Chapter 9

Optimization of the Tetrazolium Dye (MTT) Colorimetric Assay for Cellular Growth and Viability

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Abstract

The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during MTT exposure. Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of viable cells in culture. However, various parameters have been identified that can affect cellular metabolism and other factors, which significantly modify MTT-specific activity and can result in calculated false high or false low cell counts. Therefore, it is essential to establish assay parameters with the proper controls for each cell line and/or drug treatment in order to optimize assay conditions and minimize confounding effects. These parameters should include determining appropriate cell densities, culture medium, optimal concentrations and exposure times for MTT, fresh culture medium at the time of assay to avoid nutrient depletion, and controlling for drug treatment effects that may influence cellular metabolism. By controlling these important parameters, the MTT colorimetric assay provides accurate and reliable quantification of viable cell number.

Key words: Cell growth assay, Cell viability assay, Colorimetric assay, MTT, Tetrazolium dye

1. Introduction

The discovery and development of novel drug therapies for the treatment of cancer involves the intensive research efforts of investigators in academia, the pharmaceutical industry, as well as governmental agencies. Initially, disease-oriented preclinical drug discovery includes the rapid in vitro screening of candidate drugs against a panel of human tumor cell lines. In general, tumor cells are exposed to various antitumor agents for a fixed duration of time and, afterwards, cell number and/or viability is determined.

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Many methodologies exist that can perform such a function and include techniques as simple as manually counting cells or cell nuclei, to much more elaborate efforts such as measuring ^{3}H-thymidine incorporation, fluorometric DNA assay, and flow cytometry. However, the majority of these techniques are labor intensive, time consuming, produce difficult to handle waste products that are toxic or radioactive, and are expensive to perform. However, one of the most popular and convenient ways to determine viable cell number in 24- or 96-well microtiter plates is the rapid colorimetric tetrazolium dye procedure commonly referred to as the MTT assay developed by Mosmann (1).

As initially described, the MTT assay is based on the cleavage of the yellow-colored tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, into a blue-colored formazan by the mitochondrial enzyme succinate-dehydrogenase (2). The assumed advantage of this cell assay system is that this reaction can only take place in living cells with functional mitochondria. It was also assumed that the amount of formazan formed during a given exposure period is directly proportional to the number of viable cells per well. Therefore, the MTT assay system is widely viewed as a convenient, reliable, and quantitative method for measuring cell proliferation and viability (3). However, many of these early assumptions were overly optimistic regarding the specificity of this assay system. Over time, it became clearly evident that nonmitochondrial enzymes can also specifically reduce tetrazolium salts at various cellular locations outside the mitochondria, and that the level of MTT reduction varies greatly between different cell types (4, 5). Furthermore, MTT-specific activity in nearly all cell lines significantly decreased with increased cell culture duration (3). Over time, other critical conditions became evident that were required for accurate assay performance.

Information presented in this review will summarize the various metabolic and chemical factors that can alter MTT-specific activity and thereby significantly affect quantification of cell number. Although the MTT colorimetric assay is a convenient and reliable method for measuring cell proliferation and viability, these characteristics absolutely depend on the establishment of assay conditions that optimize accuracy and eliminate confounding factors that affect MTT-specific activity (see Note 1).

2. Materials

2.1. Cell Culture and Harvesting

1. Control media: Dulbecco's Modified Eagles's Medium (DMEM)/Ham's F12 Medium (F12) at a ratio of 1:1 (Sigma Chemical Co, St. Louis, MO), supplemented with 10 µg/mL

insulin, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% bovine calf serum (BCS, Hyclone, Logan, UT).

2. Phosphate-buffered saline (PBS): sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline.
3. Harvesting medium: 0.05% trypsin containing 0.025% EDTA in PBS.
4. Bovine calf serum.
5. 2,4-Dinitrophenol.
6. Crystal violet/citric acid cell lysis solution: 2.1 g citric acid (Sigma) and 0.1 g crystal violet dye dissolved in 100 mL water and filtered with #2 Whatman paper.
7. Teflon cell scraper.
8. Hemocytometer
9. 2 mM D-glucose (dissolved in PBS or nutrient-depleted culture medium and then sterilized by filtration through a 0.2-µm filter).

