonic shock.
3. Return the cells to a 37°C water bath for exactly 1 min. The staining procedure relies on an osmotic shock of the cells during the 1 min at 37°C; FDG is being taken up into the cells by passive osmotic loading.
4. Stop FDG loading at the end of 1 min by adding 2-mL of ICE-COLD staining media with 1 μ g/mL propidium iodide. Use ice-cold pipets or tips to aliquot the staining media into the tube containing the cells. The uptake of FDG is stopped and the substrate and products locked in the cells by a rapid dilution into cold isotonic staining medium. This one step brings the cells back to isoosmotic conditions, stops the loading, and "freezes" the cell membrane, thereby locking the substrate and products inside the cell. Use propidium iodide to facilitate live/dead

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to what? or
equal volumes
of cells + FDG?

cell discrimination on FACS. It is necessary to gate out dead (propidium iodide bright) cells as these can interfere with the analysis.

5. Keep the cells on ice until ready to perform FACS analysis. The enzymatic conversion of FDG to fluorescein proceeds even though the cells are on ice. Any amount of enzyme will hydrolyze all of the available substrate, given enough time, yielding a homogeneous fluorescence distribution representing the amount of FDG loaded per cell. If one is interested only in qualitative discrimination of β -gal-expressing cells from nonexpressing cells, the above protocol is sufficient. For fluorescence-to-enzyme-content correlations, one must ensure that each sample is analyzed at the same time after loading and before the substrate levels are exhausted. Alternatively, one can use a competitive inhibitor of *E. coli* β -gal to stop or slow the reaction (*see Note 3*).

3.4.3. Special FACS Requirements for FACS-Gal

1. Set up and calibrate the machine to detect fluorescein, propidium iodide, and forward scatter. The FACS facility should be able to handle most requirements as outlined for FACS-Gal. A useful reference is Parks et al. (30, *see also* Chapter 29).
2. Using unstained cells identical to the type to be analyzed, set the autofluorescence compensation using the method of Alberti et al. (31). Cultured cells, adherent cells especially, and some cell types from isolated tissues have high levels of autofluorescence when excited at 488 nm. This autofluorescence is caused primarily by cellular compounds (e.g., NADPH, complex hydrocarbons, and so on) that are excited at 488 nm and can emit across a broad range of wavelengths. It is essential, therefore, that autofluorescence compensation is set to perform accurate quantitation of β -gal activity. This is especially true when low levels of β -gal activity are being analyzed.

4. Notes

4.1. ONPG Analysis

1. To generate accurate readings for β -gal activity, it is important that the substrate should never be limiting during the reaction. To ensure that this is the case, duplicate reactions should be performed for each sample with two concentrations of protein extract. Absorbance values should be directly related to the concentration of cell extract used in each sample. For best results, the use of a spectrophotometer with a kinetics software accessory that permits the calculation of rates of

substrate conversion is recommended (e.g., Beckman DU-60 series with Kinetics Soft-Pac™ module and thermostatted cuvet jacket).

2. At pH 11, β -gal activity continues, albeit extremely slowly. Samples should be read shortly after completing assays and not left for extended periods (e.g., overnight) before reading.
3. Repeated freeze-thawing can lead to inactivation of β -gal. It is also possible to lyse cells by an addition of Triton-X100, to a final concentration of 0.1% (see MUG procedure).

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"Beckman"
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4.2. MUG Analysis

1. This protocol is designed for use with a fluorescence microtiter plate reader, although it can be easily adapted to be read by any fluorometer. The advantages of such a plate reader are many, including the ability to easily read 100- to 300- μ L vols and to read 96 wells in 2-3 min. Thus, fluorescence microtiter plate readers are well-suited for performing large-scale screenings and/or quantitations of cell lines or reaction conditions. For use with a fluorometer, the reaction volume may be scaled up appropriately.
2. Cells also may be sorted directly from a FACS into wells. The advantage of this method is that a precise number of cells can be deposited, allowing extremely accurate quantitation. When using this method, deposit no more than 50 μ L of cells in each well (generally about 20,000 cells, depending on the nozzle diameter), and dilute to 105 μ L with Z-buffer. As a control well, deposit an equal volume of sheath fluid from the sorter and dilute with Z-buffer.
3. Include appropriate controls for MUG autohydrolysis. Although boiling the MUG does not result in significant hydrolysis, it can convert at a slow rate in Z-buffer during extended reactions.
4. The autofluorescence contribution of the lysed cells is negligible. However, autofluorescence can become significant with a large number of lysed cells/well (usually greater than 200,000 cells/well). An appropriate control for all experiments is to deposit an equal number of parental (*LacZ*⁻) cells and measure their activity. This also controls for the contribution of the endogenous β -gal to the hydrolysis.
5. Because of inner-filter effect (quenching of fluorescence at high fluorescent product concentrations), it is important to standardize the measurements to ascertain that they are within a linear range. The reactions are easily standardized against dilutions of purified enzyme.

