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Measuring β-Galactosidase Activity in Bacteria: Cell Growth, Permeabilization, and Enzyme Assays in 96-Well Arrays

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We describe a high-throughput procedure for measuring β-galactosidase activity in bacteria. This procedure is unique because all manipulations, including bacterial growth and cell permeabilization, are performed in a 96-well format. Cells are permeabilized by chloroform/SDS treatment directly in the 96-well blocks and then transferred to 96-well microplates for standard colorimetric assay of β-galactosidase activity as described by Miller [J. H. Miller (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY]. Absorbance data are collected with a microplate reader and analyzed using a Microsoft Excel spreadsheet. The β-galactosidase specific activity values obtained with the high-throughput procedure are identical to those obtained by the traditional single-tube method of Miller. Thus, values obtained with this procedure may be expressed as Miller units and compared directly to Miller units reported in the literature. The 96-well format for permeabilization and assay of enzyme specific activity together with the use of 12-channel and repeater pipettors enables efficient processing of hundreds of samples in an 8-h day.

***Key Words:* β-galactosidase assay; Miller units; microtiter; 96-well arrays; plate reader; gene regulation.**

β-Galactosidase, the product of the *lacZ* gene of *Escherichia coli,* is one of the most widely used reporters of gene expression in molecular biology. Since β-galactosidase activity can be monitored using a variety of chromogenic and fluorogenic substrates, it is a versatile reporter for both prokaryotes and eukaryotes. The standard assay for quantitating the amount of β-galactosidase activity in cells, originally described by Miller (1) for assay of bacterial cultures, involves spectrophotometric measurement of the formation of the yellow chromophore *o*-nitrophenol (ONP) as the hydrolytic product of the action of β-galactosidase on the colorless substrate *o*-nitrophenyl-β-D-galactoside (ONPG). The amount of ONP produced as a function of reaction time per volume of cell culture is divided by the optical density of the culture to generate a value of specific enzyme activity in Miller units (1). To increase the number of samples that can be easily assayed, investigators have adapted the Miller method to a 96-well format employing microplate readers, including an integrated system incorporating a robotic pipetting device and a microplate reader (2, 3).

A critical step in the Miller procedure is the permeabilization of cells. Rather than preparing cell extracts, e.g., by sonic disruption, the bacteria are treated in a way that partially disrupts the cell membrane such that small molecules like ONPG can freely diffuse into the cell. In the standard Miller assay (1), toluene or chloroform and SDS are used to permeabilize the cells. However, because toluene and chloroform etch plastic microplates, the standard permeabilization methods cannot be used directly for assay of β-galactosidase activity with a microplate reader. To take advantage of the high-throughput capabilities of microplate assays without having to resort to robots, other methods for cell disruption have been developed. For example, Arvidson *et al.* (4) lysed cells by infecting them with bacteriophage T4. However, although lysis by phage T4 is extremely efficient, large amounts of a high titer phage stock are required, which may not be ideal when large numbers of samples are to be analyzed, and the lysate needs to be prepared on a *lacZ* deleted strain to keep background values low (5). In addition, Schupp *et al.* (6) described a procedure for permeabilization of gram-negative bacterial cells using the cationic cyclic polypeptide antibiotic Polymyxin B in conjunction with Triton X-100. Although permeabilization by this procedure is as effective as that obtained by chloroform/SDS treatment, we found polymyxin B to be unsuitable

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in the microplate format because excessive bubble formation interferes with absorbance measurements.

In this paper we describe a method for measuring β-galactosidase specific activity in large numbers of samples. The procedure takes advantage of 2.2-ml 96-well polypropylene blocks, which are not only resistant to chloroform treatment, but provide an excellent format for simultaneously growing multiple cultures. Cells are grown and subsequently permeabilized by chloroform/SDS treatment directly in the polypropylene blocks. Absorbance data are collected with a microplate reader and exported into a Microsoft Excel spreadsheet for rapid analysis. The microplate reader is versatile in that either kinetic or end-point assays can be performed. To our knowledge, this is the first report of a method for assay of β-galactosidase activity in which all manipulations, including cell growth and permeabilization, are carried out in a 96-well format and where the values obtained are identical to those produced by the standard, single-tube method of Miller (1).

MATERIALS AND METHODS

Strains. Bacterial strains used in this study are derivatives of *E. coli* K-12 strain HB301 [W3110 (Δ*argF-lac*) U169] containing protein–*lacZ* fusions of SoxS-inducible genes carried on single-copy λ prophages integrated into the bacterial chromosome.

