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WST-8 assay for high throughput screening of cell viability: comparison studies with MTT assay for monitoring cell growth

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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. There is an increasing interest to find rapid and accurate cell counting methods to the successful cell and tissue culture studies.

The objective of this research was to compare accuracy, reliability, and reproducibility of a common cell counting method, MTT assay to the relatively new method, WST-8 assay for monitoring cell growth.

Standard curve method was made by loading serially diluted CHO-K1 cells into a 96 well plate. The cell number was correlated with absorbance signals produced by MTT and WST-8 assays. With initial density of 1×10^3 to 4×10^3 cells/well, the cell growth of CHO-K1 cells was monitored either using trypan blue exclusion method or MTT and WST-8 assay for certain period of time (n=3).

Results showed that WST-8 assay had a better correlation with cell number compared to MTT assay for monitoring cell growth. Using acid isopropanol as solubilisation agent, MTT assay gave a linearity of 0.9578 in the range of 400 to 1×10^5 cells/well with a maximum standard error of 11 %. DMSO did not increase the sensitivity and reliability of the method (r^2 value was 0.9819 within a range of cell concentration of 1500 to 1×10^5 cells/well and has a standard error of 14 %). The WST-8 assay gave a better linearity with r^2 value of 0.995 in the range of 400 to 8×10^4 cells/well with standard error less than 11%. During the cell growth, WST-8 assay is superior compared to MTT assay.

It was concluded that its low sensitivity, high variability and technical consideration made MTT assay inappropriate to be regarded as the best method for cell counting. The soluble and ready to use tetrozolium salt, WST-8 assay offered higher accuracy and linearity for determining viable cell, making this assay more reliable and convenient for high throughput cell counting.

Keywords: WST-8 assay – MTT assay – cell number and viability

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.

A variety of methods and instruments have been developed to quantify mammalian cells. Conventionally, counting cells is performed by haemocytometer, using vital stain such as trypan blue, which provides

information of both cell number and viability. However, to count a large number of samples is laborious and fraught with personal error (Cook and Mitchell, 1989). An electronic counter, such as Coulter^R counter, or a portable flow cytometer, Microcyte, can also be used to estimate both cell number and viability (Al-Rubeai *et al.*, 1997). However, again both these methods are inconvenient for a large number of samples. In addition, samples from cell cultures often show a tendency to form cell aggregates, which makes it difficult to count individual cells.

Numerous reports have suggested the increasing interest of measuring absorbance signals in microplates for high-throughput applications (Finlay et *al., 1984;* Lowick *et al.*, 1993; Mohler *et al.*, 1996*).* In these studies we introduce WST-8 assay in comparison with other absorbance-based cell viability method, MTT assay for determination of cell growth.

Materials and Methods

Cell line

Cell line used in this project is CHO K1 attached cells (ATCC CCL 61). 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°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 absorbance signals of indirect cell counting methods by following the growth of cells over a period of time. The inoculum density ranged between 1 x 10^3 and 4 x 10^3 cells/well.

Standard Curve

Cell number was correlated with absorbance-based indirect methods by dilution of cells in a range of 50 to 10^5 cells/well in 10% FCS-containing medium a 96-well plate. Following cell attachment, the cell number was counted by MTT and WST-8 assays.

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).

MTT assay. The MTT assay was adopted from Mosman (1983) and Carmichael *et al.*, 1987). Ten µl of MTT solution at concentration 5 mg/ml (Sigma) was added into each well of 96 well plate and followed by 4 hours incubation at 37°C. One hundred µl of acid isopropanol (0.04 N HCl Isopropanol) (Mosman, 1983) or DMSO (Carmichael, 1987) was added to solubilise the dark blue crystal. Pipetting up and down the culture in each well was necessary to dissolve the formazan into the medium. The absorbance was measured using Emax Molecular Devices plate reader at 540 nm with 650 nm reference.

