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Recommended Citation

Qureshi, S. A. (2007). Beta-lactamase: An ideal reporter system for monitoring gene expression in live eukaryotic cells. *Biotechniques*, 42(1), 91-96.

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β -Lactamase: an ideal reporter system for monitoring gene expression in live eukaryotic cells

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BioTechniques 42:91-96 (January 2007)
doi 10.2144/000112292

To gain insightful information about the mechanisms through which genes are activated and repressed requires gene reporter systems that are sensitive, robust, and cost-effective. Although numerous reporter gene technologies are commercially available, none are as sophisticated and user-friendly as β -lactamase (BLA) when it comes to studying gene expression in live cells. This article presents an overview of the BLA technology and describes how it can be exploited for studying rare events such as homologous recombination in somatic cells and be used to deliver any DNA sequence of choice anywhere within the genome.

INTRODUCTION

Over the past several years, the combination of functional genomics, proteomics, and bioinformatics has contributed significantly to the identification of novel molecules that now serve as valid targets for therapeutic intervention against various diseases. Rapid generation of lead compounds that will inhibit or, in some cases, potentiate the activities of these target molecules necessitates the availability of robust, sensitive, and cost-effective assays. Previously, biochemical assays were used more than cell-based assays, but, due to emerging technologies, this trend is changing, and the demand for cell-based assays is gradually increasing. Although biochemical assays are well-defined, sensitive, and easy to miniaturize, they are nonphysiological and require purified protein. Moreover, biochemical assays can be challenging to develop for difficult-to-purify (but nonetheless therapeutically important) proteins, such as membrane receptors, ion channels, and G protein-coupled receptors (GPCRs). In contrast, cell-based assays offer investigators the luxury of probing biological activity of a protein or a pathway in its natural

physiological milieu, and therefore are closer to recapitulating the actual in vivo situation.

CURRENT REPORTER GENE DETECTION SYSTEMS

The success of cell-based assays depends entirely on the quality of the reporter gene. To date, numerous reporter genes have been used for monitoring gene expression (1). While useful, most commercially available reporter technologies are not versatile enough to cover every aspect of cell-based assay development and screening (see Table 1). For instance, although luciferase-based assays provide acceptable sensitivity and an excellent dynamic range, they cannot be used for fluorescence-activated cell sorting (FACS), because the bioluminescent emission produced after the breakdown of D-luciferin is only transient. Thus, deriving stable cell lines from single cells expressing luciferase necessitates labor-intensive serial dilutions and makes this system ill-suited for cell-based assay development (1). Secreted alkaline phosphatase (seAP) is another reporter system in which

the expressed seAP is detected when it activates a dioxetane derivative, which decomposes and emits light during the decomposition process. Like luciferase, the seAP system is incompatible with FACS, and developing seAP expressing cell lines from single cells also requires time-consuming and labor-intensive serial dilutions. The oldest reporter system, chloramphenicol acetyl transferase (CAT), requires a radioactive substrate; this feature reduces its utility for many researchers.

Another usable reporter gene is the *lacZ* encoded enzyme β -galactosidase (1). Historically, this reporter gene has served as a valuable tool for cell and developmental biologists. The activity of β -galactosidase, comprised of a homotetramer of 116-kDa subunits, in a cell or fixed tissue can be monitored colorimetrically with its substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), which produces a blue precipitate upon cleavage. This enzyme's activity may also be detected fluorometrically in live cells with the synthetic substrate, fluorescein- β -digalactopyranoside (FDG) (2). Whereas the native substrate does not fluoresce, β -galactosidase cleavage of FDG produces free fluorescein

Table 1. Characteristics of Gene Reporter Systems

Reporter Gene	Enzyme Amplification	Miniaturizable	Ratiometric	FACS-Compatible	Stable Cell Lines
β -Lactamase	+++	+++	+++	+++	+++
Luciferase	+++	++	—	—	+
Secreted AP	+++	+	—	—	+
CAT	+++	—	—	—	—
β -Galactosidase	+++	++	—	++	++
GFP	—	+	—	+++	+++

FACS, fluorescence-activated cell sorting; AP, alkaline phosphatase; CAT, chloramphenicol acetyl transferase; GFP, green fluorescent protein; +++, highly appropriate; ++, appropriate; +, time-consuming or labor-intensive; —, inappropriate.

that can be detected in live cells by epifluorescence microscopy or flow cytometry. Although the β -galactosidase/FDG combination has been used in FACS, it has shortcomings (3). First, because FDG is polar, it does not readily cross the plasma membrane. To introduce FDG inside cells, they must be subjected to a harsh hypotonic shock procedure, which leads to uneven substrate loading. Since fluorescence signal intensity from the cells depends on both enzyme and substrate concentrations, it is affected by uneven substrate loading. Second, the free fluorescein produced by FDG cleavage rapidly diffuses out of the cell. To minimize this efflux, cells must be kept on ice during the entire loading period. Currently, no other commercially available lipophilic

substrate exists that can readily cross the cell membrane and quantitatively report the expression levels of β -galactosidase within a cell. In spite of the problems associated with the β -galactosidase/FDG system, it has been used as a tool for studying protein-protein interactions in live cells (4).