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Time-points (kinetics) should be chosen to ascertain the linearity of the reaction.

6. The reaction can be run at temperatures between 4 and 37°C. The hydrolysis rate is approx six-fold faster at room temperature than at 4°C, and again about four-fold faster at 37°C than at room temperature.
7. For 20,000 cells of a typical stably transfected cell line, a 1-h incubation at 37°C should be sufficient to obtain a reasonable fluorescence signal. The assay has been used successfully to quantitate β -gal activity in cells with an average of 5 mol of enzyme/cell, as well as in cells with over 800,000 mol of enzyme/cell. In the latter case, <100 cells/well were needed to quantitate the level of enzyme.
8. For transient assays, in which the number of transfected cells can be a small fraction of the total cell number, keep in mind that it is necessary to scale up the reaction to have sufficient activity for measurement.

4.3. X-Gal-based Histochemical Assay

1. Often we have noted a heterogeneity of staining within cell populations, both clonal and polyclonal (5; Figs. 1 and 2, this work). This is not a result of local variations in the permeability of cells for the stain, but reflects a true fluctuation in the level of β -gal activity on a per-cell basis. However, as quantitation of β -gal is relatively inaccurate using this technique, with cultured cells the investigator is advised to utilize the FACS-gal method for more accurate quantitation.
2. Fixation of whole tissues is performed essentially as described (19). Briefly, fixation is prolonged for 1-2 h, and sodium deoxycholate and NP-40 are added to the fixative, to final concentrations of 0.01 and 0.02%, respectively, to enhance permeability of the tissue. In addition, tissues are incubated with X-gal stain at 30°C instead of 37°C in order to reduce background staining and minimize tissue damage. Following a rinse with 3% DMSO in PBS, tissues are stored in PBS prior to imbedding and sectioning.
3. With certain tissues we have encountered problems with a high background resulting from nonspecific hydrolysis of the substrate that leads to weak false positives. For example, with a transgenic line expressing β -gal under control of the Rous sarcoma virus promoter, although expression of the β -gal gene could be detected in the testes of transgenics by Northern analysis, no difference could be detected in the intensity of blue color observed between transgenic and nontransgenic

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littermates' testes (G.R. MacGregor and P.A. Overbeek, unpublished observations). Certain tissues, for example, kidney, testes, spleen, and pancreas of the mouse, appear to have higher backgrounds of β -galactosidase activity than others.

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4. Commercially available antibodies (from Promega Biotech, Madison, WI) have been shown to have greater sensitivity in the detection of *E. coli* β -gal activity than the X-gal stain (5). We have found that the antibody (a mouse monoclonal) works well for detection of β -gal within cultured cells (5), but has failed to detect the enzyme in serial tissue sections from transgenics that have been shown to be expressing β -gal by the histochemical (X-gal) method.
5. The indigo precipitate prepared by the above procedure is electron-dense and can be clearly revealed by transmission electron microscopy (EM) (29). It is therefore possible to obtain precise subcellular localization of β -gal fusion proteins. To prepare cells for EM, stain for X-gal as above. Carry out standard cell preparation of EM. However, since the precipitated indigo dye is soluble in propylene oxide (a general reagent used in the preparation of sections for transmission EM), transfer cells from 100% ethanol directly to resin without propylene oxide treatment.
6. We have been unable to detect β -gal immunocytochemically following treatment of the cells with X-gal.
7. The fixative described here works well for NIH 3T3 cells (see Fig. 1). However, for other cell types, alternative fixatives may give better retention of cell morphology.

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4.4. FACS-Gal Analysis

1. Keep cells as healthy as experimental conditions permit. We routinely maintain cells in a mid-log growth state for 1-2 d prior to analysis. Certain cell types, especially adherent cells, such as 293 or NIH 3T3, appear to have higher than usual endogenous β -D-galactosidase activity if they are abused or allowed to go to confluency. However, it is possible to reduce this background with a chloroquine treatment prior to analysis (see Note 4).
2. No dependency on the cell concentration has been found for staining patterns (using concentrations ranging from 10^5 /mL to 10^7 /mL).
3. Phenylethyl- β -D-thiogalactoside (PETG) (1 mM) has been found to be an ideal competitive reversible inhibitor of *E. coli* β -gal in viable mammalian cells (7,8). It has a low K_i (c_a , 1 μ M); thus very little is required

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β -Galactosidase Reporter Gene

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to inhibit the reaction. The thiol ester bond renders the reagent practically nonhydrolyzable by the enzyme, thereby simplifying its influence on the kinetics of the FDG-to-fluorescein conversion. Finally, it is hydrophobic and can cross the cell membrane readily, even at 4°C.