2.2. MTT Preparation

1. MTT assay solution: 2–5 mg/mL MTT (thiazolyl blue tetrazolium bromide) in PBS (see Note 2).
2. Isopropanol or DMSO.

3. Methods

Viable cell number can be determined most easily in 24- or 96-well culture plates using the MTT colorimetric assay. The number of cells/well is calculated against a standard curve prepared at the start of each experiment, so that the same cell and MTT preparations are used for both the standard curve and the experiment. Standard curves are prepared by plating various concentrations of isolated cells (10^4–10^6), as previously determined by hemocytometer, in triplicate in the culture plates. Depending on the type of cell to be counted, standard curves are prepared so that MTT absorbance readings of experimental samples are obtained in the linear part of the curve. Sample absorbance readings that near the minimal or maximal detection levels will not provide accurate viable cell number counts.

3.1. Isolation of Cells

1. Many cell lines, such as the highly malignant +SA mammary epithelial cells (6–8), attach and grow on the surface of plastic culture plates. The MTT assay method is best suited for monolayer culture, but can also be used in 3-dimensional cell cultures using collagen or matrigel. Isolation of cells prior to experimentation can be routinely accomplished with a

trypsin-EDTA solution. Feed-stock cells can be grown in 100 mm culture plates and maintained on culture medium for a period of time until they reach 80% confluence (still in log phase of growth). In order to harvest cell for experimentation, medium is removed and cells are then rinsed in sterile PBS or just base medium that is free of serum or bovine serum albumin, in order to remove any remaining protein. After rinsing, PBS is removed, and trypsin-EDTA solution is added to cells. The amount of trypsin added depends on size of cell culture plate or flask. For example, the amount of trypsin to harvest cells grown in 100 mm plates is usually 2 mL, while 1 mL of trypsin would be used to harvest cells grown in a T-25 culture flask. Once cells are exposed to trypsin, culture plates or flasks are then returned to the incubator for 5 min. Time of exposure to trypsin is very critical. If exposure time is too short, isolation and harvest efficiency is poor, whereas if exposure time is too long, trypsin will induce cell lysis and death. After the 5 min incubation period, an equal amount of BCS as the amount of trypsin solution used (1–2 mL) is added to each plate in order to stop (quench) the trypsin digestion. Cells can then be isolated using a teflon cell scraper or rubber policeman and by using a pipette to flush the bottom of the plate.

2. The cell suspension is then collected from all plates or flasks, placed in a sterile conical tube, and centrifuged at $300 \times g$ for 5–10 min to pellet the cells. Digestion medium is then removed, and cells are resuspended in 5–20 mL of culture medium (depending on the number of cells isolated). A 100 µL of this cell suspension is then placed in a 12×75 mm test tube (sterility of tube is not required) and 900 µL of crystal violet/citric acid solution is added to the tube to bring total volume to 1 mL. The tube is then vigorously vortexed for 2 min. This procedure will cause cell lysis, but leave nuclei intact. Since cell size and shape display a wide range of variance, it is much simpler and more accurate to count the darkly crystal violet-stained round nuclei of each cell instead of the irregularly shaped intact cells.
3. Add 10 µL of the lysed cell mixture to each side of a hemocytometer and count the cells, remembering that the cells have been diluted tenfold in order to determine the number of cells/mL in the harvested cell suspension.

3.2. Preparation and Construction of MTT Standard Curve

1. Once cells are counted, cells can be diluted to a concentration that allows easy preparation of the MTT standard curve. For example, if the isolated +SA cell concentration is 5×10^6 cells/mL, then cells, medium, and MTT can be pipetted into the wells

Table 1
Typical cellular dilutions and media volumes used to construct a MTT standard curve (density of isolated cells in suspension is 5×10^6 cells/mL)

Group number	Cell number (1×10^5)	Volume of cell suspension (μL)	Volume of medium (μL)
1	0	0	1,000
2	0.5	10	990
3	1.0	20	980
4	1.5	30	970
5	2.0	40	960
6	2.5	50	950
7	3.0	60	940
8	4.0	80	920
9	5.0	100	900
10	6.0	120	880
11	8.0	160	840
12	10.0	200	800

of a 24-well culture plate in triplicate (appropriate adjustment can be made when using 96-well plates): Table 1 shows an example of cell and medium dilution that would be typical for preparation of a MTT standard curve at the start of a given experiment. After completing the standard curve and plating cells at the appropriate concentration (5×10^4 per well in 24-well plates for neoplastic +SA mammary epithelial cells), cells are returned to the incubator for a 24 h period to allow for suspended cells to reattach to the surface of the plastic culture plate.