Conditions for cell growth. A repeater pipette (Eppendorf) was used to introduce 1 ml of LB broth aseptically into previously autoclaved 2.2 ml 96-well polypropylene blocks (Marsh Biomedical Products, Catalog No. AB-0661). Each well of the arrays was inoculated with a scraping from a glycerol stock frozen at −80°C using a disposable inoculating loop; uninoculated wells containing only LB broth served as a control for cross-contamination. After covering the arrays with a sterile mat cap (Marsh, Catalog No. AB-0675), the cultures were placed on a rotating platform shaker at 250 rpm and incubated at 37°C for at least 10 h. A 12-channel multichannel pipettor was used to transfer 5 μl of the overnight cultures into a second block containing 1 ml LB broth and the arrays were incubated on the platform shaker at 37°C until the A_{600} of the culture reached ~0.1–0.2. Note that each strain to be assayed is inoculated into four separate wells in a vertical column of the block with column 1 reserved as a blank lacking cells. Thus, 11 duplicate strains can be analyzed in the top half of the block (rows A–D), while an additional 11 duplicate strains can be analyzed in the bottom half of the block (rows E–H). Using a repeater pipettor, two wells of each strain were treated with 10 μl of paraquat (50 mM) to induce SoxS expression while the other two were treated with water. The arrays were incubated for an additional 1–1.5 h, at which time cell growth was arrested by the addition of 20 μl of chloramphenicol (5 mg/ml) using a repeater pipettor and the blocks were placed on ice.

Measurement of culture density in a microplate reader. To determine the culture density, which is proportional to the protein content of the culture, a 12-channel pipettor was used to transfer 50 μl of culture from each well of the block array to a flat-bottom microtiter plate (Marsh, Catalog No. N2-9091) containing 150 μl of sterile water in each well. The A_{600} of the diluted culture was determined using a Molecular Dynamics Model SpectraMAX 340 PC microplate reader. As discussed below, dilution of the culture was required because cell density is not proportional to A_{600} when the values are above ~0.25–0.5.

Cell permeabilization. For cell permeabilization, 1 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) was dispensed into the wells of a second polypropylene block. After adding 20 μl of freshly prepared 0.1% SDS and 40 μl of chloroform into the wells with a repeater pipette, a 12-channel pipettor was used to transfer an aliquot of the cell cultures (50–100 μl) from the original block into the block containing the permeabilization mixture. Permeabilization was accomplished by aspirating and dispensing the mixtures 10–15 times with a 12-channel pipettor. In the standard Miller method (1), permeabilization is achieved by vortexing, one at a time, test tubes containing the cell-chloroform/SDS mixture for 10 s. As shown below, our permeabilization method is just as efficient as the Miller method, but it is faster because cell samples are permeabilized 12 at a time.

Assay of β-galactosidase activity with a microplate reader. After allowing the chloroform to settle to the bottom of the wells of the permeabilization block, a 12-channel pipettor was used to transfer 100-μl aliquots of permeabilized cells in Z-buffer from the permeabilization block to duplicate flat-bottom microplates. Thus, the four wells inoculated with a given strain are assayed in duplicate, which produces 4 values for each uninduced culture and four values for each induced culture. At zero time, the assay is initiated by using a 12-channel pipettor to add 20 μl of ONPG (4 mg/ml) to each well of the microplate. In the endpoint assay, the microplates are incubated at room temperature for the appropriate length of time, e.g., 10 min, before the reaction is terminated by the addition of 50 μl of 1 M Na_2CO_3. Then, the microplates are introduced into the plate reader and the A_{420} and A_{550} values are determined. The absorbance data for A_{420}, A_{550}, and A_{600} are transferred to a Microsoft Excel spreadsheet and β-galactosidase specific activities in Miller units are calculated (1). A sample spreadsheet may be found at http://www.research.umbc.edu/umbc/~wolf/b-gal. Alternatively, the kinetics of ONP formation can be measured by introducing the microplates into the plate reader and following the A_{420} as a function of time (and omitting the addition of Na_2CO_3).