WST-8 assay. The protocol was employed as per the WST-8 (Cell Counting Kit-8) specification sheet (Dojindo, Japan). The frozen solution (Dojindo, Japan) was thawed in a water bath at 37°C (5 minutes) or on the

bench top (30 minutes). Ten µl of the solution was added to each well of the plate containing 100 pi of media and be careful not introduce bubbles to the well since the bubbles interfere with the OD reading. Note: To measure the absorbance later, 10 µl of 1% w/v SDS could be added to each well and cover the plate to keep away from the light and store at room temperature. The plate was incubated for 2 hours at 37°C. The absorbance was measured at a wavelength of 450 nm using an Emax Molecular Devices plate reader with a reference wavelength of 650 nm.

Results and Discussion

Correlation of absorbance to cell number

In the first experiment, cell number was correlated with absorbance of coloured formazan product using microplate reader at 595 nm wavelength with reference of 650 nm. This was performed by dilution of cells in a range of 50 to 10^5 cells/well in 10% FCS-containing medium and were loaded into a 96-well plate. After incubation for 4 hours in the presence of MTT solution, solubilisation agent (acid isopropanol) was used to dissolve the formazan. The isopropanol was acidified to convert the indicator phenol red to yellow, which did not have high absorbance at 570 nm. Studies using CHO-K1 cells demonstrated that the lowest detection limit (i.e., the number of cells that give signal above background within reasonable standard error) of this method was 400 cells/well. As shown in Figure 1a, within maximum cell density tested (1 x 10^5 cells/well) the curve generated r^2 value of 0.9578 with maximum standard error of 11%. However, lower cell counts (1.25 x 10^4 cells/well), gave a better r^2 value of 0.9888 (Figure 1b).

For determining cell number, MTT assay was found to have high variability. Laborious pippetting procedures during solubilisation of formazan product which is time consuming and prone to error, yet could not be avoided. Acidified isopropanol was replaced with DMSO as the solubilisation of formazan was reported to be much easier (Carmichael *et al.*, 1987; Twentyman *et al.*, 1987; Marionnet *et al.*, 1997).

Studies using DMSO as solubilisation agent, however, did not give better results. lt was foulid that the sensitivity of M'l"T assay using this solvent to detect cell number was poorer. The minimum cell number that could be detected within reasonable standard error was 1500 cells. A r^2 value of 0.9819 was observed within a range of cell concentration of 1500 - 1 x 10^5 cells/well (Figure 1c). Higher r^2 value of 0.9953, however, was observed with maximum cell density of 1.25 x 10^4 cells/well, the same value as that observed using acid isopropanol (Figure 1d). Although DMSO offered a rapid and easier step in dissolving the formazan, compared to acid isopropanol greater standard error (± 14%) was observed. This error was primarily found at low cell number.

Growth Study

In subsequent experiments, absorbance was compared with cell number by following the growth of cells over a period of time. The relationship between absorbance and viable cell count is illustrated in Figure 2. Good correlation between absorbance and cell number was observed at initial cell growth. However, of the three assays trialed, there was no observable correlation.

WST-8 assay

Correlation of absorbance to cell number. As mentioned in the original protocol (Tominaga *et al.*, 1999), WST-8 gives absorption at 450 nm by cellular reduction and a yellow formazan product which is soluble

in the tissue culture medium. In the presence of electron mediator,1-methoxy PMS, the production of WST-8 formazan increased with increasing incubation time for up to 4 hours as measured by absorbance. For this experiment, a 2 hours incubation time was used.

At first, dilutions of 50 to 10^5 cells/well in 10% FCS medium were loaded into a 96-well plate. The result demonstrated that the amount of formazan generated by dehydrogenase in cells was directly proportional to cell number of living cells in the range of 400 - 8 x 10^4 cells/well with linearity r^2 = 0.995, as shown in Figure 3. The standard error observed using this method was less than 11 %.

