

Microtiter plate assay of yeast cell number using the fluorescent dye Calcofluor White M2R

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The development of a fast, simple, sensitive, and accurate method for counting cells in culture in various testing conditions is important for many fields of biology and biotechnology research. Several methods are widely used, each of which has advantages and limitations. One commonly used technique for counting yeast cells is measurement of optical density by spectrophotometry at an absorbance of 600 nm (A_{600}). This method is simple and easy to handle, but the tendency of cells to settle down is often a limiting factor. Moreover, the narrow quantitation range necessitates laborious concentration adjustment that is unsuitable for high processivity of numerous culture samples with various cell densities. The estimation of cell density by cell counting in a hemocytometer is too laborious and time-consuming to be useful in high-throughput screening. The development of fluorescence indicators of cellular DNA content has made the process of quantitating cell number faster and more convenient. There are several dyes used in cell quantitation that exhibit fluorescence enhancement upon binding to DNA, such as Hoechst 33258 (1-4), Hoechst 33342 (5,6), DAPI (diaminophenylindole) (6,7), propidium iodide (8,9), and SYBR[®] Green I (10). Several methods have been described that quantitate cell number in the microtiter plate, although almost all require cumbersome and lengthy preparative steps or extraction of the bound dye from the cell or are not applicable to unfixed cells. A large number of assays employing quantitation of the cell number that rely on the cellular metabolic activity have also been developed (11,12); however, their utility is limited because the cellular

metabolic processes vary over the time. Also, the method of quantification of dye metabolism is often incompatible with other assays.

Here, we describe the correlation between A_{600} and fluorescence of Calcofluor White M2R dye binding to the cell surface of budding yeast. Fluorescent dye Calcofluor White M2R [also known as Fluorescent Brightener 28 (Sigma, St. Louis, MO, USA)] is frequently used in cell biology to stain plant cell walls and other cellulose-containing structures. In yeast, calcofluor binds to the chitin-rich bud scars present on a mother cell after cell division (13). Figure 1 presents spectral characteristics of bound calcofluor.

We developed and quantified a rapid, simple, fluorometric assay, which is readily adaptable to automation. The accuracy of the calcofluor binding was compared to A_{600} using a UV-Vis spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA) (Figure 2). The correlation coefficient 0.99 is a result of linear regression of the emitted fluorescence as a function of A_{600} , indicating that emitted fluorescence is directly proportional to the number of cells in the well.

Cell number determination can be easily performed according to the protocol shown in Table 1. *Saccharomyces cerevisiae* strains were cultured in sterile 96-well microtiter plates (Porvair Sciences, Shepperton, UK) in the standard media YEPD and YEP-Gal (BD Difco, Sparks, MD, USA) as previously described (14). Calcofluor White M2R stock solution was prepared by dissolving 48 mg solid dye in 10 mL water and filter sterilized. Stock solution can be stored at -20°C for up to 6 months. Calcofluor staining solu-

tion in buffer A was made fresh daily and maintained at room temperature in the dark. Unbound calcofluor emitted very low fluorescence, although better results were obtained if dye solution was removed before fluorescence reading. Therefore microplate cultures were harvested by centrifugation and gently inverted onto the paper towels to remove dye solution. Cells were resuspended in TE buffer [10 mM Tris-HCl, pH 8.0, 10 mM EDTA (Sigma)]. Fluorescence intensity was measured using a fluorescence spectrophotometer (LS55 microplate reader; Perkin Elmer, Wellesley, MA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, respectively (2.5- and 5-nm slit widths). A 5-s reading was set for each microwell. Measurements were taken in triplicate and averaged. TE buffer was used as a blank standard. Fluorescence of unstained cells resuspended in TE buffer was negligible over the complete range of cell densities (almost the same as TE buffer). In each experiment, a standard calibration curve was generated by plotting fluorescence intensity versus A_{600} as determined previously by UV-Vis spectrophotometer.