RESULTS

Our studies of the mechanism of DNA binding and transcription activation by SoxS, the direct regulator of the *E. coli* superoxide regulon, employ genetic fusions of *lacZ* to the member genes of the regulon and assay of β-galactosidase in cultures grown under various conditions or containing various mutations in the SoxS DNA binding site responsible for SoxS-dependent transcription activation of target genes. To facilitate our ability to determine β-galactosidase specific activity in large numbers of cultures, we developed a high throughput procedure employing 96-well polypropylene deep-well arrays for culture growth and cell permeabilization and 96-well microplates for assay of culture density and enzymatic activity. We have successfully used this procedure on strains carrying *lacZ* fusions on single-copy λ prophages or on multi-copy plasmids. Moreover, the procedure may be used for either end-point or kinetic assays, although large numbers of samples are more easily handled by the end-point method. Figure 1 is a flow chart depicting the use of 96-well arrays for the growth and end-point assay of large numbers of samples; each step of the procedure is detailed under Materials and Methods.

For each experiment determining the effect of SoxS on expression of β-galactosidase from SoxS-inducible

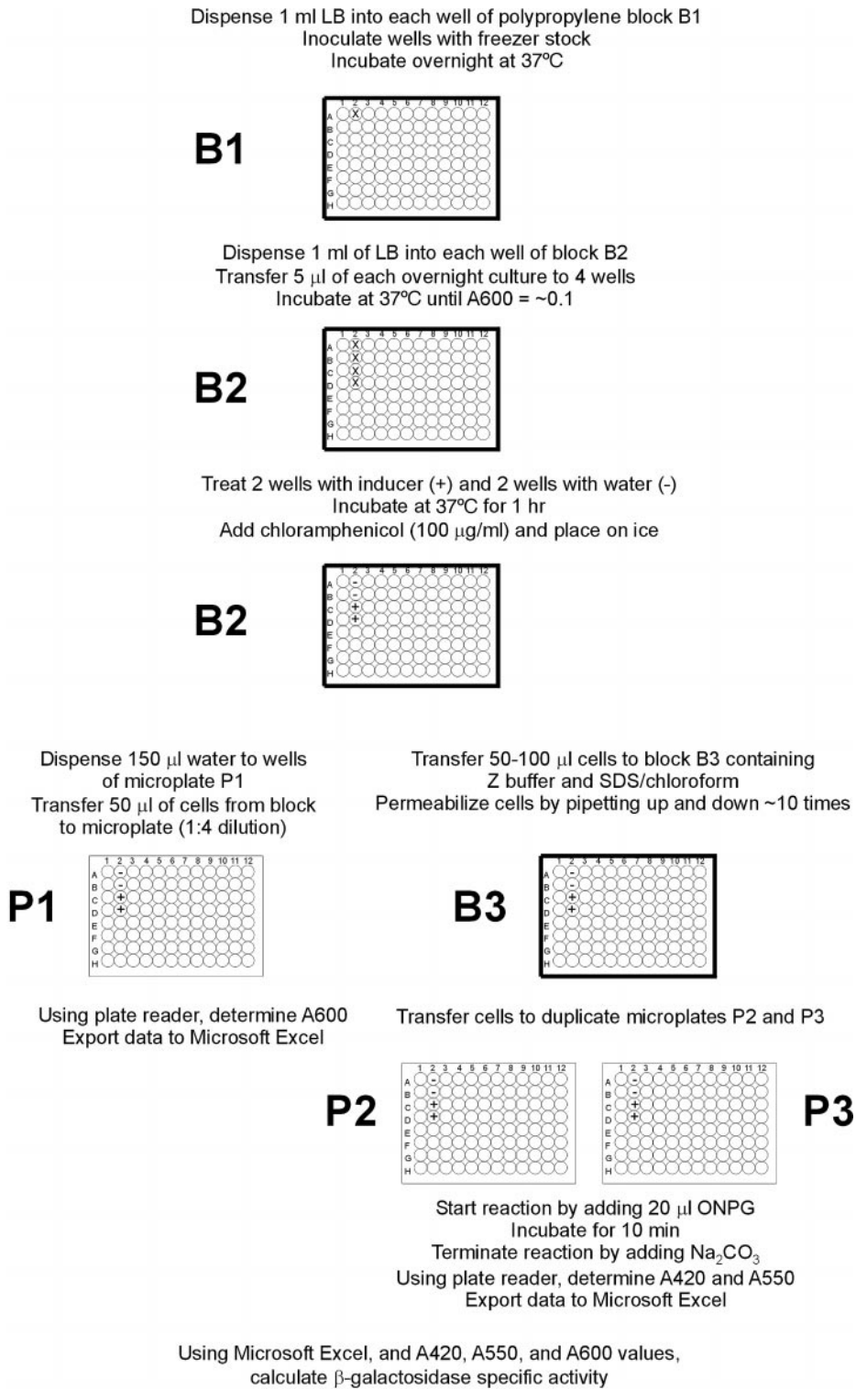

FIG. 1. Flow chart of culture growth and determination of β-galactosidase specific activity using 96-well block arrays. Overnight bacterial cultures are grown in 2.2-ml polypropylene block arrays (B1), and subcultured into a second block (B2) in which induction of gene expression is carried out; aliquots are transferred to a third block (B3) for cell permeabilization. For cell density determination, aliquots are transferred to a microplate (P1) and A_{600} measurements are made with the plate reader. For assay of β-galactosidase activity, aliquots of permeabilized cells are transferred from block B3 to duplicate microplates (P2 and P3); ONPG assays are performed and A_{420} measurements are made with the plate reader.

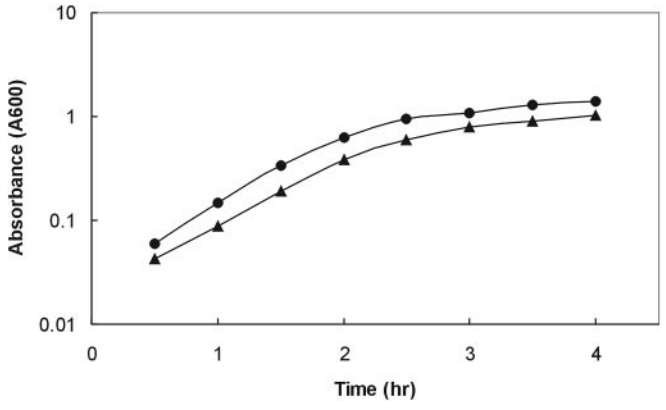

FIG. 2. Comparing the bacterial growth rate in a 96-well polypropylene block to that in 15-ml culture tubes. A saturated overnight culture of strain HB301 was diluted 1:200 into LB medium and grown in a 2.2 ml 96-well block or in 15-ml culture tubes on a platform rotating at 250 rpm in a 37°C incubator. Aliquots were taken every 30 min and A_{600} values determined using a spectrophotometer. Symbols: ●, culture growing in test tube; ▲, culture growing in 96-well polypropylene block.

