Enzyme-Generated Intracellular Fluorescence For Single-Cell Reporter Gene Analysis Utilizing Escherichia coli β-Glucuronidase

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We report the development of a new fluorescence-activated cell sorter (FACS)-based reporter gene system utilizing the enzymatic activity of the E. coli β-glucuronidase (gus) gene. When loaded with the Gus substrate fluorescein-di-β-D-glucuronide (FDGlc), individual mammalian cells expressing and translating gus mRNA liberate sufficient levels of intracellular fluorescein for quantitative analysis by flow cytometry. This assay can be used to FACS sort viable cells based on Gus enzymatic activity, and the efficacy of the assay can be measured independently by using a fluorometric lyse assay. Furthermore, both the β-glucuronidase and the previously described E. coli β-galactosidase enzymes have high specificities for their cognate substrates, allowing each reporter gene to be measured by FACS independently.

Key terms: β-Glucuronidase, β-galactosidase, reporter gene, fluorescence-activated cell sorting, selectable marker

The measurement of lacZ reporter gene activity on the fluorescence-activated cell sorter (FACS-Gal) has proven to be a useful tool in studies of gene regulation at the single-cell level (5,15,17). The FACS-Gal system permits the quantitative, sensitive analysis of gene expression on a single-cell basis and allows for simultaneous measurement of other cellular parameters, such as cell size, surface immunophenotype, DNA content, etc. (6). In addition, the sorting capability of the FACS can be used to isolate viable single cells based on lacZ expression, making the FACS-Gal assay a nonlethal selectable marker system as well as a quantitative reporter gene system. Recent technological advances in the methodologies of introducing genes into whole organisms has made the study of gene expression in vivo a reality. FACS-Gal can be used to isolate cells expressing lacZ from transgenic or chimeric animals, as long as those cells can be processed into single-cell suspensions (15). Soriano et al. have generated several murine transgenic strains with the lacZ gene under the control of endogenous promoters with ubiquitous expression patterns (7,26). β-gal Activity was detected in the lymphocytes of several of these transgenic lines, using the FACS-Gal system (26; W.T. Kerr, personal communication).

Although lacZ has proven to be of general utility in the study of developmentally regulated gene expression in mice (1), β-gal activity has not been detected in lymphocytes isolated from a large number of independently generated transgenic or chimeric mice, in which the lacZ gene was inserted into the genome under the control of promoters/enhancers known to be expressed in lymphocytes. This absence of activity has been described for lacZ under the control of tissue-specific (25) or ubiquitous (for review, see 5,22) regulatory elements. In several mice that were examined, lacZ mRNA was not detected, suggesting that the block is due to transcriptional silencing or to mRNA instability (5). The clear lack of β-gal expression in developing lymphocytes in many systems is an impediment to analysis of immune function. Establishment of a reporter system not subject to this limitation would be of general use to those interested in studying lymphocyte gene expression in vivo.

There are several requirements for an enzymatic reporter system to be useful for flow cytometry studies. These include 1) an enzyme with stable in vivo activity; 2) a fluorogenic substrate that can be loaded into viable

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cells which, upon hydrolysis, yields a highly fluorescent product that is retained in cells. 3) a low level of endogenous enzymatic activity for this fluorogenic substrate; and 4) low levels of cellular fluorescence at the wavelengths at which the product fluorescence is detected. We limited our search for new reporter genes to hydrolyses due to the availability of greater than 300 cloned and characterized members of this class of enzyme (10). Furthermore, interest in hydrolyses has led to the development of a number of commercially available fluorogenic hydrolytic substrates.

Of ten cloned hydrolyses, including cellulases, β-glucosidases, and E. coli β-glucuronidase (gus) only the Gus enzyme and a fluorescein-based substrate satisfied the above criteria. The gus gene has been used extensively as a reporter gene in plants, because there is no endogenous cellular glucuronidase activity in the majority of plants tested (11,12). Although high levels of lysosomal β-glucuronidase activity have been reported for a number of mammalian tissue types (19), we find very low Gus activity in lysates of several mammalian cell lines. In this study, we show that, in combination with FACS, a gus reporter gene system can be used both in quantitative expression studies and as a selectable marker. Furthermore, we show that the β-glucosidase and β-glucuronidase enzymes will hydrolyze only their cognate substrates, allowing for the development of a dual reporter system based on these hydrolyses.