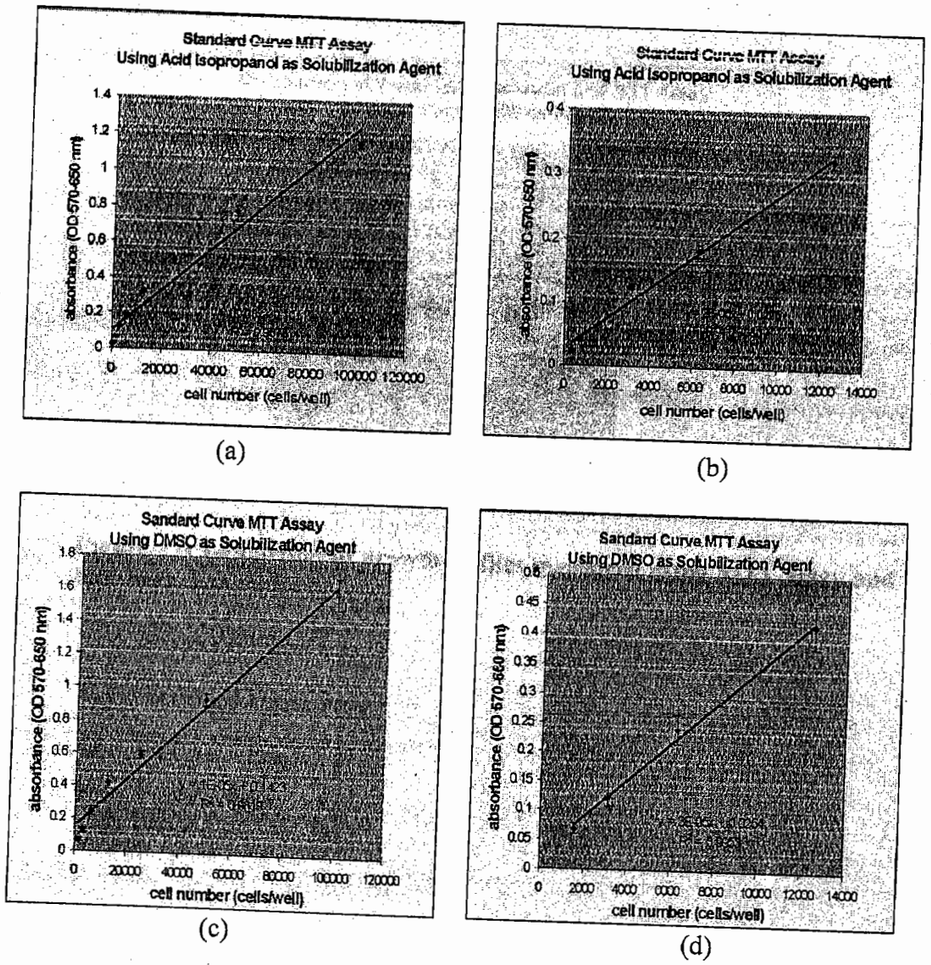

Figure 1. Standard curves correlate cell numbers and absorbance signals of MTT assay. (a) using acid isopropanol as solubilisation agent, at high cell number, (b) using acid isopropanol as solubilisation agent, at lower cell number, (c) using DMSO as solubilisation agent, at high cell number, (d) using DMSO as solubilisation agent, at lower cell number

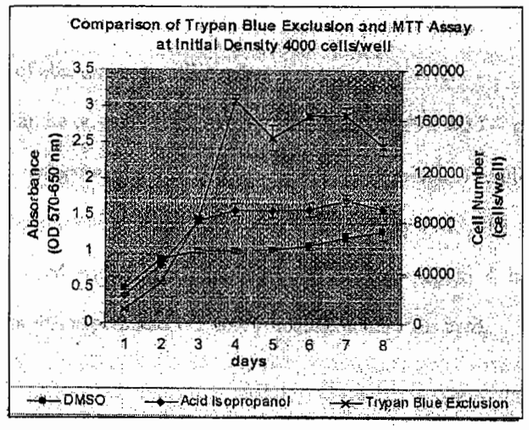

Figure 2. Comparison of growth profiles measured using MTT assay and trypan blue exclusion method