2. The next day, medium is removed from the wells of the standard curve and experimental groups that will be counted (Day 0), and fresh medium is added to these wells. As discussed later in this chapter, the refeeding of cells fresh medium prior to MTT exposure is an absolute requirement in order to obtain accurate viable cell number. Afterwards, 200 μL of the sterile MTT stock solution (2 mg/mL in PBS) is then added to each well of the standard curve and each well of the experimental groups to be counted that day. If the stock MTT

solution used in the above example is 2 mg/mL, the final MTT concentration in medium is 0.83 mg/mL. The concentration of the stock MTT solution can be modified (2–5 mg/mL) depending on the type of cell that is being used and culture plate (24- or 96-well) in order to optimize MTT-specific activity.

3. The standard curve and experimental cell culture plate containing cells exposed to MTT are returned to the incubator at 37°C for a 4 h period. This incubation period was determined to be optimal for +SA mammary tumor cells to produce the greatest amount of formazan reduction in the least amount of time. Different cell types with higher or lower metabolic rates may require adjustment in length of the incubation period in order to optimize the MTT assay for that particular type of cell.
4. Afterwards, the MTT containing medium is removed from all wells and the remaining cells containing formazan crystals are dissolved in 1 mL of isopropanol. Again, this volume of isopropanol can be adjusted depending on the type of culture plate being used (200 µL for 96-well plates) in order to optimized absorbance intensity.
5. Optical density of each well can then be determined with a microplate reader using an absorption spectrum that can range between 565 and 630 nm against the blank wells in the standard curve. For +SA mammary tumor cells, maximum absorbance was observed using a 570 nm filter.
6. The MTT standard curve is constructed by plotting the dependent variable (number of cells) on the *x*-axis against the independent variable (optical density) on the *y*-axis using linear/linear or log/log regression analysis, depending on what type of regression provides the best fit for a particular cell line and range of the standard curve.
7. The number of viable cells in experimental treatment groups can then be determined by reading absorbance values from individual wells in control and treatment groups against the standard curve. If treatment effects on the growth of cells are to be monitored over a period of days, then each day another set of wells (multiple treatment group plated at the start of the experiment) can be exposed to MTT and cell number in each group can be calculated from the original standard curve prepared at the start of the experiment. Again, it is essential that the same preparation of cells and MTT are used for both the standard curve and the experiment, and fresh standard curves must be prepared at the start of each subsequent experiment. An example of a MTT standard curve similar to one described above is shown in Fig. 1.

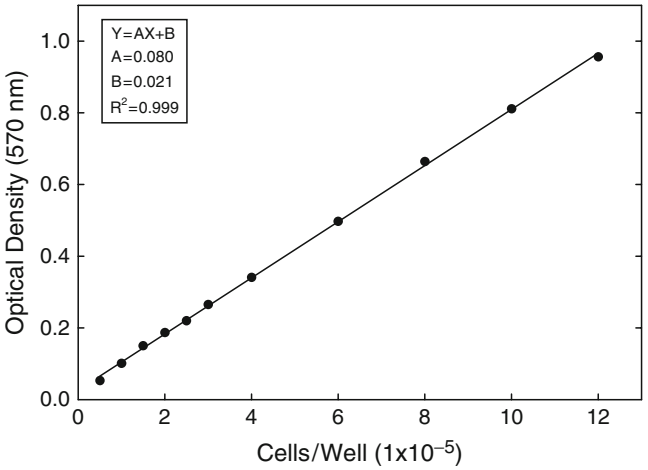

Fig. 1. A representative MTT standard curve for neoplastic +SA mouse mammary epithelial cells (8). Isolated cells were counted manually with a hemocytometer, and then various concentrations (5×10^4 to 1×10^6) were plated in triplicate into 24-well culture plates, fed 1 mL of control culture media, and returned to the incubator for a 24 h period to allow for cell to reattach to the plastic culture plates. The next day, media was removed and cells were fed 1 mL fresh media, and then 200 μL of 5 mg/mL MTT was added to each well so that final concentration was 0.83 mg/mL MTT. Plates were returned to the incubator (37°C) for a 4 h incubation period. Afterward, media was removed and 1 mL of isopropanol was added to each well to dissolve the formazan crystals. Optical density was measured at 570 nm zeroed against the blank wells (0 cells/well) for each well and a linear/linear plot created showing the relationship of the concentration of formazan produced vs. number of cells per well.