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is another commonly used reporter system (5). The main advantage of GFP is that it is noninvasive and inherently fluorescent (i.e., it requires no substrate for detection). In addition, using different spectrally resolved mutants of GFP enables an investigator to track the expression of two (or more) genes in the same cell (multiplexing). As a non-enzymatic reporter, however, GFP's limit of detection is approximately 10^5

molecules/cell. The low sensitivity of GFP undermines its effectiveness as a good reporter system.

β -LACTAMASE: A REPORTER FOR ALL SEASONS

The β -lactamase reporter system makes use of the enzyme TEM-1 β -lactamase (BLA), which lacks the N-terminal 23 amino acid periplasmic secretory signal sequence. BLA is a 29-kDa enzyme encoded by the ampicillin resistance gene (*amp^r*) that is active either as a monomer or when fused N or C terminally to a heterologous protein (6) and can cleave β -lactam-containing molecules like penicillins and cephalosporins with simple kinetics and high catalytic efficiency. No ortholog of

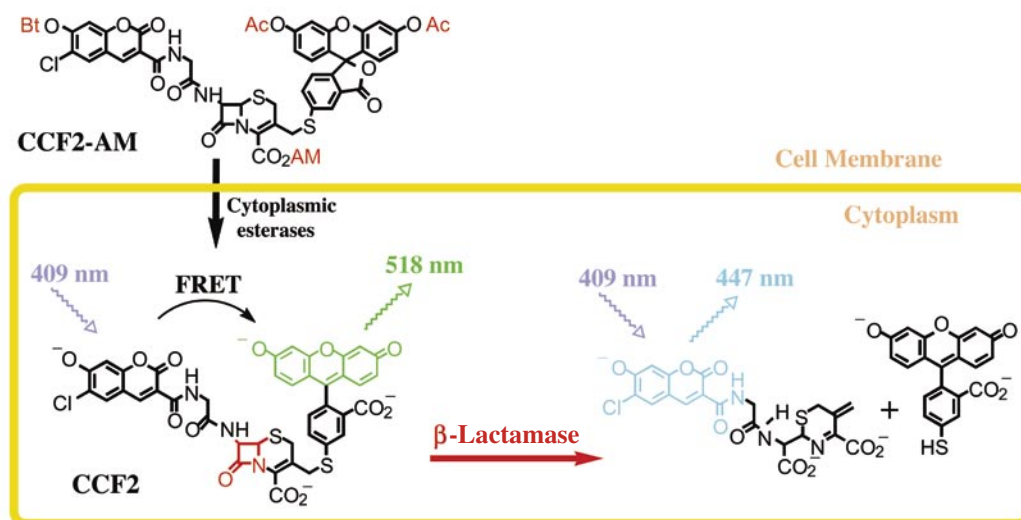


Figure 1. Illustration of the β -lactamase reporter system. After an esterified (acetoxymethylated; AM) form of this substrate CCF2/4 (CCF2/4-AM) enters a cell, endogenous esterases convert it to CCF2/4, thereby trapping it inside the cell. Exciting CCF2/4 at 408 nm leads to efficient FRET from the coumarin moiety to the fluorescein derivative and produces green fluorescence detectable at 530 nm. After β -lactamase cleaves CCF2/4, the two fluorophores separate, causing loss of fluorescence resonance energy transfer (FRET); excitation at 408 nm now results in blue fluorescence detectable at 460 nm.

