Bioquimia

Reporte breve
Biología molecular

A modified method for cloning adherent mammalian cells

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ABSTRACT

Dilution cloning of adherent mammalian cells is a very frequent practice in most molecular and cell biology laboratories. It is commonly used for isolation of specific biochemical mutants, and cell strains with marker chromosomes. In this report, we present a modified and simple dilution cloning method that allows even inexperienced workers to successfully clone adherent mammalian cells.

Key words: *Cell culture, dilution cloning, mammalian cells.*

RESUMEN

La clonación por dilución de células de mamífero en cultivo es una práctica muy frecuente en la mayoría de los laboratorios de biología molecular y celular. Esta técnica se utiliza comúnmente para el aislamiento de mutantes bioquímicas específicas y cepas celulares con marcadores cromosómicos. En el presente reporte presentamos un método de clonación por dilución modificado muy simple que permite incluso a trabajadores no experimentados clonar exitosamente células adherentes en cultivo.

Palabras clave: Cultivo celular, clonación por dilución, células de mamífero.

INTRODUCTION

Dilution cloning of adherent mammalian cells is a very frequent practice in most molecular and cell biology laboratories. It is commonly used for isolation of specific biochemical mutants, mycoplasma-free cells,[1] and cell strains with marker chromosomes or to separate individual transfected cells in order to obtain specific clones with higher expression of the interest gene. Although direct colony cloning using rings or discs are also frequently used in less experienced hands it is prone to contamination and it leads to loss of colonies due to drying. Our particular interest is to obtain specific clones that express a transfected gene and maintain the original phenotype, since a large number of the commonly used cancer cell lines are reputedly cross contaminated (estimated globally to be between 17 and 35%).[2,3] In this report we present a modification of the technique, which produces reproducible higher cloning efficiencies.

The modification presented here adds a previous seeding step, which allows the cells to recover one day from the centrifugation step and aids with a visual verification of the correct number. A subsequent serial dilution step reduces the need of repeatedly pipetting small number of cells in a large volume and reduces the centrifugation step required.

MATERIALS AND METHODS

Reagents

Dulbecco's Minimal Essential Medium (DMEM) was purchased from Invitrogen (MD, USA), fetal

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bovine serum from MP Biomedicals (CA, USA), trypsin, phosphate-buffered saline (PBS) and EDTA were purchased from Sigma (MO, USA) All plastic ware used was obtained from Corning (NY, USA).

Cell culture

Cervical cancer cells (HeLa) were maintained in Dulbecco´s modifier Eagle's medium (DMEM) with 5% fetal bovine serum. Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Modified dilution cloning technique testing design

To analyze if the proposed modifications could indeed improve the cloning efficiency, we used a double blind study, in which four newly incorporated MSc students were instructed to perform the classical technique (termed Number 1 method) and the modified technique (termed Number 2 method) to analyze the efficiency of both assays. The workers performed the assays by quadruplicate. The four workers came from different BSc backgrounds, and have not spent more than three weeks in the cell culture facility. Neither of them knew before hand the techniques nor were advised of the particulars of the study. The cloning efficiency was analyzed by two independent laboratory workers in a blind fashion.

Analysis of cloning efficiency

To analyze the cloning efficiency, cells were seeded as described in results and, after 24 hours, screened to count the number of wells with none, one, two or more than two cells. In order to verify that all the cells developed colonies, the cells were then incubated for one additional week, fixed with 70% ethanol, stained with 0.1% crystal violet in phosphate-buffered saline, washed four times in water and visualized in an inverted microscope. Routinely under our conditions, 100% of the cells produced an equal amount of colonies.

Statistical analysis

Statistical analysis was performed using ANOVA to compare all the groups. To corroborate a Bonferroni *t* test was used to compare wells with 1 cell between both methods.