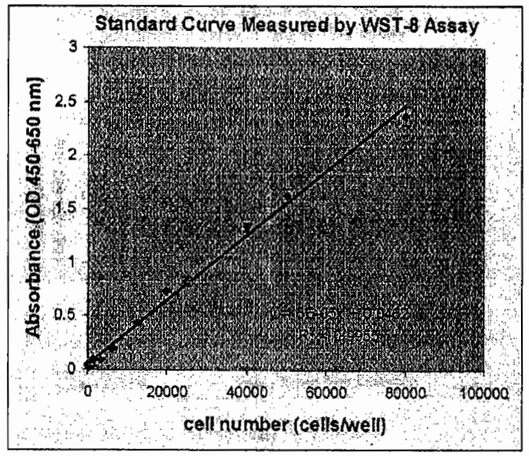

Figure 3. Standard curve correlates cell number and absorbance signal of WST-8 assay. Serially diluted CHO-K1 cells in the range of 50 to 1 x 105 cells/well loaded into a 96 well plate. Following cell attachment, the cell number in each well was determined by WST-8 assay as described in the text.

Growth Study. Having a good correlation between absorbance signal and cell number, WST-8 assay was used to monitor the cell growth. Studies using various initial cell densities demonstrated that good correlation between cell number and absorbance was observed during the first 3-4 days of cell growth. However, as depicted in Figure 4, once the density reached high cell number (more than 8 x 10^4 cells/well), the absorbance level was so high that it could not be detected by plate reader (maximum absorbance value of microplate reader was 3).

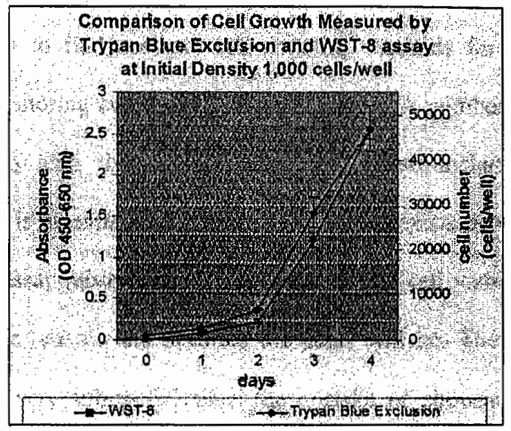

Figure 4. Comparison of cell growth profiles measured using WST-8 assay and trypan blue exclusion using two different initial densities, (a) 1000 cells/well, (b) 2000 cells/well.

There is the need to find accurate cell counting methods for various experiments which involve either monitoring cell growth or proliferation. A number of properties are important for conversion to high throughput techniques. These include accuracy, relia-

bility and reproducibility. It is essential that a linear relationship exists between cell number and signal end point of the assay (absorbance signal) in assessing cell viability. The higher the level of linearity, the more reliable and accurate the assay will be. The high level of accuracy and reproducibility is very substantial since the mistake of choosing inappropriate method for determining cell number may lead to erroneous interpretations of results.

In this study linearity and sensitivity are determined by the changes in absorbance which should reflect cell numbers. Cell growth was also monitored using a similar approach over a period of time in standard plastic tissue culture, 96 well plates. For these particular experiments, the effects of culture conditions such as pH and protein concentration to the reproducibility of the methods were examined. All the experiments were conducted at least in triplicate and repeated at least twice to assure the reproducibility.

For this study trypan blue exclusion method was employed to determine cell number. It is a direct method for cell counting and has been routinely used for cell viability assessment (Mishell *et al.*, 1980). In this study, this method was used in comparison with indirect cell counting methods MTT and WST-8 assay for determining cell numbers and cell viability.

In this experiment we examine MTT assay for monitoring cell growth in a 96 well plate. This system, with its convenient plate format, provides a high througput method and is promoted as a preferable alternative to techniques such as trypan blue exclusion. However, we found that laborious procedures and high variability made this method inappropriate to be regarded as the best method for cell counting.

The following explanations were likely to be responsible for the occurrence of high variability. At first, this experiment was intended to adopt a method that has been widely used in the laboratory in which isopropanol was acidified using 0.04 N HCl. This organic solvent was needed to dissolve the insoluble formazan product. However, the results demonstrated that the solubility of formazan product in final acid isopropanol-medium mix was extremely difficult and prone to high variability. In addition, the use of acid in the final solvent could alter the spectral properties of the t'ormazan (Denizot and Lang, 1986). In another aspect, serum content in the media was suggested to also cause high variability due to its precipitation when the organic solvent was added (Denizot and Lang, 1986).