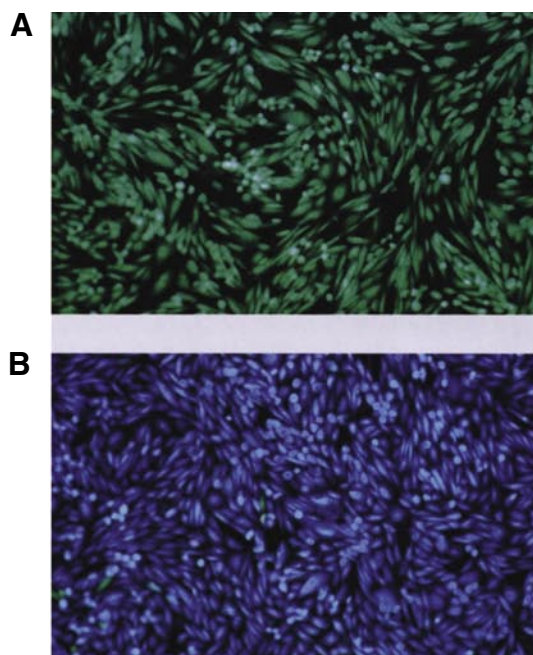


Figure 2. Monitoring the expression of β -lactamase in living cells. After loading the cells with an esterified (acetoxymethylated; AM) form of this substrate CCF2/4 (CCF2/4-AM), cells from which the enzyme TEM-1 β -lactamase (BLA) is absent are green (A), whereas the BLA-expressing cells are seen as blue (B).

BLA exists in eukaryotes. Additionally, overexpression of BLA is not accompanied by any apparent toxicity in eukaryotic cells.

The effectiveness of BLA as a reporter gene was not realized until Zlokarnik and colleagues reported the synthesis of a fluorogenic BLA substrate CCF2 (7). Another BLA substrate, CCF4, is relatively more soluble than CCF2 in aqueous solutions and is therefore preferred over CCF2 for some applications. CCF2/4 is composed of two fluorescent dyes, 7-hydroxycoumarin-3-carboxamide and fluorescein, bridged by cephalosporin. The esterified (acetoxymethylated; AM) form of this substrate, CCF2/4-AM, is lipophilic and readily traverses cell membranes without stressing or damaging live cells. As shown in Figure 1, once CCF2/4-AM enters a cell, endogenous esterases convert it to CCF2/4. Because CCF2/4 is negatively charged at physiological pH, it becomes trapped inside the cell. CCF2/4 can be easily detected by fluorescence resonance energy transfer (FRET). In the intact molecule, excitation of coumarin at 408 nm leads to efficient FRET (quantum yield 0.74)

to the fluorescein derivative and produces green fluorescence ($\lambda = 530$ nm) (Figure 2A). Cleavage of CCF2/4 by β -lactamase, however, separates the two fluorophores, causing loss of FRET; excitation of coumarin now results in blue fluorescence ($\lambda = 460$ nm) (Figure 2B). Thus, based on the change in the color of the fluorescence emission signal, live cells expressing BLA can be unequivocally, simply, and rapidly (in as little as 1 h) distinguished from those that lack the enzyme by epifluorescence microscopy, fluorescent plate reader, or flow cytometry.

In the BLA-CCF2/4 system, the green fluorescence observed immediately after delivering (loading) the substrate indicates that the intact substrate has been successfully loaded in each cell. This unique and powerful feature distinguishes

BLA from other reporter systems, in which registering delivery of the substrate is impossible (e.g., loading the β -galactosidase substrate FDG into cells). Equally important, the CCF2/4 substrate enables ratiometric data analysis (the ratio of the net blue fluorescence signal intensity and the net green fluorescence signal intensity; em. 460/em. 530), which eliminates problems arising from well-to-well variations in cell number and fluorescence signal intensity.

Because neither BLA nor its substrate (intact or cleaved) is toxic, this reporter system can be used to not only monitor gene expression in live animals (8), but is also amenable for FACS, which can process as many as 10^4 cells/s (9). Consequently, the BLA reporter system facilitates rapid development of stably transfected clonal cell lines. With the CCF2/4 substrate, as few as 50 molecules BLA can be detected within a cell (7). This combination of enzymatic fluorescence signal amplification and ratiometric data analysis produces exquisitely sensitive and specific BLA-based assays that are ideally suited for ultraminiaturizable

high-throughput screening (HTS) applications (10).

Besides being useful as a traditional reporter that allows easy monitoring of signaling pathways in live cells (6), BLA-based cell-based assays have been developed for various classes of gene families including GPCRs and nuclear hormone receptors (11–15). This biosensor has also been used for studying protein folding (16), adaptive and targeted gene evolution (17,18), protease activity (19), gene trapping (20,21), fusion of human immunodeficiency virus (HIV) virions to primary CD4⁺ T lymphocytes (22,23), RNA splicing (24), and protein-protein interactions by fragment complementation (25–28). In one report, the BLA reporter system was used for identifying inhibitors of hepatitis C viral replication (29).

In spite of its many attractive features, some of the shortcomings associated with the BLA-CCF2/4 system are that it has poor dynamic range and its substrate CCF2/4 has a high molecular weight and poor solubility in aqueous solutions. In light of this, there is a need for superior new-generation BLA substrates, such as CC1 (30), that will further improve the quality and effectiveness of this reporter.