RESULTS

The procedure starts with tripsinizing, counting and seeding approximately 15,000 cells in one well of a 96-well microplate. The next day the cells should be near confluent. Adjustments in the initial number should be performed depending on the cell line used. The medium is aspirated, 50 μL of phosphate buffered saline/EDTA (PBS/EDTA 1 mM) and trypsin to 0.25% are added, and the plate is incubated at 37°C for 5 minutes in order to detach the cells with repeated pipetting. If the cells are difficult to disperse, a 1 mL sterile syringe can be used by passing the suspension one or two times. One hundred μL of complete medium is then added to 9 adjacent wells. Fifty μL of complete medium is added to the first well containing the suspended cells, mixed by careful pipet-

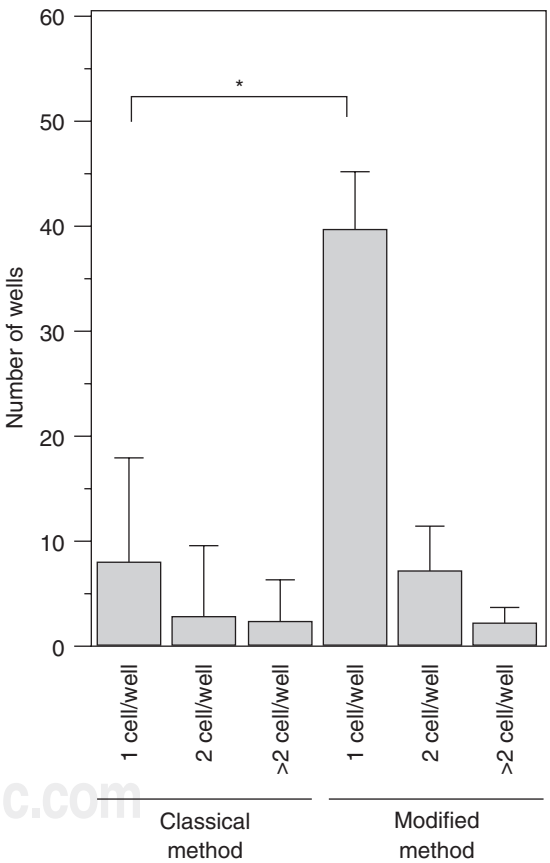

Figure 1. Efficiency of the cloning procedures. Cells were processed as described and the number of wells containing one, two or more than two cells counted. The results are the media and standard deviation for quadruplicate results by each student (i.e. 16 assays).* $p < 0.01$ by ANOVA.

ting and a serial dilution is performed by passing 100 μL of each well successively to the nine prepared wells. Final well should have approximately 54 cells in 200 μL of medium. The cells are further diluted in 10 mL of complete medium or, if the cells have a low cloning efficiency, conditioned and filtered medium. This last step is easily performed in a 50 mL tube, which for the last seeding should be maintained with constantly manual shaking. Aliquots of 100 μL are seeded in the rest of the 96-well microplate. Stand the microplate at a 45° inclination to make the pipetting easier and leave the plate for 15 minutes at this position. This will make for a rapid latter inspection, since the cells will be located at roughly the same position in the bottom. After one week the colonies can be seeded in 24 or 12-well microplates.

DISCUSSION

Dilution cloning[4] involves detachment of the adherent cells cultivated at low density, centrifugation and resuspension of the pellet in a relatively small volume. After counting and diluting to achieve a concentration suitable for obtaining single colonies, the cells are seeded in multiwell dishes or semisolid media such as agar[5] or methocell.[6] For a 96-well dish, usually 40 to 60 cells per 10 mL are used to increase the probability of obtaining single cells in the wells. This technique has the inconvenient that pipetting a small number of cells is always difficult and poorly reproducible, perhaps due to the centrifugation step. The procedure presented here has shown most useful with recently incorporated laboratory workers. Avoiding the shock of centrifugation just before the seeding, increasing the number of dilution steps and using visual aids for the number of cells initially seeded could be accounting for the difference noted.

Although a strict scientific conclusion cannot be obtained from these results due to the limited laboratory worker sample, the results presented here are encouraging, considering that not only a double blind approach was used, since that students with different background, but similar experience, improved their cell cloning ability.

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