Application Note:
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Using a Thermo Scientific Microplate Photometer to Choose an Optimum Reagent for Cytotoxicity Assays

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Key words

- Cytotoxicity Assays
- Thermo Scientific Multiskan FC
- Multiplate Photometer
- Photometric Cell Proliferation
- Tetrazolium Salts
- alamarBlue Dye
- Resazurin Dye

Abstract

A number of different reagents can be used to perform photometric cell proliferation and viability assays. A microplate photometer can quickly determine which reagent is the best choice for an assay, and then optimize the assay. Significant time savings and productivity gains can be achieved with this tool.

This paper explains how three different cell proliferation reagents were tested on MG87 murine fibroblast cells. The tested reagents were commonly used tetrazolium salts and alamarBlue® dye.

Reduction and metabolism rates were detected photometrically using the Thermo Scientific Multiskan FC microplate photometer.

Introduction

Many photometric cell proliferation assays require the production of colored metabolites by living cells. Tetrazolium salts, such as3-{4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-l(phenylamino) carbonyll-2//-tetrazolium hydroxide (XTT) are widely used to estimate cell quantity and viability in cultures. Cells reduce colorless MTT or XTT tetrazolium into colored formazan compounds (Figure 1 and 2), which can then be photometrically detected.

The use of MTT results in a non-soluble formazan metabolite that has to be dissolved in order to quantify it, whereas the use of XTT yields a water-soluble formazan. Thus, the MTT test includes an additional solubilization step, which makes MTT a poor choice for high-throughput toxicity screens. The XTT reagent offers a simplified, *in vitro* cell proliferation assay with a possible applicability to a variety of problems in cellular biology and pharmacology.

The third assays used in this study used alamarBlue resazurin dye, an inexpensive alternative to formazan salts in cell proliferation and toxicity studies.

Assay mechanism: MTT

The assay is based on the cleavage of the tetrazolium salt, MTT (Figure 1), in the presence of an electron-coupling reagent to the water-insoluble formazan salt (Figure 1). Live cells catalyze the reduction of MTT, therefore studied cells are incubated for several hours to produce the formazan dye. After solubilization, the formazan dye is quantified using a microplate photometer. The absorbance reflects the viability of the cells.

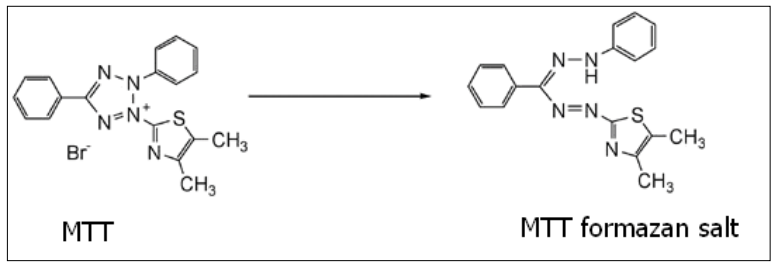

Figure 1. Structure of MTT and the correpondent formazan salt

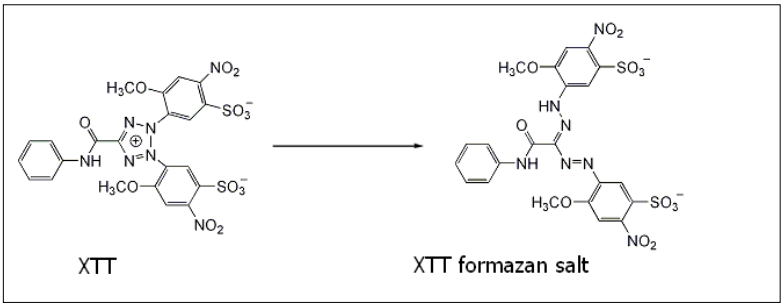

Figure 2. Structure of XTT and correspondent formazan salt

Assay mechanism: XTT

The tetrazolium salt XTT is reduced to XTT formazan by living cells (Figure 2). The reduction occurs in viable cells only and is primarily related to glycolytic NAD(P)H production. The amount of formazan directly correlates to the number of metabolically active cells in the culture. In contrast to MTT, the cleavage product of XTT is water-soluble. Therefore, a solubilization step is not required. The studied cells are incubated with the XTT labeling mixture for several hours. After incubation, the formazan salt is quantified using a microplate photometer and the absorbance reflects the cell viability.

Assay mechanism: alamarBlue

AlamarBlue resazurin dye incorporates an oxidation-reduction (REDOX) indicator that is both fluorescent and colored. The optical properties of the dye in responds to changes in REDOX levels in the media, thus data may be collected using either fluorescence-based or absorbance-based instrumentation. Live cells cause a chemical reduction of blue and nonfluorescent rezazurinto red and fluorescent resorufin. Continued cellular growth maintains a reduced environment, resulting in accumulation of the fluorescent red resorufin. Inhibition of the growth results in an oxidized environment, and, therefore, the dye remains non-fluorescent and blue. Absorbance in the alamarBlue assay is monitored at 570 and 600 nm. Fluorescence can be monitored at 530 – 560 nm excitation wavelength and 590 nm emission wavelength.