3.3. Parameters Determining Optimal MTT Assay Performance

3.3.1. Optimal MTT Concentration

Initial studies conducted by Mosmann (1) and Denizot and Lang (9) compared the use of MTT concentration between 0 and 4 mg/mL with a constant number of cells per well and found that the amount of formazan produced showed a steep increase between 0 and 1 mg/mL, then reached a plateau between 1 and 2 mg/mL. Both investigators concluded that standardizing the MTT concentration to 1 mg/mL for cell viability and proliferation assays was an acceptable compromise because this concentration produced excellent results and was cost-effective. Using various cell lines, it was determined that a concentration of 0.83 mg/mL MTT produced essentially the same results as 1 mg/mL (data not shown) and was selected for use in studies with +SA mammary tumor cells as described in this chapter.

3.3.2. Optimal Incubation Time

1. Neoplastic +SA mouse mammary epithelial cells were initially plated at density of 5×10^5 cell/well in 24-well culture plates and maintained on 1 mL/well control media as described above and then returned to the incubator for a 24 h period to allow cells to reattach to the plastic culture plate.

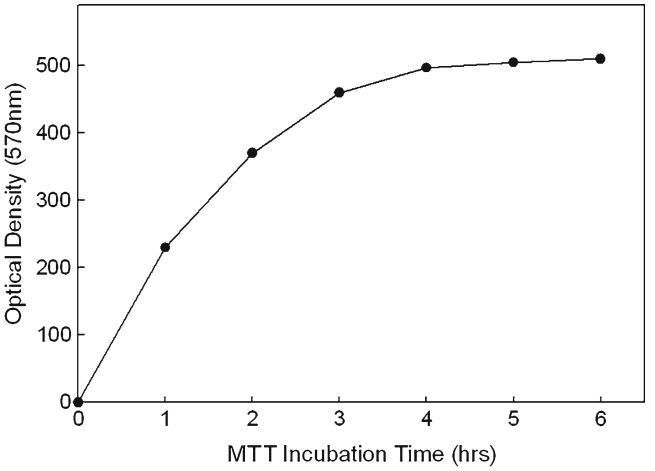

Fig. 2. Time-course relationship of formazan production after +SA cells was exposed to 0.83 mg/mL MTT. +SA mouse tumor cells were initially plated at a density of 5×10^5 cells/well in 24-well culture plates, fed 1 mL control media, and then returned to the incubator for a 24 h period to allow cells to reattach to the plastic culture plates. The next day, cell were divided into different groups with the same number of cells/well in each group, media was removed and replaced with 1 mL fresh control media, and then 200 μL of 2 mg/mL MTT was added to each well. Cells were returned to the incubator and MTT-specific activity was determine at 0, 1, 2, 3, 4, 5, and 6 h following MTT exposure (6 wells/group).

2. The next day, medium was removed and replaced with 1 mL fresh control media and 200 μL of 2 mg/mL MTT in sterile PBS was added to each well. Cells were returned to the incubator and MTT-specific activity was determined at 0, 1, 2, 3, 4, 5, and 6 h following MTT exposure (6 wells/group).
3. Figure 2 shows that there is a continuous increase in MTT-specific activity between 0 and 3 h, which then reached a plateau between 3 and 4 h after treatment exposure.
4. It was concluded from these results that a standardized incubation time of 4 h following MTT exposure was an acceptable compromise for further studies because it produced the optimal specific activity in the least amount of time (Fig. 2) (see Note 3).

3.3.3. Glucose Depletion in Culture Medium Significantly Reduces MTT-Specific Activity

1. Nutrient-depleted control medium was collected after 4 days in culture from 100 mm culture plates containing near confluent cultures of neoplastic +SA mouse mammary epithelial cells. Cells were initially plated at a density of 1×10^6 cells/culture plate and then maintained in the same 10 mL control medium for a 4-day incubation period. Afterwards, media from these culture plates was collected for use in subsequent experiments (see Note 4).

