XTT Cell Proliferation Assay Kit

Instruction Manual

Catalog Number 30-1011K *(1000 Assays)*
Store at -20°C

This product is intended for laboratory research purposes only. It is not intended for use in humans.

Introduction

Tetrazolium salts have been widely used as detection reagents for many years in histochemical localization studies and cell biology assays (1,2). The second generation tetrazolium dye, XTT, can be effectively used in cell proliferation, cytotoxicity, and apoptosis assays (2,3,4). XTT is reduced to a soluble, brightly colored orange derivative by a mix of cellular effectors. The sensitivity of an XTT assay is greatly improved by the usage of an intermediate electron carrier, PMS (N-methyl dibenzopyrazine methyl sulfate). PMS helps drive XTT reduction and the formation of its formazan derivative.

Background Principle of the XTT Assay

The XTT cell proliferation assay was first described in 1988 by Scudiero *et al*. (3) as an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange (Figure 1). This color change is accomplished by breaking apart the positively-charged quaternary tetrazole ring (2). The formazan product of XTT reduction is soluble and can be used in real-time assays.

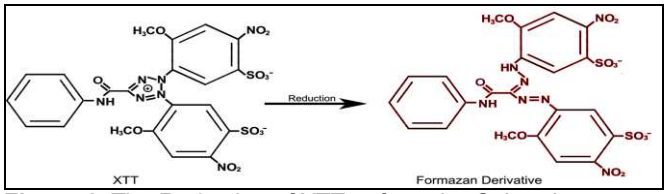


Figure 1. The Reduction of XTT to form the Colored Formazan Derivative

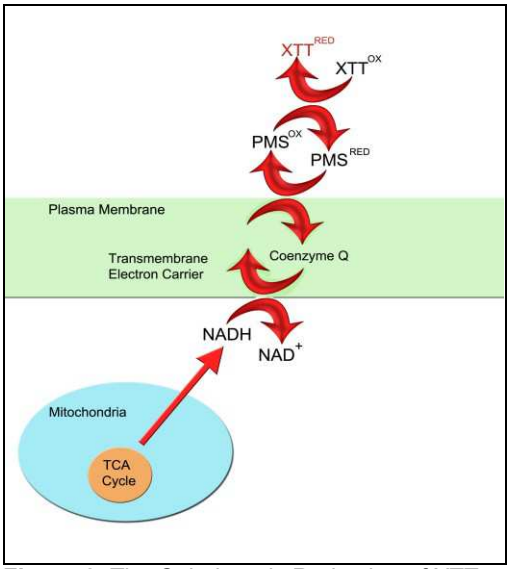


Figure 2. The Colorimetric Reduction of XTT by Cellular Enzymes

XTT is thought to be excluded from entering cells by its net negative charge (2). Considerable evidence suggests that XTT dye reduction occurs at the cell surface facilitated by trans-plasma membrane electron transport. Mitochondrial oxidoreductases are thought to contribute substantially to the XTT response with their reductants being transferred to the plasma membrane (Figure 2). It has been proposed that XTT assays actually measure the pyridine nucleotide redox status of cells (2, 4).

XTT can be used alone as a detection reaction but the results are not optimal. XTT assay results are greatly improved when an intermediate electron acceptor, such as PMS (N-methyl dibenzopyrazine methyl sulfate), is used with XTT (Figure 2). PMS is the Activation Reagent included in the XTT Cell Proliferation Assay Kit. Findings suggest that PMS mediates XTT reduction by picking up electrons at the cell surface, or at a site in the plasma membrane that is readily accessible, and forms a reactive intermediate that then reduces XTT to its highly pigmented formazan product.

Kit Components

Component	Volume	Number of Vials in Kit	Storage	Form	Stability
XTT[1] Reagent (Part No. 999-001)	5.0 mL	10 vials (Aliquot to avoid freeze-thaw damage)	-20°C in the dark	Sterile solution	9 months
Activation Reagent (Part No. 999-002)	0.5 mL	2 vials (Aliquot to avoid freeze-thaw damage)	-20°C in the dark	Sterile solution containing PMS[2]	9 months

[1]XTT = (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) inner salt; [2]PMS = (N-methyl dibenzopyrazine methyl sulfate) functioning as the intermediate electron carrier.