Materials and methods

Toxicity assays were performed using the following commercial kits and reagents:

- MTT Cell Proliferation Kit
- Roche Applied Science, cat. no. 1465007XTT
- Cell Proliferation Kit II, Roche Applied Science, cat. no. 11465015001
- alamarBlue cell proliferation indicator, AbD Serotech, cat. no. BUF012A
- 96-well transparent cell culture plates Cellstar, GreinerBio-One, cat. no. 655180

All cytotoxicity assays were performed in modified DMEM, containing 10% FBS and 100 µg/ml of normocin, and in HEPES-buffered modified DMEM, containing 10% FBS, 100 µg/ml of normocin, and 15 mM HEPES (pH 7.2). Dyes were dissolved in phenol red-free modified DMEM supplemented with 10% FBS and 100 µg/ml of normocin and 15 mM HEPES (pH 7.2). Absorbance in all assays was measured with the Multiskan® FC photometer.

Determination of an optimal assay on the Multiskan FC photometer

The graduation plots were built relating absorbance to live cells density for all three assays. A varying number of MG87 fibroblasts were plated on 96-well plates in 100 µl of modified DMEM. After incubating for 24 hours, 100 µl of HEPES-buffered modified DMEM was added into each well and cells were incubated for an additional 24 hours. Afterwards, the media was aspirated and replaced with phenol red-free modified DMEM containing the tested dye. After five hours the absorbance was measured using the following wavelengths:

- MTT: 550 nm, using 690 nm as reference
- XTT: 492 nm, using 690 nm as reference
- alamarBlue dye: 570 nm, using 630 nm as reference

XTT assay optimization

Cell density was also estimated by visual observation using a light microscope. Based on these results, the best assay was chosen for further optimization.

To determine the optimal incubation time for the XTT assay, 2,000 cells were plated and treated as described above except the last incubation step, which ranged from two to five hours.

Results and discussion

First MTT, XTT and alamarBlue dyes were compared for cell proliferation assays to select the optimal one for cytotoxity tests on MG87 fibroblasts. The absorbance values were plotted against cell density for all assays. The results are shown in Figure 3. The higher plating cell density led to higher absorbance values. However, microscopic inspection revealed that a plating cell density above 2,000 cells/well resulted in more than 95% of confluency of the cells after 48 hours in culture. As dense cultures can be less susceptible to harmful stimuli, to avoid underestimation of compound toxicity on the one hand, but to obtain the highest signal-to-noise ratio on the other hand, we selected the plating cell density equal to 2,000 cells/well for the screening.

Based on the experimental data, the XTT assay showed the best performance. Photometrically-detected alamarBlue dye reduction had the smallest/lowest/narrowest assay window. In the case of the MTT dye, cells did not appear healthy after five hours of incubation. In addition to this rather questionable performance, the MTT assay required a long solubilization step. Thus, the XTT assay was selected for toxicity screening due to its convenience and the best signal-to-noise ratio of the three tested assays.

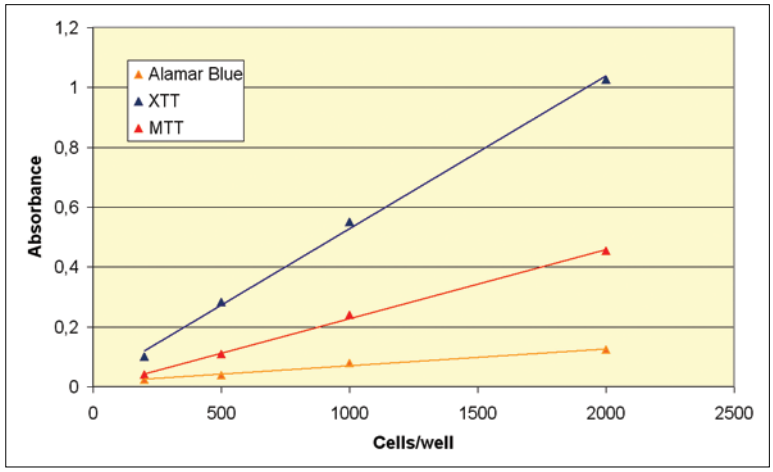

Figure 3. Absorbance versus seeding plate density plot for the three cytotoxicity assays. The absorbance values of the reference wavelength have been subtracted from the results.

