SHORT COMMUNICATION

An Alkaline Sucrose Gradient Centrifugation Method Applicable to Non-dividing and Slowly Dividing Cells

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ABSTRACT

An alkaline sucrose gradient centrifugation method applicable for non-dividing and slowly-dividing cells was developed through modification of a fluorometric method of Kissane and Robins.

One of the important requirements for alkaline sucrose gradient centrifugation is to use a small number of cells (10⁴-10⁵ cells) in order to prevent cellular DNA from forming a tangled mass (T. Ono, unpublished data). This requirement implies a necessity of quantitative detection of tens and hundreds ng of DNA in the fractions of the gradient. Up to now, the problem has been solved either by use of DNA previously labeled with radioactive isotopes¹,²,³,⁴ or by use of a zonal centrifugation.⁵ The latter permits a loading of a large number of cells, and brings DNA levels of the gradient to those analyzable by conventional methods of DNA analysis.

In the present communication, a slightly modified fluorometric method of Kissane and Robins⁶ with sensitivity of tens ng DNA was successfully applied for sucrose gradient centrifugation of cultured mammalian cells.

Mouse leukemic L5178Y cells were cultured in Fischer’s medium supplemented with 10 percent horse serum.⁷,⁸ The alkaline sucrose gradient centrifugation was essentially that of Lett et al.⁵ and carried out by a SW 25.1 rotor with a Beckman model L3-50 ultracentrifuge. A half ml. of a cell suspension (2 to 6×10⁵ cells) was added to 0.5 ml. of a lysing solution (0.5 N NaOH and 0.1 M Na₂EDTA), which was
previously layered over 28 ml. of five to twenty percent sucrose gradient in 0.1 N NaOH, 0.9 M NaCl and 0.003 M Na₂EDTA plus 1.5 ml. of twenty-five percent sucrose solution as a cushion in a nitrocellulose centrifuge tube. Lysis was carried out for 2.5 to 3 hours at approximately 29°C. The tubes were centrifuged at 20,000 rpm for 240 minutes. After centrifugation, the sucrose gradient was fractionated into every two ml. through introducing a fifty percent sucrose solution from the bottom of a centrifuge tube. To each fraction, 0.1 ml. of 1 mg/ml. bovine albumine (Nutritional Biochem. Co., Cleveland, Ohio) solution in water was added, followed by 2 ml. of 10 percent trichloroacetic acid and left overnight at 4°C. The solution was centrifuged at 1,200 G for 30 minutes with a model ‘Superior’ centrifuge (Sakuma Co. Ltd., Tokyo). The supernatant was removed by aspiration and the precipitate was subjected to washings, twice with 2 ml. of 5 percent trichloroacetic acid, and once with 1 ml. of 0.1 M alcoholic potassium acetate. Finally, 2 ml. of absolute alcohol was added to the precipitate, kept at 60°C for 15 minutes, and centrifuged. All the washing solution was removed by aspiration after the centrifugation at 1,200 G for 15 minutes. The precipitate after washing procedure was dried either by leaving overnight or by evacuating in a desiccator with a water pump for several hours. To each dried sample 50 µl. of approximately 2 M 3,5-diaminobenzoic acid dihydrochloride (0.45 g of DABA-2 HCl and 1 ml. of water) was added and kept at 60°C for 30 to 45 minutes. Lastly, 3 ml. of 0.6 N perchloric acid was added. The fluorescence at 510 nm with excitation of 414 nm was determined by a fluorospectrophotometer, model RF-502, Shimazu Co. Ltd., Kyoto. All the reagents were special grade and the water was distilled-then-deionized one.

Figure 1 is the standard curve of DNA (salmon sperm, A grade, Calbiochem. Co., La Jolla, Calif.) obtained through subjecting DNA to the above-mentioned steps after drying. When DNA in 20 percent sucrose solution was subjected to all the present regiment, the fluorometric readings of DNA were a constant, 70±5 percent of the standard DNA, independent on the amount of DNA.

In order to compare the previous radioactive labeling method and the present method, the cells were labeled with 0.1 µCi/ml. of H³-thymidine (5.0 Ci/mmol.) for 20 hours (approximately two generations) with 30 minutes of chase. They were resuspended in Puck’s saline G. (11) subjected to the alkaline sucrose gradient centrifugation and fractionated to every 2 ml. Out of 2 ml. fraction, 0.2 ml. was used for radioac-

**Fig. 1.** A standard curve of DNA by the present fluorometric method.
activity counting\textsuperscript{10} and the rest for the present analysis. Figure 2 is one of the typical experiments. It is noted that the fluorometric readings in ordinate of the figure is an arbitrary unit, and not corrected by blank readings (20 units), and the radioactivity counts were also not corrected by background counts (25 cpm). When considering these, the sucrose gradient pattern by the previous labeling method is almost identical with that by the present fluorometric method.

Previously, El-Metainy \textit{et al.}\textsuperscript{12} used a diphenylamine colorimetric method of Burton's for gradient analysis of DNA from barley seeds. However, this method requires at least $10^{-4}$ g of DNA per gradient (equivalent to $10^7$ mammalian cells or over) and is obviously not applicable to the present study. Wheeler and Lett\textsuperscript{13} have applied the fluorometric method of Kissane and Robins\textsuperscript{8} with the sensitivity of $10^{-6}$ to $10^{-7}$ g of DNA per fraction. To make this method applicable, they employed a zonal centrifugation with a larger number of the cells ($2 \times 10^7$ cells).

The present method is applicable to the commonly used gradient centrifugation, except that the rotor SW 25.1 was used to increase the DNA load about 10 times over those of SW 39, 40, 50 and 60 rotors. And the number of fractions was decreased to 18 fractions instead of 30 fractions or over. Under these conditions, the method of Kissane and Robins with the sensitivity of $10^{-4}$ to $10^{-7}$ g per fraction was successfully applied to the sucrose gradient centrifugation. It is added that the method is now applied to mouse thymocytes, hepatocytes and testis (T. Ono and S. Okada, in preparation) as well as to human lymphocytes of circulating blood (Y. Hashimoto, T. Ono and S. Okada, in preparation). They will be reported elsewhere.

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