

## Notes &amp; Tips

An artifact in studies of gene regulation using  $\beta$ -galactosidase reporter gene assaysClaudia Lefimil<sup>a</sup>, Eugenia Jedlicki<sup>b</sup>, David S. Holmes<sup>a,\*</sup><sup>a</sup>Center for Bioinformatics and Genome Biology, Fundación Ciencia para la Vida and Facultad de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile<sup>b</sup>Instituto de Ciencias Biomédicas (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile

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## ABSTRACT

Reporter gene assays are important tools for evaluating gene expression. A frequently used assay measures the activity of  $\beta$ -galactosidase ( $\beta$ -gal) expressed from *lacZ* in plasmid or genomic constructions. Such constructions are often used to interrogate the ability of DNA (query DNA), potentially encoding a transcription factor, to regulate in *trans* the expression of a promoter fused to the reporter *lacZ*. Query DNA is frequently inserted into a second plasmid within the  $\alpha$ -subunit of  $\beta$ -gal, interrupting its function. However, this plasmid can induce up-expression of  $\beta$ -gal even when void of query DNA, leading to confusion between artifact and authentic regulation.

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The reporter gene assay is an important tool for biological and biomedical research, permitting the detection of *in vivo* gene expression and the identification and analysis of *cis* and *trans* regulators that affect such expression. Several commonly used assays include the use of reporter genes encoding  $\beta$ -galactosidase ( $\beta$ -gal),<sup>1</sup> chloramphenicol acetyltransferase (CAT), alkaline phosphatase (AP), luciferases, and green fluorescent protein (GFP) (reviewed in Ref. [1]). However, artifacts have been described for the  $\beta$ -gal gene reporter system [2] and for the luciferase system [3], and it is important to recognize these and other potential artifacts in order to maximize the utility and credibility of such assays. In this note, we describe another artifact using the  $\beta$ -gal system and suggest that it can be most readily circumvented by substituting the use of one of the other common reporter genes in the offending part of the protocol.

An artifact was discovered during experiments designed to detect regulators of gene expression. The artifact was first detected in a screen for potential regulators of a candidate promoter for *fur* (ferric uptake regulator) from the extreme acidophile *Acidithiobacillus ferrooxidans*. The experimental setup used in the screen is shown in Fig. 1. Briefly, the candidate promoter for *fur* (*Pfur<sub>AF</sub>*) was cloned upstream of *lacZ* encoding  $\beta$ -gal, and the resulting

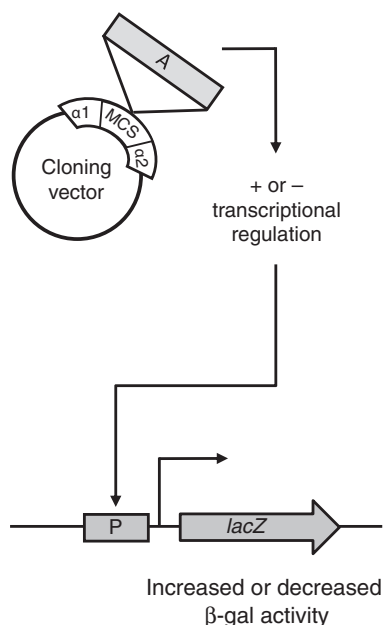
construction was inserted into the chromosome of a  $\beta$ -gal negative strain of *Escherichia coli* H2331 ( $\Delta$ *lacU169*) [4]. The modified strain, termed *E. coli* H2331 *Pfur<sub>AF</sub>-lacZ*, weakly produced  $\beta$ -gal as determined by colony color (white vs. blue) on agar plates containing X-gal [5] and by the Miller assay (70 Miller units) [6], suggesting that *Pfur<sub>AF</sub>* exhibited weak transcriptional activity. Subsequently, *E. coli* H2331 *Pfur<sub>AF</sub>-lacZ* was transformed with pUC18 containing a library of *A. ferrooxidans* genomic DNA cloned into the *Bam*HI restriction enzyme site within the multiple cloning site (MCS) of pUC18. The library was constructed by partial digest of *A. ferrooxidans* DNA with *Sau*3AI, followed by size selection (~1–4 kb) by agarose gel electrophoresis [7]. The objective was to detect colony transformants that potentially encoded up- or down-regulators of *Pfur<sub>AF</sub>* by evaluating  $\beta$ -gal production. Colonies with up-regulated expression of  $\beta$ -gal were detected, but on isolation and examination approximately 50% (14 of 27) were found to be recircularized pUC18, lacking inserted DNA, or to contain a few inserted nucleotides (3–24) that do not inactivate  $\alpha$ -peptide function.