promoters as fusions to *lacZ*, we treated duplicate cultures with or without inducer (0.5 mM paraquat, a redox-cycling compound that endogenously generates superoxide) and we performed duplicate assays of β-galactosidase activity and culture density on each sample, thereby producing four values for each condition and each strain. After transferring the data from the plate reader to a Microsoft Excel spreadsheet, we determined β-galactosidase specific activities in Miller Units from which mean values and standard errors were produced. Only mean values whose standard errors were 12% or less were retained.

With this protocol, we can easily process and assay in an 8-h day as many as 176 different strains with duplicate assays of duplicate cultures each under inducing and non-inducing conditions (i.e., four blocks of overnight cultures become 16 blocks for growth and induction, which in turn become 32 microplates of ONPG assays).

Bacterial Growth in 2.2-ml Polypropylene Blocks Compared to Growth in Test Tubes

A concern with growing cells in small volume containers such as the 2.2-ml polypropylene blocks is whether the aeration is sufficient to allow the cultures to achieve a growth rate suitable for physiological studies. Figure 2 shows growth curves comparing growth in the 2.2 ml 96-well polypropylene blocks to growth in 15-ml culture tubes. An overnight culture of strain HB301 was diluted 1:200 into the wells of a 96-well block or into 15-ml culture tubes each containing 1 ml of LB broth. Both culture formats, the polypropylene blocks covered with cheesecloth and secured with a rubber band, and the culture tubes in test tube racks, were placed on a platform rotating at 250 rpm in a 37°C incubator. Samples were taken every 30 min and the A_{600} determined using a spectrophotometer (Beckman DU-65). With both formats, the doubling time of the cultures was about 25 min and similar final culture densities were achieved (Fig. 2). Replacing the cheesecloth covering the block arrays with sterile polypropylene mats did not reduce the growth rate or the final culture density (data not shown). Thus, with bacterial growth in the polypropylene blocks being similar to that obtained with cultures growing in test tubes, data obtained with the arrays are suitable for physiological studies.