MATERIALS AND METHODS

Cells and Tissue Culture

NIH 3T3 cells (ATCC CCL 163) and BOSC 23 retroviral producer cells (21) were maintained in Dulbecco's modified Eagle's medium (DMEM). 702/3 cells (ATCC TIB 155) were maintained in RPMI 1640 medium. All media were supplemented with 10% (v/v) 1:1 fetal calf/horse serum, 50 µM 2-mercaptoethanol, 100 units/ml penicillin, 0.05 mM streptomycin, and 2 mM glutamine. All cultures were maintained at 5% CO₂.

Transient Transfection and Retroviral Infections

BOSC 23 producer cells were plated at a density of 2 × 10⁵ cells per 60 mm plate in 3 ml of fresh supplemented DMEM. After 24 h, these cells were calcium-phosphate transfected, as previously described (21), with 5 µg of plasmid DNA. Forty-eight hours later, retroviral supernatant was harvested and pelleted (5 min at 1,200 g) to remove cell debris. One milliliter of retroviral supernatant was added to 10⁶ NIH 3T3 or 702/3 cells preincubated for 5 min in the presence of 10 µg/ml polybrene (21). After 8 h, the cells were washed and resuspended in polybrene-free growth medium, and cultured for at least 48 h prior to reporter gene expression analyses.

Chemicals

5-Bromo-4-chloro-3-indolylgalactoside (X-gal) was obtained from Sigma. 5-Bromo-4-chloro-3-indo glucuronide (X-Glcu), fluorescein-di-β-D-galactoside (FDG), fluorescein-di-β-D-glucuroni-
brightfield images, the emission band-pass filter was removed, leaving a 480 nm high-pass filter. Photographs were taken with a Minolta X-370 camera.

Specific Inhibition of \( \beta \)-Gluconoridase With 1,4 Saccharolactone (1,4-SL), and \( \beta \)-Gal With PETG

Prior to FDGlcu substrate loading, 100 \( \mu \)l of NIH 3T3 fibroblasts were resuspended in staining medium plus 1,4-SL and were incubated for 10 min at 37°C. The cells were maintained in 1,4-SL at a final concentration of 5 mM (unless otherwise indicated) throughout the course of the experiment. Cells were loaded as described above by mixing with 100 \( \mu \)l of a 2 mM FDGlcu + 1,4-SL solution. The cells were washed in the presence of the inhibitor and were resuspended in staining medium, including 1 \( \mu \)g/ml propidium iodide and 1,4-SL. Diminution of \( \beta \)-gal activity with PETG was carried out as described (12) by using a final concentration of 100 \( \mu \)M PETG.

RESULTS

Analysis of Candidate Reporter Genes

Lysate assays of \( E. \) coli harboring expression vector plasmids of ten candidate reporter enzymes revealed that only \( E. \) coli \( \beta \)-glucuronidase (Gus), the thermophilic bacterium Clostridium thermocellum cellulase (CelE, 9), and the Butyrivibrio fibrisolvens \( \beta \)-glucosidase (bga; 28), isolated from high-arctic Svalbard reindeer, showed high specificity for their cognate substrates (data not shown). The gus, celE, and bga genes were subsequently cloned into the Moloney-based retroviral expression vector MFG (4) and were tested for mammalian expression. Only the gus gene showed detectable activity when transfected into the human embryonic kidney 293 cell line (data not shown), and this gene was chosen for further analysis.

Expression of the \( \beta \)-Gluconoridase and \( \beta \)-Galactosidase Enzymes

To maximize expression of the \( E. \) coli gus gene in mammalian cells, several modifications were made at the 5' end of the gene by using polymerase chain reaction (PCR) mutagenesis. The 5' primer was designed to alter 1) the nucleotides surrounding the wild type met, codon to conform to the core Kozak consensus sequence for translational initiation (CCATGG) in mammalian cells (14) and 2) the second codon to encode valine, a stability conferring residue, as established by Varshavsky's N-end rule (2; Fig. 1A). The amplified 1,809-bp-pair, full-length gene was cloned into the MFG vector (Fig. 1B), permitting expression from the long terminal repeat (LTR) enhancer. Several clones tested positive for the gus insert by using PCR and restriction analysis, and they were isolated for further study. One of these plasmid clones (MFG-Gus6), when transiently transfected into mammalian 293 cells, showed \( \beta \)-glucuronidase activity, as determined by the MUGlcu assay. This plasmid was used in all further studies.