Under these conditions, curves were linear ($r^2 > 0.99$), detecting as few as 1.5×10^5 cells per well and as many as 1.0×10^8 cells per well with a single dye concentration. Figure 2B shows that the slope is slightly lower at low

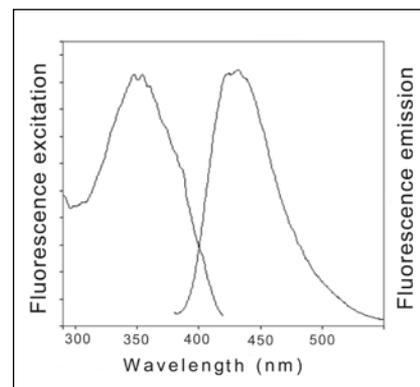


Figure 1. Fluorescence excitation and emission spectra of Calcofluor White M2R bound to the yeast cell surface. *Saccharomyces cerevisiae* cells were stained with calcofluor using protocol described in the Table 1. The excitation spectrum of stained cells was recorded at an emission wavelength of 460 nm. The emission spectrum was recorded at an excitation wavelength of 360 nm.

Table 1. Procedure for Fluorescence-Based Determination of Yeast Cell Number in Microtiter Plate

1. Cultivate yeast in the microtiter plate.
2. Centrifuge for 15 min at $\sim 1800\times g$ and remove culture medium.
3. Prepare staining solution in buffer A [2% D-(+)-glucose, 10 mM Na-HEPES, pH 7.2]: Add Calcofluor White M2R from the 5-mM stock solution to the final concentration of 25 μM . Keep in the dark.
4. Add 100 μL staining solution to each well. Mix thoroughly for 5 min at 600 rpm.
5. Incubate in the dark at 30°C for 45 min.
6. Remove staining solution by centrifugation. Add 100 μL TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) and mix.
7. Measure the fluorescence at an excitation wavelength of 360 nm and emission wavelength of 460 nm.
8. Determine the cell number per each well from the standard curve.

cell concentration, and therefore the number of yeast cells is consequently slightly underestimated. The procedure is applicable to both unfixed and 4% formaldehyde/phosphate-buffered saline fixed cells. The suitability of the assay for different yeast strains was also tested. We measured different *S. cerevisiae* strains: BY4741 (Euroscarf, Frankfurt, Germany), CBL1-20 (15), and EGY48 (16). A high degree of correlation between A_{600} and emitted fluorescence was found for all strains. The fluorescence of chitin-dye complexes correlated linearly with the cell number over a broad range of all yeast strains tested. The assay is applicable for various yeast strains with different morphological characteristics. However, the standard curve needs to be

determined for each yeast strain.

Since calcofluor predominately binds to the bud scar (13), we measured cell population at various growth stages and found that quantitation was not dependent on the senescence of the culture. Standard curves of exponentially (logarithmic) growing cells and the stationary phase cells were nearly indistinguishable (data not shown), indicating that the assay can be applied to the cell number determination at various growth stages and metabolic conditions.

One of the great advantages of the method is fluorescence stability. We exposed calcofluor-stained cells to UV light several times, and no or only very weak photobleaching was observed. Thus, several measurements of the same cells can be performed to obtain

the same value.

In this study, we evaluate calcofluor binding to the yeast cell wall and present feasibility of the resulting fluorescence for cell number determination in a 96-well format. The fluorescence-based method for cell number quantification we describe is especially appropriate when handling a number of samples with a wide range of cell densities. It offers a broad dynamic quantitation range relative to A_{600} . This method could be used in conjunction with other fluorescence-based assays in microtiter plates either simultaneously or before/after the experiment, depending on the methodology of the assay and limitation of quantitation caused by light interference.

In conclusion, high-throughput screening of cells based on fluorescence measurement of various parameters demands fast, reliable, and accurate determination of the cell number. Cell quantitation by calcofluor staining is an excellent alternative. It combines advantages of a fast, simple, and accurate technique that is low cost and reliable for a wide range of cell densities and various *S. cerevisiae* strains. It is nontoxic and noncarcinogenic, and it employs standard equipment and a simple, straightforward protocol. Accuracy, reproducibility, sensitivity, and rapid confirmation make the assay well-suited for large-scale and automated fluorescence-based applications.