Linear Range and Absorbance Units of Plate Reader Compared to Spectrophotometer

A potential issue with microtiter plate readers is the units with which specific activity may be presented and how they would relate to commonly used units, e.g., the Miller unit of β-galactosidase activity (1). This is not a problem with the Molecular Dynamics SpectraMAX plate reader because its Path Check sensor reports the microplate data as standard 1-cm cuvette values and thus the values are identical to those obtained with a standard spectrophotometer. Figure 3 compares the linear range of the plate reader and the spectrophotometer at A_{420}, the wavelength of the absorbance maximum of ONP, and A_{600}, the wavelength commonly used for monitoring culture density. Twofold serial dilutions of ONP were made with H_2O and the A_{420} was determined using both the plate reader and the spectrophotometer. Similarly, A_{600} values were also determined for each instrument using twofold serial dilutions of an overnight bacterial culture. With the plate

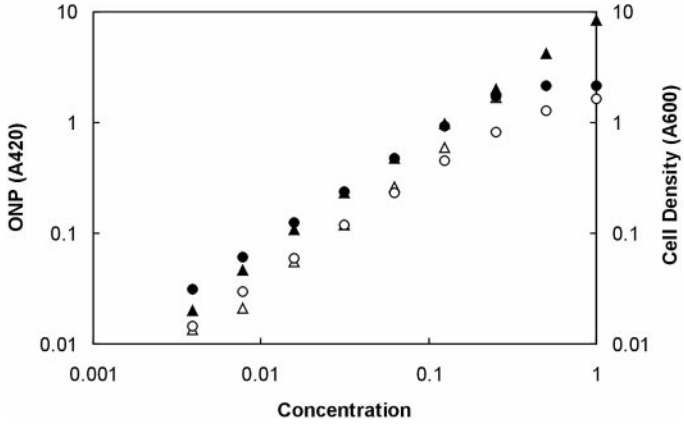

FIG. 3. Comparing the linear range of the plate reader and the spectrophotometer. Twofold serial dilutions were prepared on a solution of ONP and the A_{420} of the dilutions was determined using both a spectrophotometer (●) and the microplate reader (▲); twofold serial dilutions were also prepared on an overnight culture of strain HB301 and the A_{600} of the dilutions was determined using both a spectrophotometer (○) and the microplate reader (△).

TABLE 1

Comparing Cell Permeabilization in Polypropylene Blocks to Permeabilization in Glass Tubes[a]

	Permeabilization in blocks			Permeabilization in tubes		
	β-Galactosidase (MU)			β-Galactosidase (MU)		
Mutation	Uninduced	Induced	Induction ratio	Uninduced	Induced	Induction ratio
fpr	155 ± 10	2814 ± 13	18.1	168 ± 11	3073 ± 16	18.3
sodA	1157 ± 12	9972 ± 3	8.6	1160 ± 11	9382 ± 10	8.1
nfo	330 ± 1	2354 ± 5	7.1	340 ± 5	2436 ± 3	7.1
inaA	87 ± 11	513 ± 5	5.9	88 ± 9	498 ± 11	5.6
zwf	127 ± 8	973 ± 6	7.7	139 ± 7	1099 ± 11	7.9
Z4C	109 ± 6	153 ± 5	1.4	117 ± 3	174 ± 4	1.5
Z10A	163 ± 8	2420 ± 6	14.8	171 ± 1	2732 ± 11	16.0
Z12T	74 ± 9	820 ± 5	11.1	77 ± 8	899 ± 12	11.7

[a] Cultures of single-copy SoxS-inducible promoter fusions to *lacZ* (*fpr, sodA, nfo, inaA,* and *zwf*), as well as single-nucleotide substitutions within the SoxS binding site (Z4C, Z10A, and Z12T) fused to *lacZ,* were grown in polypropylene blocks and induced with paraquat under previously described conditions (7). Aliquots were transferred to the wells of a 96-well polypropylene block containing Z buffer and chloroform/SDS and the cells permeabilized by pipetting as described in the text; aliquots were also transferred to borosilicate glass culture tubes containing Z buffer, treated with chloroform/SDS, and permeabilized by vortexing as described by Miller (1). Permeabilized cells from both treatments were then transferred to the wells of a microplate and β-galactosidase assays were carried out as described in the text. Values are mean specific activities of β-galactosidase expressed in Miller units (MU) followed by the standard deviation presented as the percentage of the mean of at least three independent experiments.

reader, the A_{420} was directly proportional to ONP concentration over a wide range, up to $A_{420} = \sim 8$, whereas the spectrophotometer was linear up to $A_{420} = \sim 1.5$. Note, moreover, that the values obtained with the plate reader and the spectrophotometer correspond perfectly up to $A_{420} = \sim 1.5$. With measurements of culture density at A_{600}, the plate reader is linear up to $A_{600} = 0.25–0.50$ and the values at each concentration of cells correspond to those obtained with the spectrophotometer (Fig. 3); at cell densities above $A_{600} = \sim 0.25–0.50$, culture density is not proportional to A_{600} with either instrument. Thus, as long as measurements with the plate reader are made within these linear ranges (i.e., $A_{420} < 1.5$ and $A_{600} < 0.25–0.50$), the absorbance data can be used directly for accurate calculation of standard Miller units.