To study \( \beta \)-glucuronidase expression in mammalian cells, several cell lines with stable expression of the gus gene were generated by retroviral transfer. The MFG-Gus6 clone was transiently transfected into BOSC producer cells, and the viral supernatant was isolated 48 h postinfection. Subsequently, cells of the adherent murine fibroblast line NIH 3T3 were infected by using the viral supernatant. One week later, infected cells were tested for the efficiency of infection with the X-Glcu histologial stain (11,13; data not shown). Greater than 90% of the cells stained blue, indicating that most, if not all, of the target cells were stably infected with MFG-Gus6 and were expressing the reporter enzyme from an integrated provirus. In contrast, mock-infected cells showed no detectable X-Glcu staining.

Because the FDGlcu substrate is chemically similar to FDG, we reasoned that FDGlcu could be used as a protocol analogous to FACS-Gal to distinguish gus'"" from gus" cells based on the levels of fluorescein fluorescence. FACS analysis of FDGlcu-loaded, MFG-Gus6-infected cells showed two distinct populations at a ratio roughly equivalent to that revealed by the X-Glcu stain (data not shown). By using the single-cell deposition capability of the FACS (20), clones were generated by sorting MFG-Gus6-infected cells that fluoresced when loaded with FDGlcu. After expanding the cloned cells for 2 weeks, 30 clones were tested for \( \beta \)-glucuronidase activity by using the substrate MUGlcu in the quantitative cell lysate assay (22). All of the clones showed Gus activity above background levels (data not shown). The 3T3 MFG-Gus6-6 clone was chosen on the basis of a high level of expression for further study. A similar approach was used to isolate Gus" clones of the nonadherent murine...
Table 1
Determination of β-Gal, Gus, and Endogenous Enzymatic Activity in 702/3 Pre-B Cells and NIH 3T3 Fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>MFG-Gus6</th>
<th>MFG-lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>K₅₀ [µM]</td>
<td>702/3</td>
<td>3T3</td>
</tr>
<tr>
<td>MUG²</td>
<td>107</td>
<td>45</td>
<td>58</td>
</tr>
<tr>
<td>MUGluc</td>
<td>119</td>
<td>93</td>
<td>10</td>
</tr>
<tr>
<td>FDO²</td>
<td>17</td>
<td>2.9</td>
<td>1.3</td>
</tr>
<tr>
<td>FDOluc</td>
<td>133</td>
<td>7.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*K₅₀ values are expressed in µM substrate. The nonlinear least-squares regression coefficient was greater than 0.99 for each determination. β-gal, β-galactosidase; gus, β-glucuronidase.

²1 × 10⁴ 702/3 cells or 2 × 10⁴ 3T3 cells were harvested and lysed in Z-buffer. Lysates were incubated in the presence of methythumbelliferyl (MU) substrate for 24 min before the addition of stop buffer. The values indicated are relative fluorescence units, as measured by fluorometry, per lysed sample. Typically, errors in measurement are less than 10% using these assays. For substrate abbreviations, see Materials and Methods.

*Fluorescence-activated cell sorter (FACS) analysis of fluorescein substrate hydrolysis was conducted as described in Materials and Methods. Tabulated values show the median FACS fluorescence for each stained cell population, nd, not done.

pre-B 702/3 cell line as well as several β-gal¹ 3T3 and 702/3 clones.

Specificity of β-Glucuronidase and β-Galactosidase for Their Cognate Substrates

To establish whether Gus enzymatic activity can be detected independently of β-Gal enzymatic activity, and vice versa, we characterized the enzymatic specificities of both enzymes vs. several glucuronide- and galactoside-based substrates. To ensure that the initial rates of reaction were not dependent on substrate concentration, the K₅₀ values of gus with MUG and of β-galactosidase with MUGluc were in the range of 0.63 to 13.3 µM in the context of cell lysates were determined. A concentration of the substrate that was well above the K₅₀ value of either reporter enzyme, 0.63 mM (Table 1), was chosen as a suitable concentration for the lysate experiments.