2. In other studies, +SA mammary tumor cells were initially plated at density of 5×10^5 cells/well in 24-well culture plates and maintained on 1 mL control media. Cells were returned to the incubator for 24 h in order to allow cells to reattach to the plastic culture plates.
3. The next day, media was removed and cells were divided into different treatment groups and fed the following treatment media: (1) fresh control media; (2) PBS; (3) PBS supplemented with 2 mM D-glucose; (4) nutrient-depleted media; and (5) nutrient-depleted media supplemented with 2 mM D-glucose. Afterwards, 200 µL of 2 mg/mL MTT in sterile PBS was added to each well, and cells were returned to the incubator. MTT-specific activity was determined after a 4 h incubation period.
4. The results in Fig. 3 show that equal number of cells produced the highest MTT-specific activity when given fresh media prior to MTT exposure. In contrast, formazan production by an equal number of cells maintained in glucose-free PBS showed a large reduction in MTT-specific activity, and this effect was partly reversed by supplementation with 2 mM

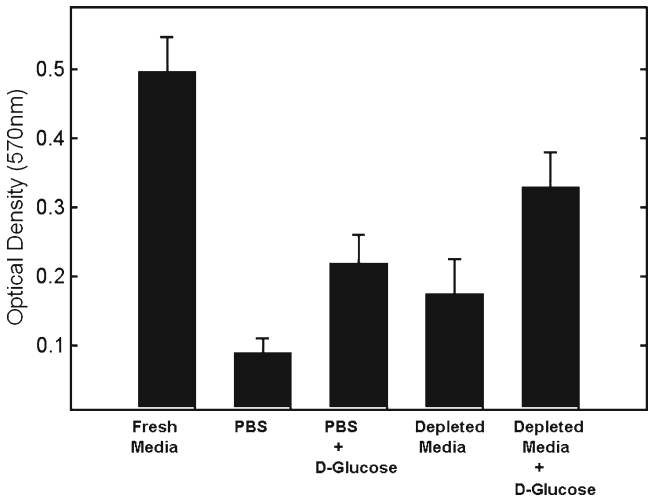

Fig. 3. Effects of glucose on MTT-specific activity. +SA mammary tumor cells were initially plated at density of 5×10^5 cell/well in 24-well culture plates, fed 1 mL control media, and then returned to the incubator for 24 h to allow cells to reattach to the plastic culture plates. The next day, media was removed and cells were divided into the following treatment groups containing the same number of cells/cell in all groups, and fed the following treatment media: (1) Fresh media, control; (2) PBS, glucose-free buffered saline; (3) PBS + D-glucose, PBS supplemented with 2 mM D-glucose; (4) depleted Media, nutrient-depleted media; and (5) depleted media + D-glucose, nutrient-depleted media freshly supplemented with 2 mM D-glucose. Afterwards, 200 µL of 2 mg/mL MTT in sterile PBS was added to each well (6 wells/group), and cells were returned to the incubator, and MTT-specific activity was determine after a 4 h incubation period.

D-glucose. Likewise, cells exposed to nutrient-depleted media showed a large reduction in formazan production as compared to an equal number of cells given fresh media, and supplementation with 2 mM D-glucose greatly increases MTT-specific activity in these cells.

5. These results demonstrate that it is essential that the MTT assay is performed in the presence of fresh media given to cells immediately prior to MTT exposure in order to obtain optimal formazan production. MTT-specific activity is greatly reduced in cells maintained in glucose-free media or nutrient-depleted media (Fig. 3).

3.3.4. Metabolic Uncouplers Greatly Enhance MTT-Specific Activity

1. Neoplastic +SA mouse mammary epithelial cells were plated at various densities (1, 2, 3, 4, and 5×10^5) cells/well in 24-well culture plates and fed 1 mL control media, then returned to the incubator for a 24 h period in order to allow cells to reattach to the plastic culture plates.
2. The next day, media was removed and replaced with 1 mL fresh control media containing 0 or 1 μM 2,4-dinitrophenol. Afterwards, 200 μL of 2 mg/mL MTT in sterile PBS was added to each well. Cells were returned to the incubator and MTT-specific activity was determined in each treatment group (6 wells/group) after a 4 h exposure to MTT.