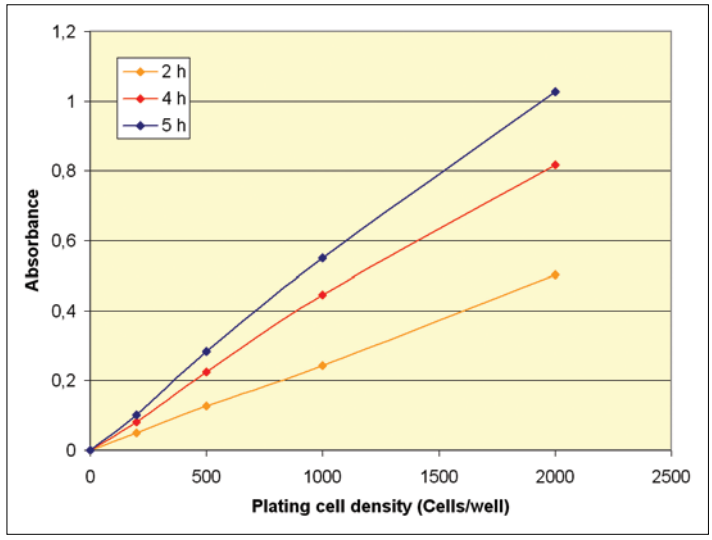

Figure 4. Absorbance versus seeding plate density plot for different incubation times with XTT dye

The results of the dye incubation time optimization are shown in Figure 4. An extension of the incubation time led to an increase in the amount of generated dye in an almost linear manner. Based on this data, the cells with XTT were incubated for four hours. This time produces high enough absorbances for a reliable assay with sufficient measurement dynamics, but is short enough for a convenient and efficient assay protocol.

Toxicity of 18 chemicals was estimated using the XTT toxicity assay and compared with the results of fluorimetrically detected alamarBlue dye reduction and microscopic observation. Compounds were applied to the cells in one fixed 100 µM concentration. As a control, cells were treated with a corresponding concentration of diluent (1% DMSO). An absorbance versus plating cell density plot was used to convert absorbance into the cell number. 100% value was assigned to the cell density of DMSO-treated samples and calculated the percentage for all the other samples. In other words, chemicals showing a 100% value are as toxic as DMSO, compounds with values below 100% are more toxic, and values over 100% represent chemicals that promote cellular proliferation. The results from all three methods (XTT, alamarBlue dye and visual microscopy) are consistent, except in the case of two chemicals where the results of the XTT assay differed remarkably from the other two assays. The observed discrepancy can be attributed to the nature of the XTT test. Both of these compounds were only partially soluble, and the limited solubility

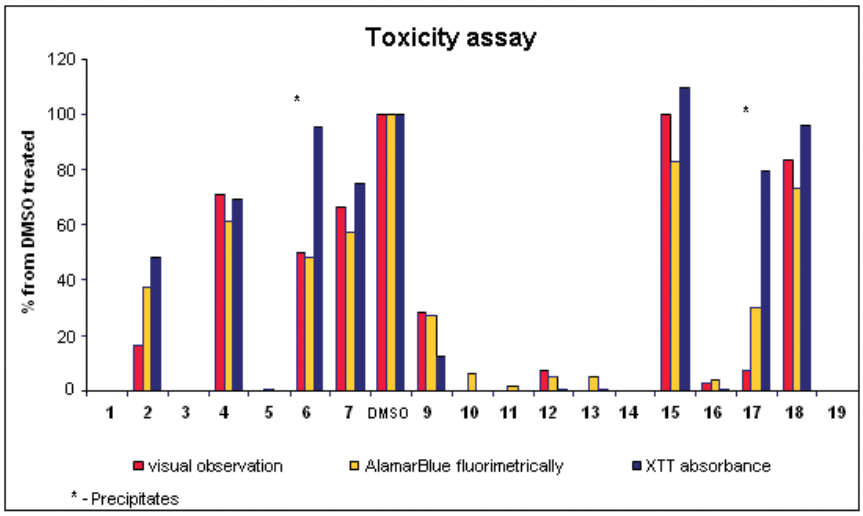

Figure 5. Cytotoxicity of the chemical compounds tested. The compounds that were only partially soluble are marked as "precipitates" in this figure.

was believed to affect the absorbance readings. Precipitate formation in the sample will have a very different effect on 492 nm and 690 nm absorbance readings, because particles scatter different wavelengths differently. Therefore, the 492/690 nm absorbance ratio will be disturbed, which consequently may lead to incorrect toxicity estimation.

Conclusion

The difference between the three cell proliferation assays was clearly shown in the comparison. The rapid and convenient XTT assay also demonstrated the best signal-to-noise ratio throughout the assay range. Thus, the XTT assay turned out to be the best alternative for absorbance-based cytotoxicity analysis in this study. The Multiskan FC microplate photometer proved to be a reliable and robust instrument with which to perform all of these assays.

References

Scudiero, D. A. et al., Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines (1988), Cancer Res. 48, 4827-4833.

Further Information

For further information about Multiskan FC, please refer to the following Web pages:

- www.thermo.com/readingroom
- www.thermo.com/mpi

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