Application Note

Cell Health and Viability

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An Absorbance-based Cytotoxicity Assay using High Absorptivity, Water-soluble Tetrazolium Salts

Cell quantitation using WST-8 and the Agilent BioTek Synergy Mx

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Abstract

Cellular proliferation and cytotoxicity studies are the mainstay for cell biology and cancer research. Cell proliferation research involves the assessment of different molecules ability to elicit cell growth and multiplication, while antitumor chemotherapy studies usually involves the investigation of the ability of agents to specifically kill tumor cells. In either case, an assessment of the change in cell number over time is a critical component in the research. Here we describe the use of an Agilent BioTek Synergy Mx monochromator-based multimode microplate reader to quantitate cells using a WST-8 cell counting kit.

Introduction

Quantitation of tissue culture cells has been the hallmark for the determination of efficacy of agents that either promote or inhibit cell growth. While cells grown in suspension can be counted directly, most tissue cultures are grown in monolayer culture, which requires dispersal using proteolytic agents such as trypsin prior to quantitation. Dispersed cells can be directly quantitated both manually with the use of a microscope and a hemocytometer or in an automated cell counter. These methods are slow and labor intensive, requiring individual samples to be quantitated individually.

There are a number of different indirect methodologies to quantitate cell number with large numbers of samples using microplates. Simple total protein and nucleic acid assays that indirectly provide information regarding cell number or more specifically changes in cell number are based on the concept that cells on average have a constant amount of these polymers. Increases or decreases of these polymers would be indicative of cellular proliferation or cytotoxicity respectively. However, these assays do not necessarily provide information regarding the viability of the cells in question and several "live cell" assays have been developed. One of the first methods developed is the incorporation of ^{3}H-thymidine. Only live cells will incorporate this traceable radioactive moiety into acid precipitable nucleic acid. While very effective and specific, this method suffers from a lengthy sample preparation procedure and from the use of radioactivity, with its inherent dangers, regulations and disposal costs. Several non-radioactive assays have been developed. These assays employ detection technologies such as absorbance, fluorescence, and luminescence.

Assays that detect the presence of ATP, a labile high energy molecule only present in living cells can be measured by the luminescence produce when it interacts with luciferase enzyme. Substrates that are converted directly from a colorless compound by cellular metabolism include the fluorescent compound calcein, as well as many tetrazolium salts. Tetrazolium salts are reduced enzymatically to produce colored formazan dyes. One of the first and most notable of these salts is MTT, which produces a colored insoluble product that has to be solublized with ethanol.[1] Improvements of the tetrazolium substrates has produced compounds that have water soluble products; the first of which is WST-1. This compound has a maximal absorbance wavelength of 438 nm. Further improvements in the tetrazolium salts have improved both reactivity and molar absorbtivity, along with decreasing toxicity.

The cell counting kit uses WST-8 in conjunction with an electron mediator, 1-methoxy PMS, to assess viable cells. The tetrazolium salt, WST-8, is reduced through a reaction with the reduced form of 1-methoxy PMS. This mediator resides at the cell membrane and reacts directly with NADH (nicotineamido adenine dinucleotide reduced form) or NADPH (nicotineamido adenine dinucleotide phosphate reduced form) (Figure 1). NADH and NADPH are generated from NAD+ or NADP+ by the reaction of dehydrogenase enzymes and their substrates, such as lactate dehydrogenase and lactic acid respectively. Therefore, the tetrazolium salt is utilized for the determination of the dehydrogenase activity or a substrate of the dehydrogenase.

The solution of a WST-8 tetrazolium salt is almost colorless while its formazan product gives a strongly colored solution with a peak absorbance at 460 nm.

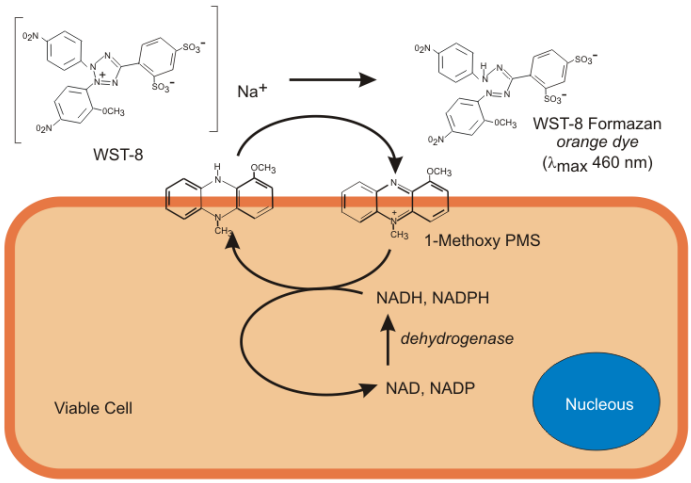

Figure 1. WST-8 assay mechanism.