NOTE: If sediment is observed in either the XTT Reagent or the Activation Reagent after defrosting, heat the reagent solution to 37°C and swirl the vial until a clear solution is obtained.

Quality Control Specifications

pH of XTT Reagent: 7.5 - 9.0
Osmolarity, XTT Reagent: 269 - 296 mOsm/kg
Osmolarity, Activation Reagent: 270 - 310 mOsm/kg
Sterility Testing: Negative for bacteria, fungi, yeast
Cell Culture Test: Proliferation and morphology (Vero cells)
Safety Precautions: Refer to the Material Safety Data Sheet (MSDS) regarding safety precautions for this product.

A Certificate of Analysis is available upon request for each lot of the XTT Cell Proliferation Assay Kit.
The MSDS is available upon request.

Equipment and Materials Required but *not* Included in the Kit

Microtiter Plate Reader with: 450-500 nm wavelength filter 630-690 nm wavelength filter	96-Well Microtiter Plate (flat-bottom)
Cell Growth Media	Sterile Vials for Aliquots
Inverted Light Microscope	Sterile Pipettes (1 mL, 5 mL, 10 mL)
37°C (5% CO_2 / 95% Air) Incubator	Multi-Channel Pipette
37°C Water Bath	Sterile Pipette Tips

XTT Pre-Assay Optimization Protocol

It is highly recommended that the optimal cell number and assay incubation time for your specific cells be determined before performing a large number of XTT assays. The XTT pre-assay optimization protocol only needs to be performed once with each cell of interest. Pre-assay optimization is important because the most sensitive differences in assay results are obtained from the linear portion of the detection curve. In general, a minimum of 5000 cells can be effectively used per assay point in the XTT assay. Some cell types, however, can exhibit low metabolic activity (e.g., resting lymphocytes, contact-inhibited cell lines, keratinocytes and melanocytes) and require higher concentrations of cells (e.g., >5 x 10^5 cells per assay point) and longer incubation times (i.e., more than 4 hours) to detect fine differences in cellular metabolic activities. *Aseptic technique should be used throughout.*

Step	Pre-Assay Optimization Procedure
1	Cell Harvest: A. Suspension culture: Collect cells by centrifugation at 200 x g for 10 minutes. Resuspend cell pellet in appropriate complete growth medium. B. Adherent culture: Aspirate medium, dissociate cells by scraping or by enzymatic treatment, neutralize trypsin and collect cells by centrifugation. Resuspend cell pellet in appropriate complete growth medium. *Note: Media containing phenol red do not appear to interfere with absorbance readings.*

XTT Pre-Assay Optimization Protocol (continued)

2	Resuspend cells at 1 x 10^6 per mL.
3	Prepare serial dilutions of cells in complete growth medium from 1 x 10^6 to 1 x 10^3 per mL.
4	Seed 100 µL per well of each cell dilution into a flat-bottom 96-well microtiter plate in **triplicate**. Include 3 control wells containing 100 µL of complete growth medium alone as blank absorbance readings.
5	Incubate the inoculated plate for 12-48 hrs based on the best-suited culture conditions for the cells.
6	Prepare the Activated-XTT Solution: A. Defrost an aliquot each of the XTT Reagent and the Activation Reagent at 37°C prior to use. B. Swirl gently until clear solutions are obtained. C. Add 0.1 mL of the Activation Reagent to 5.0 mL of the XTT Reagent, which forms enough Activated-XTT Solution for one 96-well microtiter plate assay. D. Add 50 µL of the Activated-XTT Solution to each well.
7	Return the plate to the cell culture CO_2 incubator for 2-4 hrs
8	Periodically assess the microtiter plate for the visual appearance of an **orange color**. Shake the plate gently to evenly distribute the orange color in each well. A light inverted microscope may be used to better detect the orange color.
9	Measure the absorbance of the wells containing the cells and the blank background control wells at a wavelength between 450-500 nm using a microtiter plate reader.
10	Measure the absorbance of the wells containing the cells and the blank background control wells again at a wavelength between 630-690 nm to assess non-specific readings.
11	If the readings are low, return the plate to the CO_2 incubator for a longer incubation.
12	Determine the average value from the triplicate readings and subtract the average value for the blank wells as well as the average value of the non-specific readings. Plot absorbance against the number of cells per mL or cells per well. The optimized number of cells to use in your XTT assays should lie in the linear portion of the plot.