β-Glucuronidase and β-galactosidase specificities were analyzed with the cell lysate assay using MU-based substrates and with the FACS-Gal assay using fluorescein-based substrates. Under both experimental conditions, 702/3 and 3T3 parental (untransformed) cells showed low or undetectable levels of enzymatic activity vs. these substrates (Table 1), indicating that the endogenous activity of either enzyme is very low. Furthermore, cells expressing β-gal or β-galactosidase hydrolyzed only the galactoside-based substrates (MUG and FDO) or the glucuronide-based substrates (MUGluc or FDOluc), respectively (Table 1). Similar results were found by using the chromogenic X-gal (25) and X-Gluc assays (data not shown). Thus, each of these enzymes can be quantitated independently of the expression of the other.

FACS Detection of NIH 3T3 gus* Cells Loaded With FDOluc

Our initial results showing that Gus activity could be detected in individual cells prompted us to explore the broader utility of this enzyme-substrate system. We used the FACS-Gal procedure to load NIH 3T3 parental cells, NIH 3T3 MFG-Gus6-6 cells, or a mixture of the two cells with FDOluc. We chose to use 2 mM FDOluc in the hypotonic loading step, because the K₅₀ of Gus for FDOluc is comparable to that of β-gal for FDG (Table 1), and 2 mM FDO is sufficient to detect β-gal in the FACS-Gal assay (6).

Consistent with the results of the lystate experiments, NIH 3T3 parental cells loaded with FDOluc are no more fluorescent than untransformed cells (Fig. 2A). However, gus* cells loaded with FDOluc developed fluorescence levels 40-fold greater than gus* parental cells (compare Fig. 2A and Fig. 2B). When a 1:1 mix of gus* and gus* cells is loaded with FDOluc, the two populations can be resolved clearly (Fig. 2C). Furthermore, there is no leakage of fluorescein from the gus* into the gus* cells, because the gus* cells loaded in the presence of gus* cells are no more fluorescent than gus* cells loaded alone (compare Fig. 2A and Fig. 2C). Thus, gus* cells generate significant fluorescein based on intracellular recombinate reporter protein expression. Comparable results were obtained by using β-gal in the FACS-Gal procedure (17).

Subcellular Localization of Fluorescein After Gus-Mediated FDOluc Hydrolysis

To determine the subcellular localization of the fluorescein product of Gus-mediated FDOluc hydrolysis, parental (Fig. 3A) or MFG-Gus6-6 (Fig. 3B) NIH 3T3 cells were loaded with FDOluc and analyzed by fluorescence microscopy. Parental cells show low or undetectable levels of green fluorescence (Fig. 3C). Any signal is likely to be due to autofluorescence, because cells imaged in the absence of substrate show a similar low fluorescence pattern (data not shown). In contrast, Gus* cells show bright, exclusively cytoplasmic fluorescence (Fig. 3D) when they are imaged under identical conditions.

Temperature Dependence of β-Glucuronidase Activity

An inherent difficulty in the use of fluorescein-based substrates is the fact that the cell membrane is permeable to fluorescein under physiological conditions. However, fluorescein leaks out of cells >200 times slower at 0°C than at 37°C (24). To avoid fluorescein staining of lacZ* cells as a result of leakage from lacZ* cells, the FACS-Gal protocol cells for incubation of FDG-loaded cells at 0°C rather than at 37°C after loading. Because β-gal still has appreciable enzymatic activity at 0°C (17), hydrolysis of the FDG substrate is not prevented under these conditions.

To establish conditions suitable both for β-glucuronidase catalyzed FDOluc hydrolysis and for intracellular fluorescein retention, the temperature dependence of the Gus enzyme was determined in an MU cell lysate assay (Fig. 4). Although Gus is not active at 0°C, the enzyme shows a reduction in activity of only twofold at 10°C vs. 37°C (Fig. 4). Thus, if hydrolysis beyond the first min of loading is required, then incubation can be carried on.
out at or above 10°C. To test whether fluorescein passes
the cell membrane at 10°C, 3T3 cells were loaded with
fluorescein, as described above for FDG, washed, and
incubated for up to 4 h at 0°C or at 10°C before FACS
analysis. Fluorescence levels of cells maintained at 10°C
were indistinguishable from those of cells maintained at
0°C after loading (data not shown), indicating that the
fluorescein product is retained within the cell at 10°C.