To explain this up-regulation, it is hypothesized that the  $\alpha$ -peptide of  $\beta$ -gal encoded by pUC18 is able to increase the activity of the wild-type  $\beta$ -gal expressed from the intact chromosomal copy of *lacZ* of *E. coli* H2331 *Pfur<sub>AF</sub>-lacZ*.

To test this hypothesis, several different plasmids encoding the  $\alpha$ -peptide of  $\beta$ -gal were transformed into the host *E. coli* BL21(DE3) that contains *lacZ* expressed from its native promoter (*P<sub>LAC</sub>*) [8]. The plasmids tested were pUC18 [9], pGEM-T (Promega), and pMCL200 [10]. Each transformant gave increased  $\beta$ -gal enzymatic

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E-mail address: [dsholmes2000@yahoo.com](mailto:dsholmes2000@yahoo.com) (D.S. Holmes).<sup>1</sup> Abbreviations used:  $\beta$ -gal,  $\beta$ -galactosidase; CAT, chloramphenicol acetyltransferase; AP, alkaline phosphatase; *fur*, ferric uptake regulator; MCS, multiple cloning site; RT-PCR, reverse transcription polymerase chain reaction.



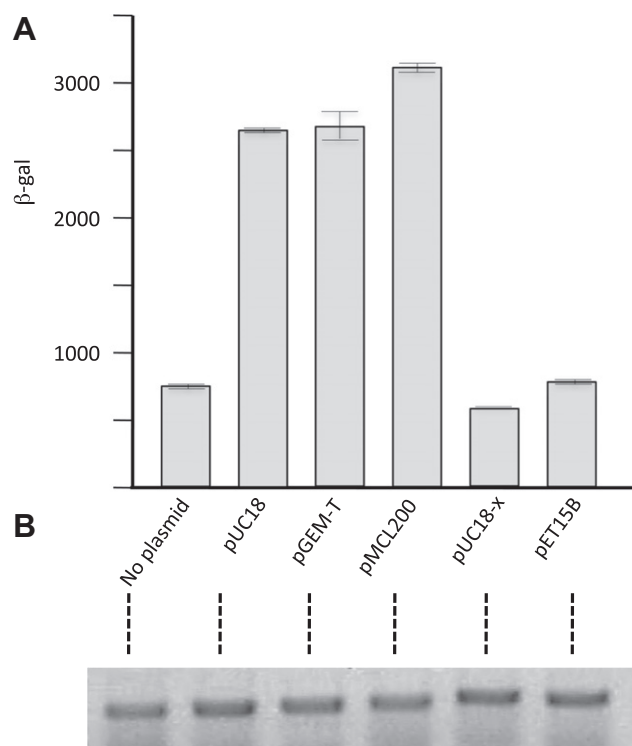
**Fig. 1.** Pipeline for the detection of genes capable of up- or down-regulation of promoters using  $\beta$ -galactosidase activity from  $lacZ$  as a measure of promoter activity. A, cloned sequence under interrogation;  $\alpha 1$ , N terminal of  $\beta$ -gal  $\alpha$ -peptide fragment;  $\alpha 2$ , C terminal of  $\beta$ -gal  $\alpha$ -peptide; MCS, multiple cloning site; P, promoter (Pfur<sub>AF</sub>) under interrogation.

activity compared with the untransformed host (Fig. 2). To evaluate whether increased  $\beta$ -gal activity required an intact  $\alpha$ -peptide, the corresponding encoding region was interrupted by the introduction of a foreign DNA fragment to give rise to recombinant plasmid pUC18-X. This construction did not induce an increase in  $\beta$ -gal activity in *E. coli* BL21(DE3), showing that an intact  $\alpha$ -peptide was required (Fig. 2). Finally, *E. coli* BL21(DE3) was transformed with the multicopy plasmid pET15B (Novagen) that does not encode the  $\alpha$ -peptide of  $\beta$ -gal, and no increase in  $\beta$ -gal activity was detected (Fig. 2), consistent with the idea that the  $\alpha$ -peptide is responsible.

A necessary component of this hypothesis is the need for the expression of the  $\alpha$ -peptide from each of the plasmids under evaluation. To test this requirement, plasmids pUC18, pGEM-T, and pMCL200 were transformed into *E. coli* JM109 (containing alpha acceptor of  $\beta$ -gal to analyze alpha complementation). Each transformant expressed functional  $\beta$ -gal, indicating that the respective plasmid encoded  $\alpha$ -peptide.