DEVELOPING A GLUCOCORTICOID RECEPTOR CELL-BASED ASSAY WITH β -LACTAMASE USING ONE-ARM HOMOLOGOUS RECOMBINATION

A member of the nuclear receptor superfamily, the glucocorticoid receptor (GR) is a ligand-activated transcriptional regulator. GR mediates glucocorticoid action that controls carbohydrate, protein, and fat metabolism, suppresses the immune/inflammatory responses, regulates cardiovascular function, and affects basal and stress-related homeostasis. Glucocorticoid therapies are used to treat asthma, chronic arthritis, inflammatory bowel disease, lymphoma, leukemia, hyperglycemia, renal and pulmonary conditions, multiple sclerosis, and headaches.

Since BLA permits an investigator to isolate a single cell expressing the

enzyme from a population, it should be the system of choice for monitoring rare events, such as homologous recombination in somatic cells that occurs at a very low frequency. That the BLA reporter system is versatile enough to be used for this purpose is demonstrated by the fact that a robust and sensitive GR cell-based assay was developed using a novel one-arm homologous recombination-based approach in HEK 293T cells; the details of how this was carried out are described in Reference 13. Briefly, a 3.6-kb region spanning the 3' end of exon 5 to the middle of intron 6 of the GR gene was amplified by PCR. This fragment was then cloned immediately downstream of the GAL4-DNA binding domain (GAL4-DBD; amino acids 1–147), such that the 151 amino acid of the resulting fusion protein represents the 500th amino acid of GR, which in turn corresponds to the first amino acid of the GR ligand binding domain (LBD) that spans from amino acid 500–777 (31). The three amino acids at positions 148, 149, and 150 arise from the plasmid polylinker codons. The plasmid was designed in such a way that, after transfection, a precise homologous recombination event would culminate in the production of the GAL4(DBD)-GR(LBD) from the strong cytomegalovirus (CMV) promoter. After digestion with a restriction enzyme, the linearized plasmid was used to transfect 100 million HEK 293T cells, which had previously been stably transfected with a BLA reporter plasmid under the control of an upstream activating sequence (UAS) containing seven GAL4-DBD binding sites (plasmid referred to as pUAS_{Gal4}-BLA). The transfection efficiency was estimated to be 0.2%. To identify stably transfected cells in which the targeted plasmid had integrated accurately, the cells were starved for 24 h and then treated with 1 μ M dexamethasone, a GR ligand, for 16 h. Subsequently, 100 million cells were loaded with CCF2-AM from which 500,000 BLA-positive cells were recovered by FACS. After eliminating cells constitutively expressing BLA from this pool, the cycle of starving cells, treating them with dexamethasone, loading them

with CCF2-AM and FACS sorting was repeated twice. From the final FACS, about 300 intense blue single cells were collected in individual wells of a 96-well plate from which approximately 200 survived. To obtain cell lines highly responsive to dexamethasone, each of the expanded 200 transgenic clonal cell lines was characterized further. This exercise led to the identification of several cell lines that were validated by reverse transcription PCR (RT-PCR) to ascertain that the targeting vector had integrated at the appropriate locus (this analysis made use of two primers—one designed to anneal within the GAL4-DBD region and the other to an endogenous GR exon not cloned in the construct). Western blot analysis on extract prepared from one of the cell lines with α -GAL4-DBD antibody revealed the presence of a 427 amino acid chimeric GAL4(DBD)-GR(LBD) protein with a molecular weight of 46 kDa.

This GR cell-based assay engineered by homologous recombination exhibits an excellent dynamic range; treating the engineered HEK 293T cells first with 1 μ M dexamethasone for 16 h and then loading with CCF4-AM for 1 h results in a >13-fold induction of BLA expression. Moreover, the high Z' factor value (0.83) demonstrates the robustness of the GR assay. Since the assay is fully miniaturizable and has been found to perform equally well in 384- to 3456-well plates, it should serve as a useful tool for drug hunters.

Development of the GR assay using homologous recombination demonstrates that such assays can indeed be developed using the BLA technology. Additionally, this powerful approach, which allows an investigator to insert a DNA sequence anywhere in the genome, is equally useful for introducing an affinity tag, overexpressing a gene, or producing a transgenic knockout cell line.

CONCLUSION

The BLA reporter system outshines other reporter gene technologies in terms of its sensitivity, robustness, ease-of-use, and because it can be used for studying gene expression in living

cells. It is for these reasons that this technology serves as an excellent tool, not only academic laboratories engaged in basic research, but also for research scientists in industry who are in the business of developing cost-effective and miniaturizable cell-based assays. From the competing reporter gene technologies, no other system offers a FACS-compatible, ratiometric, HTS-ready technology.

COMPETING INTERESTS STATEMENT

The author declares no competing interests.

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