Dissolve PETG (Sigma cat. no. P-4902 and Molecular Probes P-1692) in H₂O to give a 50 mM stock. Filter-sterilize and dispense into 500 μ L aliquots. Store frozen at -20°C. Adding PETG to a final concentration of 1 mM, stops conversion of FDG to fluorescein in live cells. After a predetermined period of incubation on ice of cells loaded with FDG (from 1 min to 2 h, depending on expected β -gal activity), add 40 μ L of the 50 mMPETG and mix thoroughly (1 mMPETG can be used directly in the ice-cold isotonic loading termination solution to inhibit the reaction at the same time FDG loading is completed). The reaction is slowed to such an extent by 1 mM that there is almost no conversion of FDG to fluorescein over a 3-h period.

- Using the FACS-gal method, most cell lines examined have exhibited little or no background activity because of endogenous β -galactosidases. These include several B and T lymphocyte lines; splenic, thymic, peripheral, or bone marrow lymphocytes; most fibroblasts; and embryo carcinoma cells. However, several mammalian cell types, including macrophages and adherent lines (such as 293 and NIH 3T3), have significant endogenous β -galactosidase activity. This activity usually stems from lysosomal β -galactosidases. Since these β -galactosidases have acidic pH activity optima, they can be partially inactivated by pre-treating cells for 15 min prior to loading FDG with 300 μ M chloroquine (a weak lysosomotropic base) (35). Chloroquine is added (to a final concentration of 300 μ M) to the ice-cold isotonic "stop-loading" medium. Otherwise, the protocol for FACS-gal analysis is identical. Alternatively, cells can be treated with 300 μ M chloroquine added directly to culture media with actively dividing cells 2-3 h prior to performing the FACS-gal analysis, with essentially similar results.

- Some cell types, bacteria and yeast, for instance, cannot be hypotonically loaded because the cell wall constrains hypotonic expansion. To overcome this difficulty, it is possible to pretreat the cells with a brief *hypertonic* shock (to shrink the cells within their cell walls) and then to load the FDG with a subsequent hypotonic treatment. Osmotic conditions should be varied depending on the organisms' resilience; it is also necessary to carry out viability tests to ensure that your procedure does not harm the cells.

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6. Molecular Probes makes available two kits (F-1180 and F-1181) containing premixed, quality-controlled reagents and a detailed protocol to carry out the FACS-gal procedure.

Acknowledgments

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References

1. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., and Chou, J. (1983) β -galactosidase gene fusions for analysing gene expression in *E. coli* and yeast. *Methods Enzymol.* 100, 293-308.
2. An, G., Hidaka, K., and Siminovitch, L. (1982) Expression of bacterial β -galactosidase in animal cells. *Mol. Cell Biol.* 2, 1628-1632.
3. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) Expression and regulation of *E. coli lacZ* gene fusions in mammalian cells. *Mol. Appl. Genet.* 2, 101-109.
4. Nielsen, D. A., Chou, J., MacKrell, A. J., Casadaban, M. J., and Steiner, D. F. (1983) Expression of a pre-proinsulin- β -galactosidase gene fusion in mammalian cells. *Proc. Natl. Acad. Sci. USA* 80, 5198-5202.
5. MacGregor, G. R., Mogg, A. E., Burke, J. F., and Caskey, C. T. (1987) Histochemical staining of clonal mammalian cell lines expressing *E. coli* β -Galactosidase indicates heterogeneous expression of the bacterial gene. *Somat. Cell Mol. Genet.* 13, 253-265.
6. Nolan, G. P., Fiering, S., Nicolas, J.-F., and Herzenberg, L. A. (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β -D-galactosidase activity after transduction of *E. coli LacZ*. *Proc. Natl. Acad. Sci. USA* 85, 2603-2607.
7. Nolan, G. P. (1989) Individual cell gene regulation studies and *in situ* detection of transcriptionally-active chromatin using Fluorescence Activated Cell sorting with a viable cell fluorogenic assay. PhD Dissertation, Stanford University.
8. Kerr, W. G., Nolan, G. P., and Herzenberg, L. A. (1989) *In situ* detection of transcriptionally-active chromatin and genetic regulatory elements in individual viable mammalian cells. *Immunology* (in press).
9. Norton, P. A. and Coffin, J. M. (1985) Bacterial β -galactosidase as a marker of Rous sarcoma virus gene expression and replication. *Mol. Cell Biol.* 5, 281-290.
10. Pennock, G. D., Shoemaker, C., and Miller, L. K. (1984) Strong and regulated expression of *E. coli* β -galactosidase in insect cells with a baculovirus vector. *Mol. Cell Biol.* 4, 399-406.
11. Fire, A. (1986) Integrative transformation of *Caenorhabditis elegans*. *EMBO J.* 5, 2673-2680.

