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GREEN FLUORESCENT PROTEIN (GFP), A QUANTITATIVE REPORTER FOR MONITORING CELL PROLIFERATION

GREEN FLUORESCENT PROTEIN (GFP), SUATU SIGNAL PENANDA QUANTITATIF UNTUK MEMONITOR PROLIFERASI SEL

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ABSTRACT

Many cell culture experiments involve large number of samples to be analyzed, either for monitoring cell growth or evaluation of the toxicity of compounds to the cells. GFP is a protein which is naturally fluorescence and has been used in a wide range of application such as for gene expression marker, quantitative reporter of heterologous protein production and analysis of transfection efficiency.
This study was aimed to compare accuracy, reliability, and reproducibility of GFP signals with a direct counting method, trypan blue exclusion during cell growth.

Standard curve was made by loading serially diluted CHO-K1-EGFP cells into a 96 well plate. The cell number was correlated with GFP fluorescence signals. With initial density of 1 x 10^3 cells/well, the cell growth of CHO-K-EGFP cells was monitored either using trypan blue exclusion method or fluorescence for certain period of time (n=3).

GFP fluorescence signals showed a good correlation with cell number and gave a r^2 value of 0.9866 in the range of 1250 to 1 x 10^5 cells/well. Similar growth profiles were obtained when the two method were compared for monitoring the cell growth (standard error maximum of 11%).

GFP allows direct measurement of fluorescence signals, reducing manipulation or preparation procedures which may influence the accuracy of the data obtained. Once the stable clones have been obtained, GFP can be used in a wide range of application.

Key words: *Green Fluorescent Protein* (GFP), *Fluorescence*, cell proliferation, *Trypan Blue Exclusion*

ABSTRAK

Banyak penelitian sel kultur yang melibatkan sejumlah besar sampel untuk dianalisis, baik yang berhubungan dengan pertumbuhan sel atau toksisitas senyawa terhadap sel. *Green Fluorescent Protein* (GFP) adalah suatu protein yang secara alami dapat berfluorescence dan banyak digunakan pada berbagai aplikasi seperti penanda untuk ekspresi gena, produksi *heterologous protein* atau monitoring efisiensi transfeksi. Penelitian ini bertujuan untuk membandingkan tingkat akurasi, taraf kepercayaan dan reprodusibilitas GFP untuk memonitor pertumbuhan sel.

Kurva baku dibuat dengan serial dilusi sel CHO-K1-EGFP dalam media 10% FCS di 96 *well plate*. Jumlah sel dalam tiap sumuran dihubungkan dengan signal fluorescence. Untuk memonitor pertumbuhan sel, signal fluoresensi dibandingkan dengan metode *Trypan Blue Exclusion* yang jumlah sel dalam tiap sumuran dihitung selama periode waktu tertentu (n=3).

Untuk monitoring pertumbuhan sel, signal dari GFP memperlihatkan korelasi yang baik dengan jumlah sel dengan tingkat linieritas 0,9866 dalam kisaran jumlah sel 1250 – 1 x 10^5 sell/sumuran (standar error maksimum 11%).

Metode ini terbukti memungkinkan pengukuran langsung signal fluoresensi sehingga mampu menurunkan kemungkinan kesalahan yang terjadi pada saat preparasi sel yang dapat mempengaruhi akurasi

data yang diperoleh. Sekali klones permanen (*stable clones*) diperoleh klons ini dapat digunakan untuk banyak aplikasi.

Kata Kunci: *Green Fluorescent Protein* (GFP), *Fluorescence*, proliferasi sel, T*rypan Blue Exclusion*

INTRODUCTION

Many cell culture experiments require accurate counting of cell number. The rapid, accurate estimation of cell viability is vital to successful mammalian and tissue culture in a wide range of applications, such as the development of high producing cell lines, evaluation of toxicological properties of compounds used experimentally and evaluation of cell cultivation process.

The green fluorescent protein (GFP) was originally discovered and isolated from the bioluminescent jellyfish *Aequorea victoria*. It is naturally fluorescent and the chromophore of this protein is made up of a ser-tyr-gly tripeptide which undergoes cyclization and oxidation reactions to become active (Prasher *et al*., 1992). It is maximally excited with blue light (395 nm with a small peak at 470 nm) and emits green light at 509 nm (Chalfie *et al*., 1994). The fluorescence signal is readily detected by means of a microscope, fluorometer or FACS machine.

The cDNA encoding this protein has been cloned and subsequently expressed in both prokaryotes and eukaryotes (Chalfie *et al*., 1994). In studies using mammalian cell lines, Gubin *et al*. (1997) found that high level GFP expression was maintained for months after over many passages in the absence of selective growth condition.

GFP has been used in a wide range of application, for example, for gene expression marker, quantitative reporter of heterologous protein production and analysis of transfection efficiency (Albano *et al*., 1998; Chalfie *et al*., 1994). An attempt to use GFP as a means to monitor biomass in mammalian cell culture was done by Hunt *et al*. (1999). They observed that the fluorescence signal of expressed GFP closely follows cell number during the exponential growth phase.