An alternate hypothesis is that pUC18, pGEM-T, and pMCL200 are able to up-regulate the expression of  $lacZ$  at the transcriptional level. To test this hypothesis, semiquantitative reverse transcription polymerase chain reaction (RT-PCR) experiments were performed as described previously [11] using template RNA prepared from *E. coli* BL21(DE3) either with or without these plasmids. Results show that the expression of  $lacZ$  RNA is the same in all cases, demonstrating that these plasmids do not up-regulate transcriptional expression of  $lacZ$  (Fig. 2B). Additional evidence for this conjecture comes from experiments with two different strains in which the native promoter of  $lacZ$  was replaced by other promoters such as *Fiu*, a promoter for a gene encoding an outer membrane ferric iron uptake protein [12], and *Fhuf*, a promoter for a gene encoding a ferric reductase [12]. Increased  $\beta$ -gal activity was observed in both cases following transformation with pUC18 (data not shown), indicating that increased  $\beta$ -gal activity is promoter independent.

All results are consistent with the hypothesis that the  $\alpha$ -peptide of  $\beta$ -gal is able to increase the activity of wild-type  $\beta$ -gal. It is not



**Fig. 2.** (A)  $\beta$ -gal activity in *E. coli* BL21(DE3) transformed with different plasmids compared with  $\beta$ -gal activity in untransformed *E. coli* BL21(DE3).  $\beta$ -gal is expressed from its native promoter ( $P_{LAC}$ ). Plasmids used were as follows: pUC18, pGEM-T, and pMCL200, which express the  $\alpha$ -peptide of  $\beta$ -gal; pUC18-X, which contains an insert that interrupts the  $\alpha$ -peptide; and pET15B, which does not contain an  $\alpha$ -peptide encoding region. All cultures were grown at 30 °C in Luria-Bertani broth with the pertinent antibiotics: ampicillin (100  $\mu$ g/ml) and/or streptomycin (100  $\mu$ g/ml). IPTG (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, 0.5 mM final) was added when the cultures reached an optical density at 590 nm of 0.5, and subsequently the cultures were incubated for an additional 2 h at 30 °C.  $\beta$ -gal activity was assayed as described previously [6] using ONPG (*ortho*-nitrophenyl- $\beta$ -galactoside) as a substrate. The reactions were performed at 30 °C, and product formation was measured by the increase in optical density at 420 nm at a specific time.  $\beta$ -gal activity is reported in Miller units. Triplicate samples of each culture were assayed with at least three repetitions of each experiment. (B) Expression of RNA from the same plasmids as in panel A, measured by RT-PCR, using primers designed to test for  $lacZ$  mRNA (messenger RNA). The RT-PCR assays were performed as described previously [11].

known how increased activity is accomplished, but we speculate that the presence of excess  $\alpha$ -peptide may help to stabilize  $\beta$ -gal, which is a tetramer of identical subunits [13]. This phenomenon is most likely different from alpha complementation, where  $\alpha$ -peptides reconstitute the dimer-dimer interface to recover active tetramers [14]. The  $\beta$ -gal activity in this case is lower than that obtained in the artifact. Alternately, the  $\alpha$ -peptide may serve as a decoy for proteases, or other cellular mechanisms that might inactivate  $\beta$ -gal, helping to extend the life of  $\beta$ -gal.

The artifact reported here can be diagnosed at the end of the experiment by sequencing candidate plasmids. However, the frequency of occurrence of the artifact reduces the efficiency of detecting bona fide transcription factors and increases DNA sequencing costs. These problems can be avoided by choosing plasmids or other vectors that do not encode the  $\alpha$ -peptide of  $\beta$ -gal as vehicles for the query DNA. The suggested artifact might have been the cause of the observation that a plasmid construction in which DNA had been inserted at a site that did not disrupt the  $\alpha$ -peptide could subsequently give rise to an increase in  $\beta$ -gal activity when it was used in experiments with *E. coli* strains carrying intact  $lacZ$  [15].

In conclusion, based on our data, an artifact can arise in gene regulation assays in which an empty plasmid encoding the  $\alpha$ -peptide of  $\beta$ -gal, but void of query DNA, can induce up-expression

of  $\beta$ -gal from a reporter gene, leading to confusion between artifact and authentic regulation from query DNA. It is suggested that this problem can be circumvented by using reporter plasmids or other vectors that do not encode the  $\alpha$ -peptide of  $\beta$ -gal for interrogating regulatory activity of query DNA.

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