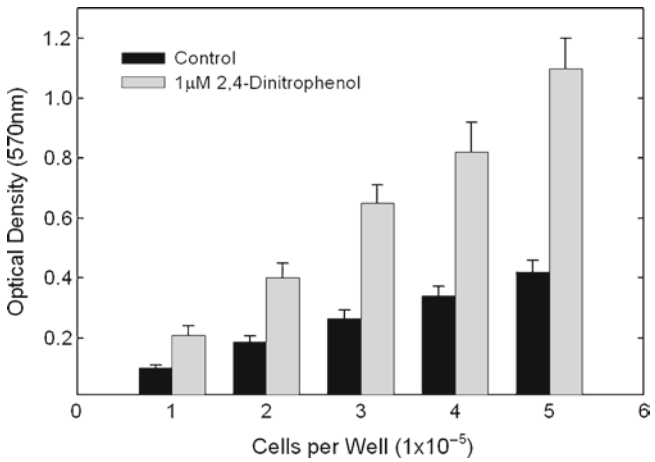

Fig. 4. Effect of the metabolic uncoupler, 2,4-dinitrophenol, on MTT-specific activity. +SA mammary tumor cells were plated at various densities (1, 2, 3, 4, and 5×10^5) cells/well in 24-well culture plates, fed 1 mL control media, and returned to the incubator for a 24 h period to allow cells to reattach to the plastic culture plates. The next day, media was removed and replaced with 1 mL fresh control media containing 0 or 1 μM 2,4-dinitrophenol. Afterwards, 200 μL of 2 mg/mL MTT was added to each well. Cells were returned to the incubator for a 4 h period and then MTT-specific activity was determined in each treatment group (6 wells/group).

3. Results in Fig. 4 show that cells exposed to 1 μM 2,4-dinitrophenol, an established metabolic uncoupler, display an increase in MTT-specific activity as compared to the same number of cells maintained in control media.
4. These results demonstrate that great care must be taken in establishing the proper controls and parameters when using the MTT assay to count cells treated with drugs that alter respiration and metabolism. Cells exposed to uncouplers, such as 2,4-dinitrophenol, displayed a very large "false high" in cell number as compared to the same number of cells from the vehicle-treated control group (see Note 5).

4. Notes

1. The MTT assay is an excellent method for quantification of viable cells in culture. The metabolic activity between different cell lines shows great variability and therefore the MTT standard curve needs to be characterized and optimized for each new cell line. It is also important when testing drug effects on proliferation and viability to include the proper controls for drug effects on cellular metabolism during treatment. By establishing proper assay conditions and controls, the MTT assay is an accurate and reliable method for counting viable cells.
2. MTT dissolves very slowly, so MTT and PBS can be placed on stirring plate and mixed vigorously for 10–20 min, and then filtered through a 0.2-μm filter to sterilize the solution and remove any insoluble particles remaining. This solution can be stored for up to a month at 4°C protected from light.
3. Other parameters have also been identified that play a critical role in modulating MTT-specific activity. Optimization of MTT concentration and exposure time is very important because different cell types display a wide variability in dose- and time-response in maximal formazan production (4, 5).
4. One of the most critical conditions that need to be accounted for when performing the MTT assay is glucose depletion in the culture medium. Previous studies have shown that cells with the highest glucose metabolism display the greatest reduction in MTT-specific activity over time in culture (3). These studies also showed that transfer of cells to a glucose-free medium was associated with an immediate decrease in tetrazolium salt reduction that was pH-independent, indicating that cellular transport and constant metabolism of glucose are required for optimal formazan production (3).

5. Phenol red, the red-colored pH indicator commonly present in many culture media, has an absorbance that overlaps with formazan and its presence in the assay can significantly affect sample absorbance and produce a high background in the blank samples and false highs in treatment cell samples (9). This problem can be eliminated with the use of phenol red-free media. Drugs and chemical agents can also significantly modify MTT-specific activity if these treatments increase or decrease mitochondrial respiration or cause metabolic uncoupling. Drug-induced respiratory uncoupling will produce a large false high, whereas agents that decrease metabolic respiration and electron transport will produce a false low in MTT-specific activity as compared to vehicle-treated controls containing identical cell numbers (3, 10).

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