In this study we compare indirect counting method based on fluorescence (GFP) and cell number counted by trypan blue exclusion during cell growth. The linearity and the sensitivity of method would be determined by the changes in the intensity of fluorescence signals in a variety of cell numbers.

MATERIALS AND METHODS

Cell line Producing GFP

The cell line used in this experiment was CHO K1 attached cells (ATCC CCL 61), transfected with pEGFP-C1 constructed by S. Pak and C. Marquis (Figure 1). The GFP fluorescence was expressed under constitutive CMV promoter. The cells were cultivated in a 1:1 mixture of Dulbecos Modified Eagles Medium (DMEM) and Coons F12 (CSL Melbourne) supplemented with 10% Fetal Calf Serum (FCS) in T-flask at 37^0 C and 5% CO_2 humidified atmosphere. Every two or three days the cells were subcultured at reduced concentration (10-30% confluence) into fresh media.

Cell Growth

All experiments were carried out in triplicates and repeated at least twice. Cell number counted by trypan blue exclusion was compared by fluorescence signals of indirect cell counting method (GFP signals) by following the growth of cells over a period of time. The inoculum density was 1×10^3 cells/well.

Standard Curve

Cell number was correlated with fluorescence-based indirect cell counting method by dilution of GFP-containing cells in a range of 50 to 10^5 cells/well in 10% FCS-containing medium in a 96-well plate. Following cell attachment, the cell number was counted by measuring GFP signals using microplate reader (f-max Molecular Devices).

Cell Counting

Trypan Blue Exclusion

In a 96 well plate the media need to be removed prior to adding of trypsin solution (Sigma). Incubation up to 10 minutes was required with pipetting up and down in between. Trypan blue was added and the culture was mixed. PBS solution was added if required. Cell counting was performed using a haemocytometer under the microscope according to Freshney (1994).

Green Fluorescent Protein (GFP)

On the day of the assay, the fluorescence produced by the cells in 96 well plate was measured using a fmax Molecular Devices microplate reader at 485 nm excitation and 530 nm emission wavelength.

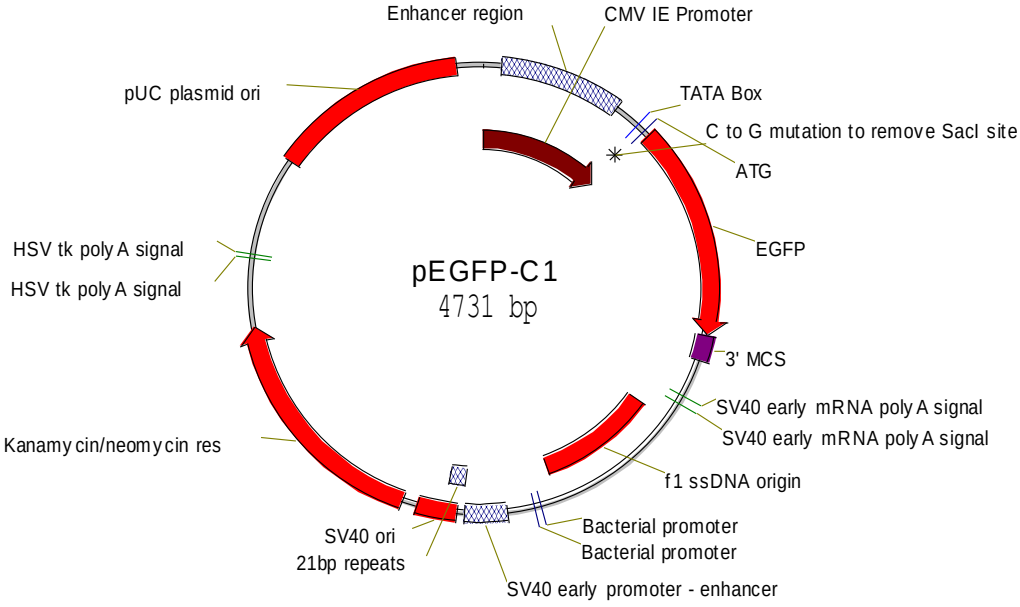

Figure 1. Map of pEGFP-C1 (constructed by S.Pak and C. Marquis)

RESULTS AND DISCUSSION

Green Fluorescence Protein (GFP) has been widely used in the laboratory as a reporter gene for recombinant protein production. It has been observed that the intensity of the GFP fluorescence correlated with protein expression (Bailey, et. al., 1999). Since green fluorescence is only detected in viable cells (Hunt, *et al*., 1999), the use of a cell line expressing GFP was advantageous for viability/proliferation studies.

In these experiments stable clones expressing green fluorescence protein were studied in which the intensity of green fluorescence was monitored during the cell growth using a microplate reader at excitation wavelength of 485 nm and emission wavelength of 530 nm. The signal intensity produced by this clone was compared to the cell number counted by manual cell counting, trypan blue exclusion method.