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12. Goring, D. R., Rossant, J., Clapoff, S., Breitman, M. L., and Tsui, L.-C. (1987) In situ detection of β -galactosidase in lenses of transgenic mice with a γ -crystallin / *lacZ* gene. *Science* 235, 456-458.
13. O'Kane, C. J. and Gehring, W. J. (1987) Detection in situ of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 84, 9123-9127.
14. Ghysen, A. and O'Kane, C. (1989) Neural enhancer like elements as specific cell markers in drosophila. *Development* 105, 35-52.
15. Lis, J. T., Simon, J. A., and Sutton, C. A. (1983) New heat shock puffs and β -galactosidase activity resulting from transformation of drosophila with an *hsp70-lacZ* hybrid gene. *Cell* 35, 403-410.
16. Bonnerot, C., Rocancourt, D., Briand, P., Grimber, G., and Nicolas, J.-F. (1987) A β -galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA* 84, 6795-6799.
17. Allen, N. D., Cran, D. G., Barton, S. C., Hettle, S., Reik, W., and Surani, M. A. (1988) Transgenes as probes for active chromosomal domains in mouse development. *Nature* 333, 852-855.
18. Kothary, R., Clapoff, S., Brown, A., Campbell, R., Peterson, A., and Rossant, J. (1988) A transgene containing *lacZ* inserted into the dystonia locus is expressed in neural tube. *Nature* 335, 435-437.
19. Sanes, J. R., Rubenstein, J. L. R., and Nicolas, J.-F. (1986) Use of recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO. J.* 5, 3133-3142.
20. Kalmins, A., Otto, K., Ruther, U., and Muller-Hill, B. (1983) Sequence of the *lacZ* gene of *E. coli*. *EMBO. J.* 2, 593-597.
21. Miller, J. H. (1972) Assay of β -galactosidase, in *Experiments in Molecular Genetics*. (J.H. Miller, ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 352-355.
22. Bondi, A., Chierigatti, G., Eusebi, V., Fulcheri, E., and Bussolati, G. (1982) The use of β -galactosidase as a tracer in immunocytochemistry. *Histochemistry* 76, 153-158.
23. Price, J., Turner, D., and Cepko, C. (1987) Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* 84, 156-160.
24. Chakrabarti, S., Brechling, K. and Moss, B. (1985) Vaccinia virus expression vector: Co-expression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell Biol.* 5, 3403-3409.
25. Geller, A. I. and Breakfield, X. O. (1988) A defective HSV-1 vector expresses *E. coli* β -galactosidase in cultured peripheral neurons. *Science* 241, 1667-1669.
26. Huang, C. H., Samsonoff, W. A., and Grzelecki, A. (1988) Vaccinia virus recombinants expressing an 11-kilodalton β -galactosidase fusion protein incorporates active β -galactosidase in virus particles. *J. Virol.* 62, 3855-3861.
27. MacGregor, G. R. and Caskey, C. T. (1989) Construction of plasmids that express *E. coli* β -galactosidase in mammalian cells. *Nucleic Acids Res.* 17, 2365.
28. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgramme quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72, 248-254.
29. Bonnerot, C., Rocancourt, D., Briand, P., Grimber, G., and Nicolas, J.F. (1987) A β -galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA* 84, 6795-6799.

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30. Parks, D. R., Lanier, L. L., and Herzenberg, L. A. (1986) in *The Handbook of Experimental Immunology*, 4th Ed. (Weir, D. M. and Herzenberg, L. A., eds.) Blackwell, Edinburgh, pp. 29.1-29.21.
31. Alberti, S., Parks, D. R., and Herzenberg, L. A. (1988) A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* 8, 114-119.
32. Wallenfels, K. and Weil, R. (1971) in *The Enzymes*, 3rd Ed. (Boyer, P., ed.) Academic, New York, pp. 617-663.
34. Hofmann, J. and Sernetz, M. (1983) A kinetic study on the enzymatic hydrolysis of fluorescein di-acetate and fluorescein - di- β -D galactopyranoside. *Anal. Biochem.* 131, 180-186.
35. Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA* 75, 3327-3331.