The Competitive β-Glucuronidase Inhibitor 1,4-SL
Inhibits Hydrolysis of the FDGlcu Substrate

In Vivo

To maintain a correlation between enzyme activity and
fluorescence, the enzymatic reaction must not lead to the
hydrolysis of all of the available substrate within a cell. In
our 3T3 gus+ clones, no time-dependent increase in the
fluorescence levels of stained cells was observed at 10°C
(data not shown), suggesting that these cells express lev-
els of Gus sufficient to hydrolyze all of the available FDG-
lcu during the loading step. Thus, the distribution of flu-
orescence levels seen in Figure 2B is not a reflection of
the enzymatic activity within the gus+ 3T3 clone but,
rather, it is an indication of the uniformity of loading of
the FDGlcu substrate.

Distinguishing between 3T3 cells expressing relatively
low vs. high levels of Gus requires slowing the reaction to
avoid the hydrolysis of all available substrate into fluo-
rescent product. A previously identified β-glucuronidase
inhibitor, 1,4-SL (16), was tested for its ability to inhibit
the reaction and its effect on the hydrolysis of FDGlcu by
β-glucuronidase in viable cells. Pools of MFG-Gus6-infec-
ted cells were hypotonically loaded with mixtures of 2
mM FDGlcu and several concentrations of 1,4-SL and
were analyzed by FACS. In the absence of inhibitor,
the median fluorescence of the gus+ cells was >30-fold
above background (Fig. 5B). In the presence of concen-
trations of 1,4-SL from 0.2 to 5.0 mM, there was a clear
concentration-dependent decrease in the fluorescence
distribution of the gus+ cells (Fig. 5C–E). These results
indicate that loading cells with 5 mM 1,4-SL sufficiently
inhibits β-glucuronidase activity to allow quantitative
analysis of expression by FACS.

Relative Gus Activity as Determined by FACS is
Correlated With Relative Activity as Determined by
the MUGlcu Lysis Assay

When loaded with FDGlcu in the presence of 1 mM
1,4-SL, the clonally mixed pool of 3T3 MFG-Gus6-in-
fected cells shows a broad distribution of FACS-measured
fluorescence (Fig. 5D). To test whether this distribution is
indicative of the relative Gus activity of cells within the
gus+ population, 13 fractions were sorted on the basis of
fluorescence fluorescence (Fig. 6A), such that each frac-
tion included cells of a unique, narrow range of fluores-
cence. The sorted populations were then lysed and as-
sayed by the MUGlcu assay to determine the relative
enzymatic activity of each fraction. Plotting the FACS-
determined mean fluorescence of each sorted sample
against the fluorescence as determined in the lysis assay
reveals a strong linear correlation between these inde-
pendent assays (Fig. 6B). However, the positive X-inter-
cept suggests that a fraction of the reporter enzyme is
accessible to substrate only upon lysis of the cell.
Fig. 3. Subcellular localization of the hydrolysis product, fluorescein. Cells were loaded with 2 mM FDGlcu for 2 min at 37°C and were processed as described in Materials and Methods. Brightfield images of loaded ST3 parental (A) or MG-Gus-G6 (B) cells were taken by using differential interference contrast optics with exposures of approximately

theless, this experiment indicates that fluorescence measured by FACS reflects Gus activity in vivo.

DISCUSSION

Several properties of the Gus reporter system are useful in studies of mammalian gene expression. The Gus gene is only 1,800 base pairs in size (vs. the 3,400-base-pair lacZ gene), allowing for its use in DNA constructs with functional size constraints, such as retroviral vectors. The Gus enzyme, like β-gal, is extremely stable under a variety of conditions (e.g., in the presence of 0.1% v/v Triton X-100) and over a broad pH range (13,14). The Gus and β-gal hydrolases are evolutionarily related (19) with a 30% amino acid identity (determined by using the FASTDB-Intelligenetics suite) over a region previously shown to be conserved in such hydrolases (81). Because they have evolved in the same organism, these enzymes have presumably been selected to operate optimally under comparable biochemical conditions. Although the Gus enzyme is tetrameric in its native state and retains enzymatic activity when fused to the C-terminus of heterologous polypeptides (13), much like β-gal (18),
allowing for translational fusions to genes of interest in heterologous promoter expression studies.