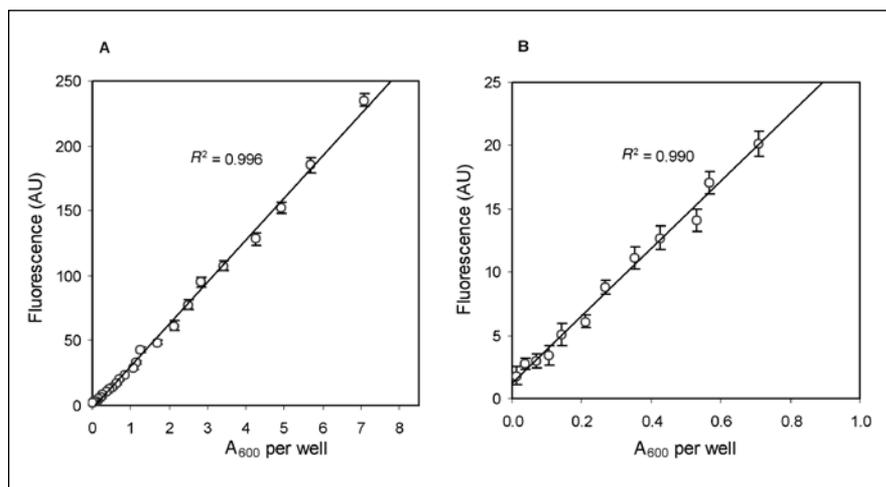


Figure 2. Correlation between A_{600} and fluorescence of calcofluor binding to BY4741 cell surface at assay linearity at (A) high and (B) low cell counts. Experiments were done in triplicates. Data points are the mean (\pm SEM) from three different experiments. Optical density was determined by absorbance measurement at 600 nm (A_{600}). Fluorescence is given in arbitrary units (AU).

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Human aldehyde reductase promoter allows simultaneous expression of two genes in opposite directions

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The simultaneous expression of two genes is becoming an increasingly important practical task for research and biotechnology. In biomedicine, it is often necessary to introduce two coding regions to achieve a therapeutic effect. Common examples include two polypeptide chains that assemble into an active protein, such as a light and heavy chain of antibodies or the two components of the interleukin 12 (IL-12) heterodimer (1,2). New systems for ensuring the expression of two sequences in the same cell would therefore be useful.

Currently, one of the most popular methods to express two sequences in a single cassette takes advantage of internal ribosome entry sites (IRES) to direct cap-independent translation (3,4). With IRES, one bicistronic RNA is transcribed and two separate proteins are translated from that single transcript. However, translation from an IRES is often reduced compared to the promoter-proximal start site, leading to unreliable expression of the downstream gene (5). Another alternative is the use of two promoters, one for each gene. However, this increases the size and complexity of the expression cassettes and may lead to construct instability or to grossly different levels of transcription (6,7). A single bidirectional promoter allows the expression of two sequences within a relatively simple expression cassette.

Several bidirectional promoters and elements have been described. Some of them lie between two recognized genes (8,9), while for others, including the aldehyde reductase (AKR1A1) described here, a second gene has not been found. Many of these promoters are TATA-less,

belong to housekeeping genes, and have CpG islands (10). However, we found only one report in the literature where the simultaneous expression of two genes [chloramphenicol acetyltransferase (*cat*) and luciferase] from a single bidirectional promoter [dipeptidylpeptidase IV promoter (DPPIV)] is described (11); other papers showed activity in forward or reverse orientations separately. The aldehyde reductase promoter (ARP) described here is, similar to the DPPIV promoter, of unusually small size. We have previously shown robust expression from promoter fragments from 153–658 bp in length. The smaller fragments (153–209 bp) were shown to possess over 50% activity of the full promoter and contain no negative regulatory sequences (12). While high expression of the DPPIV promoter has been shown in intestine and renal tubules, a major advantage of ARP is its ubiquitous expression in many different tissue types (12) and throughout embryonic development (Reference 13; unpublished observation). In the present report, we analyze expression from two sets of constructs with minimal ARP sequences.

The ability of ARP to drive expression of two genes simultaneously was tested in transient transfections using firefly and *Renilla* luciferase genes as reporters. The promoter fragment (-124 to +29 relative to the transcription start site) was PCR-amplified and cloned into the *SacI* site of the pGL3-Basic Luciferase Reporter Vector (Promega, Madison, WI, USA) upstream of the firefly luciferase gene. Clones containing the fragment in either orientation were selected and the *Renilla* luciferase gene (from pRL-TK vector; Promega) inserted