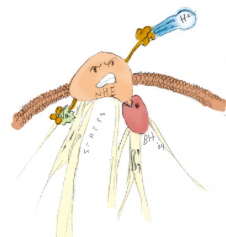
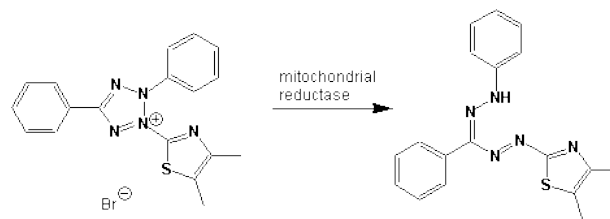


Proliferation Assay MTT Protocol



Background - Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. Several approaches have been used in the past. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high-throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The use of the MTT method does have limitations influenced by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves.



The MTT method of cell determination is most useful when cultures are prepared in multiwell plates. For best results, cell numbers should be determined during log growth stage. Each test should include a blank containing complete culture medium without cells.

Thiazolyl Blue Tetrazolium Bromide (MTT) Product Number **M 2128** Storage Temperature 2-8 oC

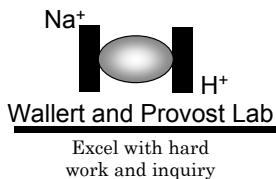
Preparation Instructions MTT is soluble in water (10 mg/ml), ethanol (20 mg/ml) and is also soluble in buffered salt solutions and culture media (5 mg/ml). Reconstituted MTT solution is stable for at least 6 months when stored at -OoC. Storage at 4oC for more than four days will result in decomposition and will yield erroneous results.

MTT Solution: 5 mg/ml MTT in PBS. Solution must be filter sterilized after adding MTT.

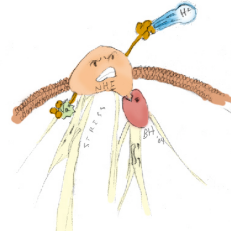
MTT Solvent: 4 mM HCl, 0.1% Nondet P-40 (NP40) all in isopropanol.

Important Assay Notes

1. Remove cultures from incubator into laminar flow hood or other sterile working area.
2. Aseptically add MTT SOLUTION in an amount equal culture volume.
3. Incubation times should be consistent when making comparisons.
4. If cells are attached to culture vessel growth surface, remove and dispose of the culture fluid. Add MTT SOLVENT in an amount equal to the original culture volume. Solvent volumes may vary but the final volumes should be consistent to facilitate comparison.
5. If cells are not attached or loss of MTT formazan occurs if culture fluid is removed, add MTT SOLVENT



Proliferation Assay MTT Protocol



directly to the culture in an amount equal to the original culture volume.

- Plates should be read within 1 hour after adding MTT SOLVENT.

6. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down (trituration) may be required to completely dissolve the MTT formazan crystals especially in dense cultures.

Note: For most tumor cells, hybridomas, and fibroblast cell lines, 5,000 cells per well to perform proliferation assays. 12 well plates need 1 ml of soln and have about 400,000 cells, 24 well plates have 0.5 ml and 200,000 cells at confluency.

Procedure:

Short 96 well assay: EACH condition should be done in triplicate or more.

1. **DAY ONE:** Trypsinize one T-25 flask and add 5 ml of complete media to trypsinized cells. Centrifuge in a sterile 15 ml falcon tube at 500 rpm in the swinging bucket rotor (~400 x g) for 5 min.
2. Remove media and resuspend cells to 1.0 ml with complete media.
3. Count and record cells per ml. Remember to remove the cells aseptically when counting.
4. DILUTE the cells (cv=cv) to 75,000 cells per ml. Use complete media to dilute cells.
5. Add 100 μ l of cells (7500 total cells) into each well and incubate overnight.
6. **DAY TWO:** Treat cells on day two with agonist, inhibitor or drug.
 - If removing media, do very carefully. This is where most variation in data may occur.
 - Final volume should be 100 μ l per well.
7. **DAY THREE:** Add 20 μ l of 5 mg/ml MTT to each well. Include one set of wells with MTT but no cells (control). All should be done aseptically.
8. Incubate for 3.5 hours at 37°C in culture hood.
9. CAREFULLY Remove media. Do not disturb cells and do not rinse with PBS.
10. Add 150 μ l MTT solvent.
11. Cover with tinfoil and agitate cells on orbital shaker for 15 min.
12. Read absorbance at 590 nm with a reference filter of 620 nm.

Storage: The MTT Reagent must be kept at 4°C in the dark. The Detergent Reagent can be stored at either 4°C or ambient temperature. If the detergent reagent is kept at 4°C, warm the bottle for 5 minutes at 37°C and gently mix by inverting before use (avoid creating bubbles).

References

- Slater, T. et al. (1963) Biochem. Biophys. Acta 77:383.
van de Loosdrecht, A.A., et al. J. Immunol. Methods 174: 311-320, 1994.
Alley, M.C., et al. Cancer Res. 48: 589-601, 1988.

POSSIBLE SOURCES OF ERROR

1. MTT SOLUTION is stable when stored frozen. Storage at 2-8 °C may result in decomposition and yield erroneous results. Development of dark color or formation of crystals indicate product deterioration.
2. Microbial contamination will contribute to the cleavage of MTT and formation of MTT formazan yielding erroneous results.
3. Uneven evaporation of culture fluid in wells of multiwell plates may cause erroneous results.
4. High protein levels (serum, albumin, etc.) in the culture medium may form a precipitate when MTT SOLVENT is added. Samples with protein concentrations equivalent to 10% fetal bovine serum seem acceptable. Sera with higher protein concentrations than fetal bovine serum may have to be used