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Estimation of Viable Cell Count after Fluorescein Diacetate Staining Using Phosphorimager Analysis

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Work with cultured cells often requires a precise knowledge of viable cell count to standardize conditions and to carry out quantitative experiments. A popular method of fluorescence for counting viable cells is based on hydrolysis of the esteric bond of fluorescein diacetate (FDA) substrate by cellular esterases, resulting in the release of free fluorescent fluorescein. Detection of fluorescence relies on flow cytometry or fluorescence microscopy. While flow cytometry requires an expensive laboratory instrument that may not always be available in common molecular biology laboratories, the standard microscopic method may be cumbersome and time-consuming when evaluating large numbers of specimens. Exceptional problems can be encountered when working with plant cells. First, plant cells tend to grow in clumps or long chains, and the counting methods often result in large errors. Second, the large size of the cells (up to 200 μm in diameter) makes conventional microscopy difficult when using glass slides with grids.

Here, we describe a simple and reliable method for routinely assessing the viable cell count in plant suspension cultures. The method is based on a classical FDA staining of viable cells (2), followed by direct measurement of the fluorescence intensity on a phosphorimager. We used a plant suspension culture of tobacco cells (TBV-2 cell line) (1) growing under standard conditions to the density of approximately 5 million cells/mL of suspension. The number of cells was estimated in a Bürker chamber, and the cell suspension was serially diluted with Murashige and Skoog (MS) medium in tubes (100–200 μL final volume). The FDA reagent was prepared by diluting a stock solution (3 mg FDA/mL in acetone; FDA from Sigma, St. Louis, MO, USA) with MS medium at 1:200 before use. Equal volumes of FDA reagent

Benchmarks

were added to each sample (final concentration of FDA was 7.5 µg/mL of reaction mixture), and the cells were incubated for 5 min at room temperature with occasional shaking. Twenty-microliter aliquots were then spotted on a piece of nylon membrane (Hybond®-N+; Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) and air-dried. The membrane was then placed upside down into a Model 860 Storm PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA) and a "press sample" function was applied. The fluorescence was scanned on a blue-fluorescence laser channel (excita-

tion wave length 450 nm) at a high resolution (100 pixel). The intensity of fluorescence in each spot was estimated using an ImageQuant™ program (Molecular Dynamics) with an ellipse integration method (Figure 1). The measured units of fluorescence were plotted vs. cell number in Figure 2.

Linearity was obtained for a wide range of cell counts, from 250–10 000 cells/spot. When the FDA reaction was conducted on cells killed by heat-treatment (70°C for 5 min), the fluorescence signal was negligible. The sensitivity limit was approximately 50 cells/spot, which yielded a distinguishable signal

from the background (ca. 0.17 vs. ca. 0.03 for the background). The fluorescence of independently stained samples was quite similar, with standard errors approximately 10%. Removal of free FDA from the reaction mixture before spotting samples on a membrane appeared to be unnecessary; different kinds of nylon-based membranes performed equally well. The fluorescence of dry samples on membranes (stored in the dark at 25°C) was stable, and there were no differences in the repeated measurements, which allowed scanning of membranes up to a week later.

In summary, we have described here a phosphorimager application for monitoring the growth and viability of plant cell cultures. Once calibrated, the system provides routine measurement of a large number of specimens with high accuracy. Moreover, the ImageQuant software enabled storage of data as Microsoft® Excel® (Microsoft, Redmond, WA, USA) files in a database. With minor modification, the described method may be applicable to other types of cells.

Figure 1. Fluorescence scan of nylon membrane spotted with serially diluted TBV-2 cell culture stained with FDA. Note that contours of cell clumps are visible at higher dilutions. Normally, each clump contains approximately 5–10 cells. The image was obtained by processing the scan with an ImageQuant program using the sensitivity range of 0–100 000 counts.

Figure 2. Standard curve depicting a linear relationship between viable cell counts and fluorescence intensity. The fluorescence intensity (IF—arbitrary units) was measured in spots containing serial dilutions of the FDA-stained TBV-2 cells. Each symbol in the standard curve represents an average value from 5 independent experiments, followed by standard deviation (SD).

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