When cells are loaded with 2 mM FDGlcu in the presence of 1 mM 1,4-SL, a strong linear correlation is found between the relative fluorescence of the gus" population as measured by FACS, with the fluorescence as measured in the lysis assay (Fig. 6). This is in contrast to the lacZ system, which, for unknown reasons, shows exponential enzyme kinetics in vivo (22). Nevertheless, the high specificities of the Gus and β-gal enzymes for FDGlcu and FDC, respectively, suggest that the "FACS-Gus" system could be used in combination with FACS-gal in sequential analyses.

The hypotonic-loading experiments indicate that FDGlcu is loaded into cells at a significantly higher concentration than the FDCglu substrate (data not shown). Because the fluorescein moiety is identical in these substrates, the distinct loading properties of the different substrates must be due to the different chemical structures of the glucuronide and galactoside sugars (a carboxyl group vs. a hydroxyl group, respectively). 702/3 MFG-GusG6 cells loaded with FDGlcu show a lower signal-to-background ratio after complete substrate hydrolysis than do the NIH 3T3 cell lines (similar to results obtained with FACS-Gal). Thus, although sufficient levels of FDGlcu are loaded into fibroblast cells, allowing for resolution of gus" from gus" cells (Fig. 2), smaller cells, such as lymphocytes, might require longer loading times to reach FDGlcu substrate levels sufficient for the complete separation of gus" from gus" cells.

We found that millimolar concentrations of the glucuronidase inhibitor 1,4-SL were required to inhibit the hydrolysis of GUS-fluorescein to levels sufficient for quantitative analysis by FACS (Fig. 5). By titrating 1,4-5L in the presence of a constant concentration of MUGlcu and Gus' cell extract, we determined the IC50 to be approximately 100 μM (data not shown). These results suggest that cells are loaded with a concentration of inhibitor significantly
Previous application of the FACS-Gal system demonstrated the utility of an assay that provides information on gene expression on a single-cell basis. Because the gus gene shares only limited homology with lacZ, and the Gus enzyme has a different glycolytic substrate specificity than β-gal, it is possible that the Gus reporter system will circumvent the limitations ascribed to lacZ in hematopoietically derived cells. The FACS-Gus system described in this study should be useful as an alternative to the FACS-Gal system in studies where β-gal is inadequate and in combination with the FACS-Gal system under conditions where both genes are expressed.

Recently, we have explored the utility of the FACS-Gus assay in measuring levels of endogenous β-glucuronidase activity in whole animals. Absence of β-glucuronidase activity in mouse and man results in the lysosomal storage disorder Muco-polysaccharidosis type VII (MPSVII; 5). By using the murine model of MPSVII (27), we have established conditions that allow for the quantitation of endogenous lysosomal activity as well as for sorting of cells based on this activity (manuscript in preparation). This approach may be used in gene transfer therapy to enrich cells that have been transduced with β-glucuronidase prior to reinjection of patients with the disease.

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**LITERATURE CITED**


**Fig. 6.** β-Glucuronidase activity determined on a cell-by-cell basis is an accurate reflection of enzymatic activity in lysates. MPG-Gal-infected NIH 3T3 cells were loaded with 2 mg FODGlu in the presence of 1 mM 4-4-SL, as described in Materials and Methods. Linear gating mode (×2) was set to sort 15 pools of 5 × 10⁵ to 2 × 10⁶ cells spanning the distribution on the basis of fluorescence fluorescence levels into a 96-well plate. Three examples (shaded bars) of the 15 sets of gates used in the experiment are shown. Bi 2-buffer was added to the sorted pools to lyse the cells followed by the addition of MUGluc to a final concentration of 0.6 mM. Each data point represents the mean fluorescence, as determined by FACS for each sorted population plotted, vs. the total β-gal activity of the sorted pools, as revealed by the methylumbelliferone (MUB) fluorescence of the corresponding cell lysate sample (normalized to 1,000 cell equivalents). The diagonal line indicates the linear least-squared regression.


