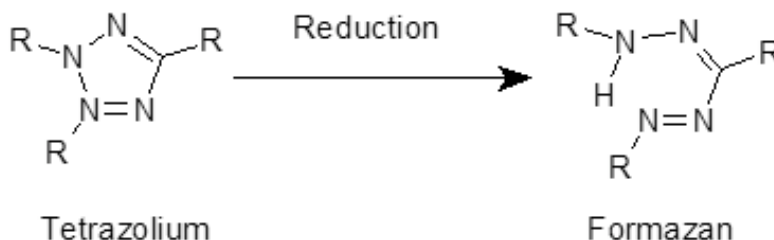


INTRODUCTION

Measurement of cell viability and proliferation comprise the underlying basis for numerous *in vitro* assays directed towards the quantitation of a cell population's response to external factors. Cell proliferation assays have utilized the uptake of radiolabeled thymidine into cellular DNA, however, this method is time consuming and involves the use of hazardous materials.



The use of tetrazolium salts, including XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide), to assay cell proliferation, cell viability, and/or cytotoxicity is a wide-spread, established practice. The XTT assay procedure avoids radioactivity, allows for rapid determination in microplates, and gives reproducible and sensitive results. Cleavage of the tetrazolium salt to formazan occurs via the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells. The reaction is attributed mainly to mitochondrial enzymes and electron carriers, but a number of other non-mitochondrial enzymes have been implicated. XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye, which can be measured by absorbance at 490 (or 450) nm in a microplate reader. Efficient reduction of XTT requires an electron coupling reagent. Trevigen's **TACSTM XTT kit** includes XTT and an electron coupling reagent for a more rapid, convenient and simple assay.

Advantages/Features:

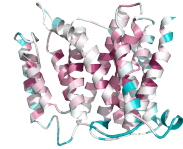
- Sensitive
- No radioactivity
- Rapid (no solubilization step as in an MTT assay)
- Ideal for high throughput assays (no washing or other steps that can cause cell loss and variability)

XTT Reagent- store at <20° C. Stock is provided at a 3x concentration (0.9 mg/mL) in RPMI media without phenol red. A volume of 5mL is sufficient to run one microplate. XTT will precipitate during storage. Therefore, the solution must be warmed to 37° C for several minutes until it is no longer opaque. Heating for unnecessary and extended periods of time will result in reduction of the XTT. Multiple freeze/thaw cycles will degrade the integrity of the solution so reagent should be aliquoted into 5 ml volumes when 25 ml bottle is first thawed.

XTT Activator-store at <20° C. Stock is provided at a 50X concentration in dH₂O. A volume of 100uL is sufficient for each 5 mL of XTT Reagent (i.e. one microplate). The XTT Activator will also precipitate during storage. Heat to 37° C for 2 to 5 min until the reagent is fully dissolved.

XTT Working Solution – Immediately before use, add 100uL of XTT Activator to 5mL of XTT Reagent to make the XTT Working Solution. XTT Working Solution should be added to cells within several minutes of preparation (be sure to check the manual for proper mixing instructions as kits may change.)

Storage: Both XTT Reagent and Activator must be kept at <20° C the dark. Warm the bottle for 5 minutes at 37°C and gently mix by inverting before use (avoid creating bubbles).



Important Assay Notes

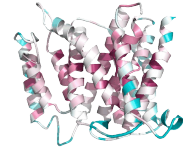
1. Remove cultures from incubator into laminar flow hood or other sterile working area.
2. Prepare XTT Working Solution by combining XTT Reagent with XTT Activator according to the above.
3. Note: if sediment is present in the XTT Solution, heat the solution to 37° C and swirl until no longer opaque.
4. Add 50uL to each well (96 well plate). Appropriate incubations times with XTT Working Solution are determined empirically, so if it is your first run take readings at interval. Continue until at least a total 2 hour incubation time.
5. Read absorbance at 450nm, with a reference wavelength of 690nm (to correct for fingerprints, smudges, ect.).
6. Suggested seeding of cells of 5,000 to 10,000 cells per well. Albeit the test is extremely sensitive so it is possible to seed using a lower concentration with longer incubation times once the XTT Working Solution is applied.

Note: For most tumor cells, hybridomas, and fibroblast cell lines, 2,000 cells per well (96 well plate) 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.

PROTOCOL

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 bucked 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 ($C_1V_1=C_2V_2$) to 25,000 cells per ml. Use complete media to dilute cells (preferably without phenol red, but it will work either way). Also it does not matter the method you just need 2500 cells per well (in a 96 well plate).
5. Add 100 µl of cells (2500 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 µl per well.
7. **DAY THREE:** Remove cultures from incubator in to laminar flow hood or other sterile working area.
8. Prepare XTT Working Solution by combining XTT Reagent with XTT Activator according to the above.
 - **Note:** if sediment is present in the XTT Solution, heat the solution to 37° C and swirl until no longer opaque.
9. Add 50uL to each well (96 well plate). Appropriate incubations times with XTT Working Solution are determined empirically, so if it is your first run take readings at interval until at least a 2 hour incubation.
10. Read absorbance at 450nm, with a reference wavelength of 690nm (to correct for fingerprints, smudges, ect.).



POSSIBLE SOURCES OF ERROR

Problem	Solution
Low absorbance readings	<ul style="list-style-type: none"> ◆ Add activator immediately before use ◆ Increase incubation time with XTT ◆ Increase seeding density of cells ◆ Ensure XTT is in solution before beginning assay
Poor replicates	<ul style="list-style-type: none"> ◆ Ensure no bubbles present in wells ◆ Pipet cells and/or XTT solution accurately ◆ Check accuracy of pipettor ◆ Ensure XTT Reagent and/or XTT Activator are fully dissolved before use
High Background	<ul style="list-style-type: none"> ◆ Check proper storage of XTT at -20 °C ◆ Use freshly made XTT solution ◆ Decrease incubation time with XTT ◆ Ensure media is free of microbial contamination ◆ Serum will contribute to reduction of XTT; if possible eliminate or reduce serum before adding XTT Working Solution

References:

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2. Stevens MG, Olsen SC. (1993) Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. *J Immunol Methods* **157**:225-31.
3. Scudiero DA, Shoemaker RH, Paul KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* **48**:48274-33.
4. Puissant A, Grosso S, Jacquet A, Behacene N, Colosetti P, Cassuto J-P, Patrick A. (2008) Imatinib mesylate-resistant human chronic myelogenous leukemia cell lines exhibit high sensitivity to the phytoalexin resveratrol. *FASEB J* **22**:1894-904.