Determination of Specific Absorbance

When performing the XTT assay, ATCC scientists used the following parameters:

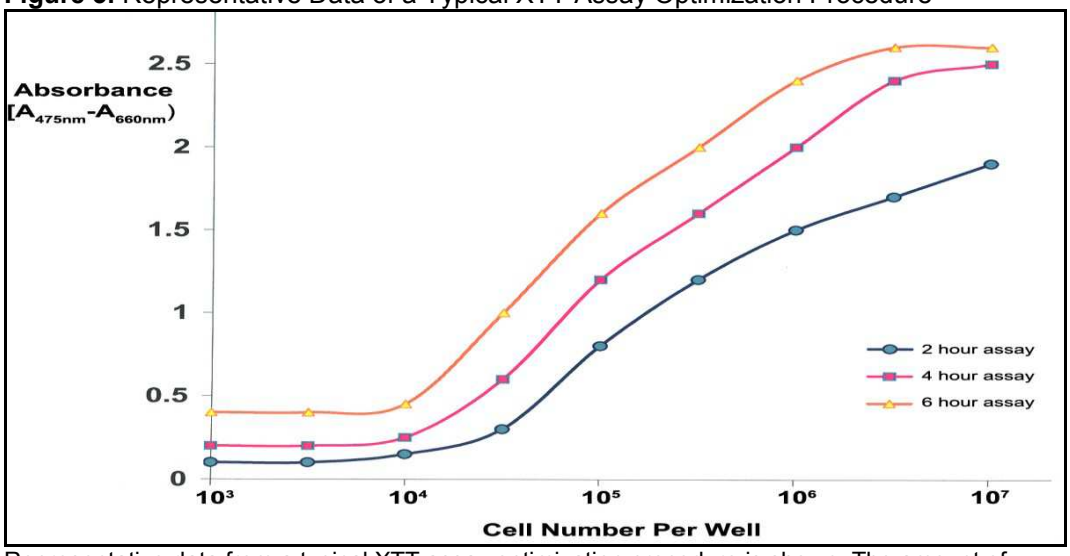
Specific absorbance filter: 475nm
Non-Specific absorbance filter: 660nm

The specific absorbance of the sample is expressed mathematically as follows:

$$\text{Specific Absorbance} = A_{475nm}(\text{Test}) - A_{475nm}(\text{Blank}) - A_{660nm}(\text{Test})$$

Representative data of a typical XTT assay optimization procedure is shown in Figure 3. The optimized number of cells to use in your XTT assays should lie in the linear portion of the plot.

Figure 3. Representative Data of a Typical XTT Assay Optimization Procedure



Representative data from a typical XTT assay optimization procedure is shown. The amount of specific absorbance detected increases with cell number and incubation time from 2-6 hours until a maximal absorbance level is achieved. The linear region of the XTT assay curve should be used in experiments. This region provides the greatest sensitivity to detect changes induced by experimental parameters.

XTT Assay Protocol

After you have optimized the number of cells to use in your XTT assay and determined the optimal amount of incubation time for activity measurements, the XTT assay protocol is easy to perform.