Materials and methods

A cell counting kit (CCK-8) was obtained from Dojino Molecular Technologies (Rockville, MD). Sterile 96-well clear-bottom microplates (part number 3603) were obtained from Corning (Corning, NY). Culture media, fetal bovine serum, insulin, and antibiotics were purchased from Invitrogen (Carlsbad, CA).

Mouse C-10 lung epithelial cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). Cells were trypsinized and resuspended in fresh media at a concentration of 75,000 cells/mL. Serial dilutions (1:2) were made using DMEM/F12 with 10% FBS as the diluent. Aliquots (200 µL) of each cell dilutions were then pipetted into 96-well microplates. Cells were allowed to grow overnight

at 37 °C in a humidified 5% CO_2 environment. The following day the cells were quantitated using a cell counting kit CCK-8. Cell quantitation was performed according to the assay kit instructions. Briefly, 20 µL of the ready to use kit reagent is added directly to cell cultures in 96-well plates that have 200 µL of media. Cells are incubates in a humidified environment at 37 °C, 5% CO_2 for 2 hours. After incubation, the absorbance at 460 nm was determined using a Synergy Mx multimode microplate reader.

For cytotoxic agent studies, primary human mesothelioma cells were seeded at 5,000 cells per well in a 200 µL volume in DMEM/F12 media supplemented with 10% FBS. After allowing cells to grow for 48 hours at 37 °C in a humidified 5% CO_2 environment, various concentrations (0 to 40 µM) of the antibiotic, thiostrepton were added to the cells. Following a 24 hour exposure to the drug, 10 µL of the cell counting reagent was added directly to the cell cultures. The plates were incubated for 120 minutes at 37 °C in a humidified 5% CO_2 environment. The WST-8 formazan product was measured at 460 nm using a Synergy monochromator-based multimode microplate reader as described previously.

Spectral scans of reacted and unreacted cell cultures were performed using a Synergy Mx multimode microplate reader. Reacted wells contained approximately 20,000 primary mesothelioma cells while unreacted wells only had media. Absorbance measurements were made from 300 nm to 700 nm in 1 nm increments. In all experiments, the reader was controlled by and the data collected using Agilent BioTek Gen5 data analysis software.

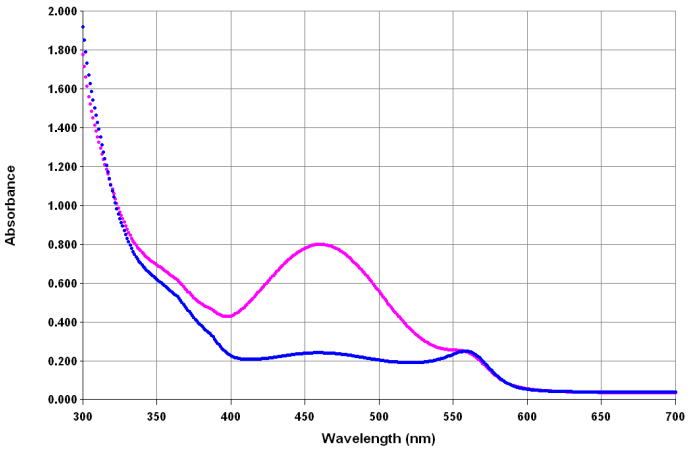

Figure 2. Spectral scan of WST-8 formazan. The absorbance spectra from 300 nm to 700 nm in 1 nm increments was measured in reacted samples with WST-8 formazan product (magenta) or nonreacted WST-8 (blue).

Results and discussion

An absorbance spectral scan of reacted WST-8 formazan product, as well as unreacted WST-8 in media demonstrates the unique peak in absorbance at 460 nm. Note that the absorbance peak for phenol red indicator dye can be seen as a small in crease in absorbance at 560 nm. The phenol red absorbance peak can easily be distinguished from the WST-8 formazan product peak at 460 nm. This allows the user the option of using media containing phenol red. Any increase in absorbance at 460 nm as a result of the presence of phenol red should be the same for all wells and can be subtracted by using a phenol red only blank well.

Figure 3 demonstrates the ability of the assay to determine different numbers of cells within the wells. When serial dilutions of mouse C-10 lung epithelial cells are seeded into 96-well microplates and assayed the following day a linear response in absorbance at 460 nm is observed.