Apart from the factors mentioned above, it has been observed that the use of reference of 620 or 630 nm caused reduction in sensitivity of about 45% (620 nm) and 24% (630 nm), respectively (Denizot and Lang, 1986). These investigators recommended the use of higher reference (690 nm) to correct such artefacts as scratches on the bottom of the wells in order to increase the sensitivity. However, at the time these studies were carried out a 595 nm filter and 650 nm of reference wavelength were available, therefore, this problem could not be overcome.

Studies on solubilising solvents (Carmichael *et al.*, 1987) demonstrated that mineral oil and DMSO were better than acid isopropanol. This was especially true when they were used for non-adherent cells in which the media could not be removed from the wells. In this experiment, the relatively toxic DMSO was chosen as mineral oil had the disadvantage that all media had to be removed completely from the wells prior to its addition (Carmichael *et al.*, 1987). However, the results showed that using DMSO, the MTT assay was less sensitive compared to the original protocol described by Mossman (1983). A detection limit of 1500 cells/well was found compared to 400 cells/

well of the original protocol. Poor sensitivity of this method has been reported by Marionnet and colleagues (Marionnet *et al.*, 1997).

Growth studies demonstrated that in 96 well plate absorbance did not correlate with cell number. During cell growth, as the cells reach a concentration of around 1×10^5 cells/well, there was no further significant increase in absorbance, although the cell number increased as observed by trypan blue exclusion. A number of factors may have contributed to this lack of correlation including pH and media volume. Although the plate was sealed during the cell growth and was incubated in a CO_2 incubator, the loss of medium due to evaporation over one-week period of incubation could not be avoided. A volume change as small as 10 µl from a total volume of 200 ptl has been reported to cause a shift in the shape of the absorbance spectrum of formazan in DMSO[10]. In addition, the sodium bicarbonate content in the medium could also impact the absorbance due to a change in the spectral properties (Jabbar *et al.*, 1989). This may have been the case as the medium was used for cellular growth for several days. Also, it has been a major disadvantage of OD measurement in which at high cell concentration saturation of absorbance signal would occur.

WST-8 was employed in these studies to overcome the difficulties found in the application of MTT such as in solubilisation of formazan product and its sensitivity. Since the reduction process of WST-8 was found to be highly water soluble (Ishiyama *et al.*, 1997), it was expected that the high variability observed in the MTT assay would be reduced and a better correlation of cell number and absorbance would be obtained.

The results demonstrated that WST-8 assay was found to be as sensitive as the MTT assay. After the signal obtained was subtracted from background absorbance (signals of the wells containing media only), a minimum detection limit of 400 cells was observed. Cell numbers below 400 cells/well yielded an absorbance signal which was low and variable and resulted in very large standard error. However, compared to MTT assay, a higher upper limit of cell number (8×10^4 cells/well) could be detected. Although a complete profile of cell growth was not obtained, even when a lower initial cell density (1,000 cells/well) was employed (Figure 4a), higher accuracy and linearity were observed. On the other hand, as can be seen in Figure 4b, there is a low level of standard error indicating that WST-8 assay could be regarded as a reasonable choice assay for determination of cell viability.

Studies using CHO-K1 cells demonstrated that WST-8 assay was convenient for determining cell number. The available reagent (purchased from Dojindo, Japan) was only added into the whole culture in a 96 well plate without addition of soluble agent, therefore avoiding error due to manipulations prior to absorbance measurement.

Conclusion

It has been shown that absorbance of MTT assay did not correlate well with cell number in a high cell number and therefore should not be used for high-throughput applications. However, the results suggest that this assay could be used for monitoring cell proliferation as long as the culture conditions were kept stable in terms of pH, serum content and volume of medium. On the other hand, WST-8 assay offered more accurate, reliable and reproducible results. The commercially available of this reagent in one bottle, the readily to use and no need

of organic solvents made this method convenient to be used for determining viable cells.

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