Step	XTT Assay Protocol Action
1	Inoculate cells in a flat-bottom 96-well microtiter plate in triplicate as follows: A. Seed in 100 µL the optimized number of cells (determined from the pre-optimization procedure) for the assay (usually 5 x10^3 to 2 x10^5 cells) into wells of a flat-bottom 96-well microtiter well plate in triplicate. B. Include 3 control wells containing 100 µL of complete growth medium alone as blank absorbance readings.
2	Incubate the inoculated plate for 12-48hrs based on the best-suited culture conditions for the cells and the experiment you are performing.
3	Prepare the Activated-XTT Solution: E. Defrost an aliquot each of the XTT Reagent and the Activation Reagent at 37°C prior to use. F. Swirl gently until clear solutions are obtained. G. Add 0.1 mL of the Activation Reagent to 5.0 mL of the XTT Reagent, which forms enough Activated-XTT Solution for one 96-well microtiter plate assay. A. Add 50 µL of the Activated-XTT Solution to each well.
4	Return the plate to the CO_2 incubator for the optimized-assay incubation time (usually 2-4 hrs; determined from the pre-optimization procedure).
5	Shake the plate gently following the incubation period to evenly distribute the **orange** color in the positive wells.

XTT Assay Protocol (continued)

6	Measure the absorbance of the assay wells containing the cells (step 1A) and the blank background control wells (step 1B) at a wavelength between 450-500 nm wavelength using a microtiter plate reader. Either zero the microtiter plate reader using the blank control wells or subtract their average value from the specific results.
7	Measure the absorbance of all the assay wells again at a wavelength between 630-690 nm and subtract the values from the 450-500 nm values obtained. This second absorbance determination will help eliminate non-specific readings from your assay results.
8	Plot the absorbance values of your corrected data on the ordinate (Y-axis) and your experimental parameters (e.g., number of cells) on the abscissa (X-axis).

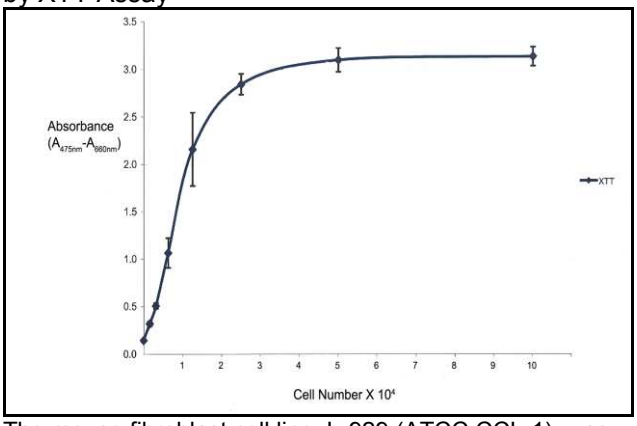
XTT Assay Data Interpretation

The plot of the XTT assay data should provide a curve with a linear portion. This is the area that will show the greatest sensitivity to changes induced by the experimental parameters. Absorbance values that are higher than control conditions indicate an increase in cell proliferation and viability. Absorbance values that are lower than control conditions indicate a decrease in cell proliferation and may be the result of cellular necrosis or apoptosis. It is recommended that assay results be confirmed by assessing microscopic morphological changes using an inverted light microscope to observe the cells.

XTT Assay Kit Results

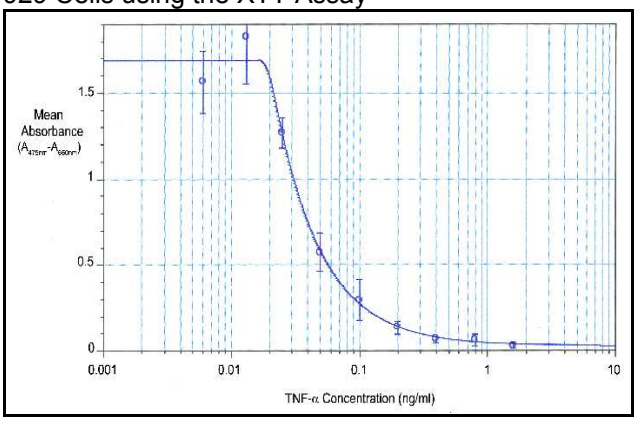
Scientists at ATCC performed two assays using the XTT Cell Proliferation Assay Kit with L-929 cells. The studies assessed cellular proliferation and viability of L-929 cells (Figure 4) and TNF-α-mediated cytotoxicity of L-929 cells (Figure 5).

