Single-step method for β-galactosidase assays in *Escherichia coli* using a 96-well microplate reader

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**ABSTRACT**

Historically, the lacZ gene is one of the most universally used reporters of gene expression in molecular biology. Its activity can be quantified using an artificial substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG). However, the traditional method for measuring LacZ activity (first described by J. H. Miller in 1972) can be challenging for a large number of samples, is prone to variability, and involves hazardous compounds for lysis (e.g., chloroform, toluene).

Here we describe a single-step assay using a 96-well microplate reader with a proven alternative cell permeabilization method. This modified protocol reduces handling time by 90%.

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Various β-galactosidase protocols for bacteria have been described, adapting some of the Miller method [1] steps for use in plate readers [2–5]. However, these methods include many of the drawbacks inherent to the original method and remain labor-intensive.

One of the challenges in further speeding up this assay is the cell permeabilization stage, which is required for the o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate to enter the cell and interact with β-galactosidase. This typically requires the transfer of cultures due to the fact that permeabilization is normally performed using chloroform/sodium dodecyl sulfate (SDS) or toluene [1,6], which can interfere with the optical density readings in standard microtiter plates. Deep well nonreactive polypropylene blocks have been suggested [5]; however, the organic solvents were reported to be difficult to manipulate using multichannel pipettes [7].

An alternative permeabilization method was proposed using PopCulture reagent [7], a compound used in protein purification. PopCulture reagent punctures the cell wall without denaturing soluble proteins or interfering with optical density readings, with the β-galactosidase remaining stable for up to 18 h [7]. The cell lysis efficiency can be further enhanced by the addition of chicken egg white lysozyme, which hydrolyzes the peptidoglycan in cell walls [8]. This protocol was shown to produce similar results to the traditional chloroform/SDS method used for cell lysis [7]. This approach allowed for kinetic readings rather than endpoint readings, obviating the need for stopping the reaction with Na2CO3 and thereby improving accuracy.

Although this new permeabilization method has improved accuracy of the assay and reduced handling time for a large number of samples, the time taken to process smaller numbers of samples remains largely unchanged. Here we describe a streamlined version of these methods to condense the assays from several liquid handling steps into a single-step assay, decreasing the labor intensity irrespective of sample size.

The one-step approach aims to combine (i) OD₆₀₀ measurement, (ii) cell permeabilization, (iii) ONPG breakdown, and (iv) kinetic OD₄₂₀ quantification into a single step. The approach involves transferring 80 µl of cells and 120 µl of custom β-galactosidase (Bgal) mix (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 36 mM β-mercaptoethanol, 166 µl/ml T7 lysozyme, 1.1 mg/ml ONPG, and 6.7% PopCulture reagent) to a microtiter plate, followed by kinetic OD₄₂₀ and OD₆₀₀ quantification on a FLUOstar Omega Microplate Reader (BMG Labtech). These are then converted into Miller units using MARS Data Analysis software. A more detailed protocol to run this assay (including FLUOstar Omega Microplate Reader script) is also available (see Ref. [9], Supplementary Data A and B).

**ARTICLE INFO**

Article history:
Received 25 January 2016
Received in revised form 7 March 2016
Accepted 21 March 2016
Available online 29 March 2016

**Keywords:**
LacZ
β-Galactosidase (Bgal)
β-Galactosidase
Microplate reader

Notes
&

Abbreviations used: ONPG, o-nitrophenyl-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; Bgal, β-galactosidase.

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http://dx.doi.org/10.1016/j.ab.2016.03.017

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To combine several liquid handling steps into a single one, cell permeabilization must be rapid. Slow permeabilization could otherwise reduce ONPG availability within the cell and have adverse effects on OD420 production and Miller units. We showed that permeabilization is immediate using the one-step β-galactosidase method, with a linear OD420 increase over time being observed throughout the assay until ONPG is depleted (Fig. 1A). Therefore, any lack of cell permeabilization does not impact ONPG availability significantly given that no initial lag phase is observed for OD420 readings (Fig. 1A). Moreover, similar Miller unit values were obtained using the diluted cultures (Fig. 1B), suggesting that the results from this modified assay are consistent and scalable.

Second, the one-step assay also determines OD600 readings at the start of the assay to avoid additional liquid handling steps. We demonstrated that cell cultures diluted in rich medium (LB medium) versus the B-gal mix are comparable over an OD600 range of 0.1–1.2 (Fig. 1C). This covers the range of cell densities used in a typical β-galactosidase assay, indicating that the OD600 measurement of cells diluted B-gal mix could potentially be used as a substitute for its quantification in LB medium.

Finally, data comparisons between the traditional fully manual assay and the one-step method were not significantly different over a range of activities (Fig. 1D; see Ref. [9], Table 1). Moderate differences observed between the two methods can likely be attributed to minor discrepancies in OD600 measurements and the lack of Na2CO3 (stop solution), which increases the OD420 slightly.

Overall, the single-step β-galactosidase assay yields both consistent and accurate results over the range of cell densities and LacZ levels typically tested with a standard β-galactosidase assay and is a suitable faster and safer alternative to the traditional method (or current methods used).

Acknowledgments

We thank Milija Jovanovic and Nan Zhang for their advice and comments. This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC, BB/J00717X/1) and U.K. Medical Research Council (MRC, MR/M017672/1) project grants.

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