An important issue with any end point enzyme assay is to ensure that the rate of the reaction is directly proportional to the amount of enzyme, i.e., that the substrate is in excess and that the activities obtained are within the linear range of the enzyme under the conditions employed. Miller reported that β-galactosidase activity is within the linear range as long as the A_{420} produced by enzymatic hydrolysis of ONPG remains below 0.6–0.9 (1). Thus, if assays produce activity values outside the linear range, they should be repeated using a smaller amount of permeabilized cells or a shorter reaction time.

Cell Permeabilization in 2.2-ml Polypropylene Blocks Compared to Permeabilization in Test Tubes

A final important issue is how well our method of cell permeabilization with chloroform/SDS in polypropylene block arrays compares to the traditional method of permeabilization achieved by vortexing cell-chloroform/SDS mixtures in test tubes (1). To examine this, lysogens carrying prophages containing transcription fusions between SoxS-regulated promoters (*zwf, fpr, sodA, nfo,* and *inaA*) and *lacZ* and also lysogens carrying *zwf–lac* fusions harboring single-nucleotide substitutions (Z4C, Z10A, Z12T) within the SoxS DNA binding site were grown in the 2.2-ml block arrays on a platform rotating at 250 rpm in a 37°C incubator until the culture density reached $A_{600} = \sim 0.1$. Induction of SoxS was achieved by adding paraquat to 0.5 mM, and incubation of the blocks was continued for 1 h, at which time protein synthesis was arrested by adding chloramphenicol to 100 μg/ml. A 50-μl aliquot of culture from each well was transferred to a block containing 1 ml of Z buffer and chloroform/SDS and cell permeabilization was carried out by pipetting the mixtures up and down ~10–15 times with a 12-channel pipettor; cells in a second set of 50-μl aliquots were permeabilized by vortexing the mixtures in borosilicate tubes, as described by Miller (1). Cells permeabilized by the two treatments were transferred to microplates and colorimetric assays were initiated by the addition of 20 μl ONPG (4 mg/ml); after 10 min, the reactions were terminated by the addition of 50 μl 1 M Na_2CO_3. Table 1 shows a typical experiment comparing the specific activities of β-galactosidase in cultures permeabilized by the two methods. The specific activities and the induction ratios obtained by permeabilizing cells in the 96-well polypropylene blocks were virtually identical to the values obtained when cells were permeabilized by the Miller method. Accordingly, it is appropriate to

express the specific activities obtained by our method in the standard Miller units. Moreover, cell permeabilization in the 96-well block arrays is much faster than permeabilization by vortexing in test tubes because in our method cell mixtures are permeabilized 12 at a time with a multichannel pipettor.

DISCUSSION

This highly optimized procedure for measuring β-galactosidase activity from large numbers of cultures has many advantages over the traditional method described by Miller (1). A primary advantage of the method described here is that hundreds of samples (assayed twice and in duplicate) can be processed by one person in an 8-h day. Second, bacterial growth and cell permeabilization are performed in compact 2.2-ml polypropylene block arrays, thereby eliminating the use of hundreds of culture tubes and switching back and forth from culture tubes to microplates; moreover, the doubling times obtained with the polypropylene blocks are equivalent to those obtained with standard glass culture tubes. Third, permeabilization by aspiration in the block arrays is as complete as the traditional method of vortexing in culture tubes. Therefore, the β-galactosidase specific activity can be expressed in Miller units. Fourth, manipulations are made simple with the use of multi-channel and repeater pipettors. Fifth, the plate reader permits immediate and accurate absorbance determination, the data are easily exported to and analyzed with Microsoft Excel, and the values are the same as those obtained with a standard spectrophotometer. Finally, this procedure yields highly reproducible results from day-to-day, with standard errors less than 12%.

ACKNOWLEDGMENTS

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