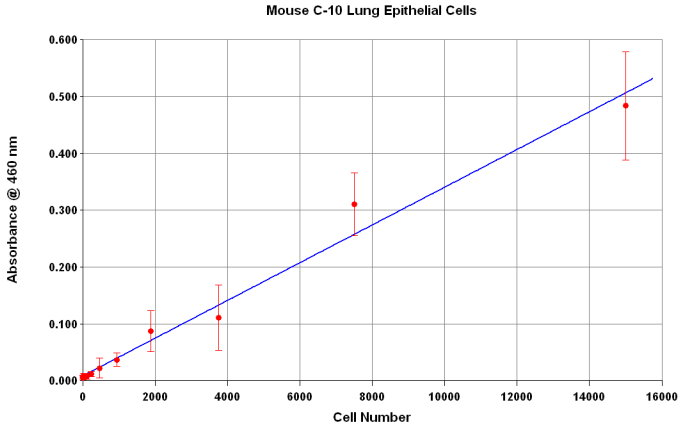

Figure 3. Cell titration.

The effect of the antibiotic thiostrepton is observed in Figure 4. Thiostrepton is believed to work by down regulating FoxM1 expression and inducing apoptosis.[2] Increasing concentration of the drug results a decrease in absorbance at 460 nm indicative of smaller numbers of viable cells. The observed dose-response is a classical sigmoidal shaped curve when plotted on semilog axis. The calculated EC_{50} under these conditions was calculated to be 7.4 µM.

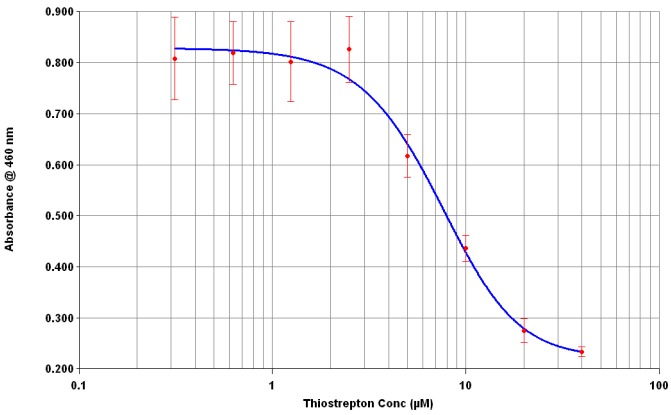

Figure 4. Thiostrepton cytotoxicity curve.

The WST-8 cell quantitation kit is an ideal solution to the rapid determination of cell numbers for cell proliferation or cytotoxicity studies. The kit provides a single ready to use reagent that can be added directly to the cell cultures, without the need to harvest or wash the cells. The end product is highly soluble in aqueous solutions, as such is nontoxic and does not require solubilization prior to measuring the absorbance. Its high aqueous solubility and low toxicity also allows for repeated measurements over time. This is of particular advantage with small numbers of cells, which may require a lengthy incubation to generate sufficient signal.

Cell numbers from proliferation or cytotoxicity studies can easily be quantitated. By comparing the absorbance obtained under experimental conditions to a preexisting calibration curve with known numbers of cells the number of cells can be calculated. Note that the assay's reaction time and incubation conditions for both the experimental and the calibration experiment must be the same for accurate comparison. Alternatively, the relative amount of absorbance generated under different conditions can be compared within an experiment independent of actually determining cell number. For example, as observed in Figure 4, one could conclude that there are significantly fewer viable cells with 24 hours exposure to 40 µM thiostrepton as compared to a 1 µM.

There are a few precautions with using tetrazolium slats for cell quantitation. Since the basis of the assay is the inherent dehydrogenase activity of viable cells, treatments that affect dehydrogenase activity may result in a discrepancy between the actual viable cell number and that determined using WST-8. Tetrazolium salts such as WST-8 can also directly interact with reducing agents such as DTT or β-ME; resulting an increase in absorbance at 460 nm. If the use of these agents cannot be avoided, background subtraction of a media only (no cells) reaction can be used to negate this. A slight spontaneous increase in absorbance of media incubated with CCK-8 reagent has been reported. The increase in background absorbance is dependant on the culture media, pH, incubation time, and exposure to light. Again, this can be corrected for by subtraction of a no-cell blank. Note that the CCK-8 reagent by itself does not under go any increase in absorbance.

The Synergy Mx monochromator-based multimode reader is an ideal multipurpose reader. The reader uses monochromators to provide wavelength specificity for both absorbance and fluorescence measurements. These hardware features allows the user to measure the fluorescence or absorbance of samples without the worry of having the necessary filters. In addition, spectral scans in either read mode can be performed automatically by the microplate reader. The reader is used in conjunction with Gen5 data analysis software. Besides controlling reader function, Gen5 collects and stores the generated data. Gen5 is also capable of automatically plotting calibration curves, calculation unknown concentrations, validating assay performance, and providing assay reports.

References

1. Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunological Methods* **1983**, *65*, 55–63.
2. Kwok, J. *et al.* Thiostrepton Selectively Targets Breast Cancer Cells through Inhibition of Forkhead box M1 Expression. *Molecular Cancer Therapeutics* **2008**, *7*, 2022–2032.

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