Figure 4. Assessment of L-929 Cellular Proliferation by XTT Assay



The mouse fibroblast cell line, L-929 (ATCC CCL-1), was incubated overnight at 37°C in a 5% CO_2 / 95% air incubator. No additional agents were added during the cell culture. The cell proliferation and viability of L-929 cells were assessed by the XTT assay (3hr assay incubation time). The XTT assay (measured at 475 nm) exhibited a loss of linearity when greater than 2.5 x 10^4 L-929 cells were used in the cell proliferation assays.

Figure 5. Determination of TNF-α Cytotoxicity on L-929 Cells using the XTT Assay



Mouse L-929 cells were plated in 96-well microtiter plates and incubated overnight at 37°C in a 5% CO_2 / 95% air incubator. Different concentrations of TNF-α (in 25 µl) were added to designated wells and the L-929 cells were sensitized with 0.4 µg actinomycin D (in 25 µl). After incubating the microtiter plate for 18 hours at 37°C in a 5% CO_2, 95% air incubator, the XTT Assay was performed (3 hour assay incubation). The results show a clear dose response effect of increasing concentrations of TNF-α on L-929 cytotoxicity.

Troubleshooting Guide

Problem: XTT Reagent is blue-green.

Potential Cause	Remedy
Contamination from bacteria/cells	Discard. Aliquot XTT Reagent using aseptic technique in future.
Excessive exposure to light	Store solutions in the dark at -20°C

Problem: Blanks (growth medium only) give high absorbance readings.

Potential Cause	Remedy
Contamination from bacteria/cells	Discard. Aliquot XTT Reagent using aseptic technique and perform assay in biological hood with sterile microtiter plates.
The complete growth medium contains reducing agents such as ascorbic acid.	Find alternative media if possible; incubate plate in dark during assay.

Problem: Absorbance readings are too high.

Potential Cause	Remedy
Contamination from bacteria/cells	Discard. Aliquot XTT Reagent using sterile technique and perform assay in biological hood with sterile microtiter plates, tips, etc.
Cell numbers per well are too high	Reduce number of cells plated per well.

Problem: Absorbance readings are too low.

Potential Cause	Remedy
Cell numbers per well are too low	Increase number of cells plated per well.
Assay incubation time is too short	Increase incubation time with XTT- Activation Reaction Solution.
XTT Reagent was used alone without Activation Reagent	Prepare combined XTT-Activation Reaction Solution and repeat assay.
Cells not proliferating well	Check and optimize culture conditions; increase time in culture after plating for cell recovery.

Problem: Replicates have widely varying values.

Potential Cause	Remedy
Inaccurate plating or pipetting	Ensure accuracy of cell plating; check accuracy of pipettes used; ensure homogeneous cell suspension.

Related Products

MTT Cell Proliferation Assay (ATCC No. 30-101K), ATCC® Genuine Cultures™, ATCC® Cell Culture Media

References

1. F. P. Altman, Tetrazolium salts and formazans. *Prog. Histochem. Cytochem.* **9**, 1-56 (1976).
2. M.V. Berridge, P.M. Herst, A.S. Tan, Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review* **11**,127-152 (2005). *A comprehensive and highly recommended review of tetrazolium-based assays. Figure 2 was adapted from the article.*
3. D.A. Scudiero *et al.*, 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**, 4827-4833 (1988).
4. N.J. Marshall, C.J. Goodwin, and S.J. Holt. A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. *Growth Regulation* **5**:69-84 (1999).

ATCC Warranty & Disclaimers

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