A stable clonal cell line was obtained by Lipofectamine^{TM} transfection of CHO K-1 cells with pEGFP-C1 under the control of cytomegalovirus intermediate early (CMV_{IE}) promoter. This promoter is strong and constitutive, meaning that any cells containing this plasmid should fluorescence brightly and its fluorescence should be relatively independent of environmental factors. This cell line was given by L. de Boer and was developed by S.Pak and C. Marquis.

Correlation of Fluorescence to Cell Number

In these experiments the cells were seeded at different densities (50 to 10^5 cells/well) into 96 well plate to assure a wide range of cell numbers. GFP fluorescence linearly correlated to cell number when serially diluted CHO-EGFP-C1 cells were plated (r^2 values of 0.9866 over a range of 1,250 to 1 x 10^5

cells/well). As demonstrated in figure 2, whilst this method offered high linearity, the GFP signal was found to be sensitive with minimum detection level of as a few as 1,250 cells/well. A standard error of maximum 11 % was observed. This relatively high error was suggested due to pippeting error during cell manipulation. With a high accuracy of pipetting technique, lower standard error was obtained (data not shown).These results demonstrated that pipetting manipulations such as dilution or sampling greatly contribute to the reliability and reproducibility of the method.

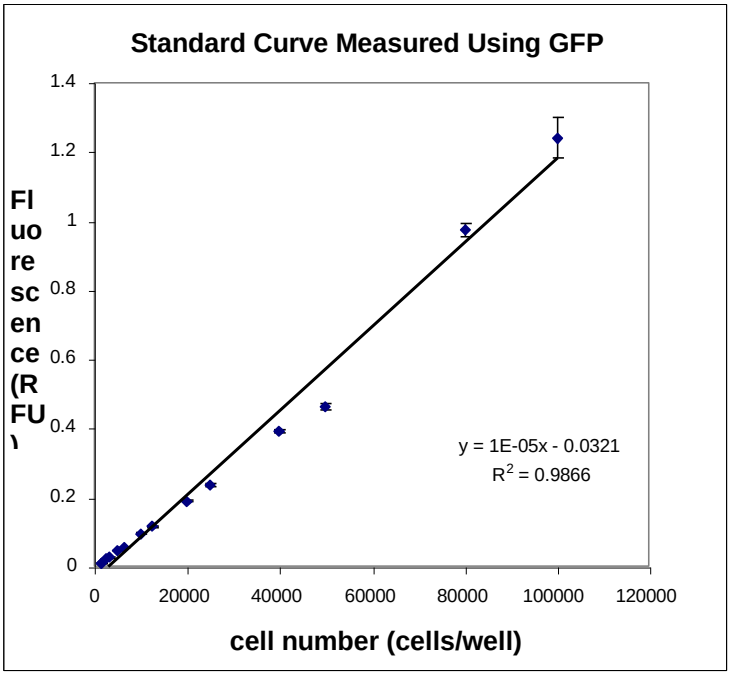

Figure 2. Standard curve correlates cell number and green fluorescence signals

Growth Study

A close correlation of GFP signals and cell number was observed (figure 3). Comparison of cell growth measured using GFP and trypan blue exclusion showed similarity when the cells were plated at initial density of 1,000 cells/well. Statistical analysis demonstrated that the GFP signals measured over time during the cell growth correlated with the cell numbers. This method was found to be statistically reproducible (t-test with $p < 0.05$, data not shown).

It was found that during the cell growth the higher the cell number, the higher the fluorescence intensity was produced by the cells. When the cells were at high cell density, the fluorescence intensity was still proportional to viable cell number indicating that layering of the cells in the bottom of the plates over the range of confluency did not impair the fluorescence signal detection by a fmax microplate reader. This method was found to be reproducible for determining cell number.

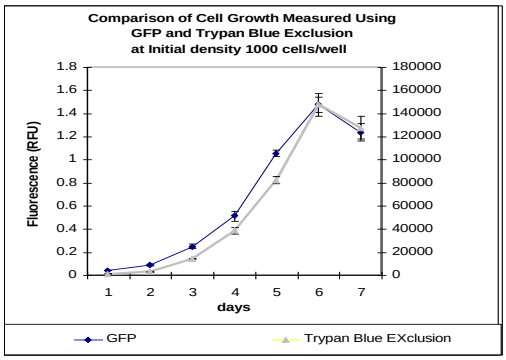

Figure 3. Comparison of cell growth profiles measured by GFP and trypan blue exclusion

CONCLUSION

Stable cell lines which constitutively express high level of GFP can be used to determine viable cell number and to monitor cell growth. This method allows direct measurement of fluorescence signals, reducing manipulation or preparation procedures which may influence the accuracy of the data obtained. Once the stable clones have been obtained, GFP can be used in a wide range of application.

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