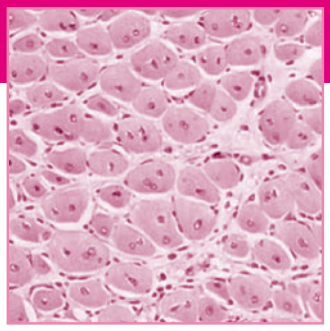
AppliCations
No.12

Cell Proliferation Assay XTT



Cell proliferation assays are widely used in cell biology for the study of growth factors, cytokines or media components. They are also applied in the screening of cytotoxic agents and lymphocyte activation. In order to determine the number of viable cells *Cell Proliferation Kit XTT* employs 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT). Only in living cells mitochondria are capable to reduce XTT to form an orange colored water soluble dye. Therefore, the concentration of the dye is proportional to the number of metabolically active cells.

AppliChem

Keywords

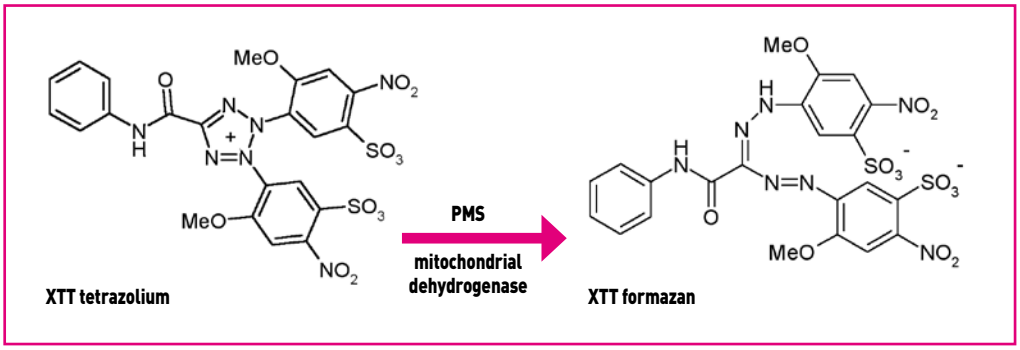
XTT assay

cytotoxicity testing

non radioactive assay

quantitating and viability testing of cells

The need for a reliable, sensitive and quantitative assay that would enable analysis of a large number of samples led to the development of methods, such as incorporation of radioactively labeled ^{3}H-thymidine into DNA or the use of 5-Bromo-2'-deoxyuridine (BrdU) as a substitute for radioactive thymidine to label DNA in living cells.
The above methods have a number of disadvantages, including: use of radioactive materials and relatively complex techniques. The use of tetrazolium salts, such as MTT, commenced in the 1950s, is based on the fact that living cells reduce tetrazolium salts into colored formazan compounds.



The tetrazolium salt of XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide salt) is an inner salt, that is cleaved to formazan by the succinate dehydrogenase system of the mitochondrial respiratory chain. Only living cells, possessing an intact mitochondrial membrane and also an intact cell membrane, do have active dehydrogenase. Agents that disrupt the membranes and destroy the respiratory chain will inactivate the enzyme and therefore the formation of the soluble orange formazan by reduction of the yellow tetrazolium salt. The reaction requires the presence of an electron coupling reagent, which is phenazine methosulfate, serving as an intermediate electron acceptor.

The biochemical procedure is based on the activity of mitochondrial enzymes which are inactivated shortly after cell death. This method was found to be very efficient in assessing the viability of cells. A colorimetric method based on the tetrazolium salt, XTT, was first described by P.A. Scudiero in 1988. Whilst the use of MTT produced an insoluble formazan compound which required dissolving the dye in order to measure it, the use of XTT produces a soluble dye. Even if MTT is better metabolized by many cell lines, XTT is sufficiently reduced by the cells in the presence of phenazine methosulfate (PMS). PMS does not influence the turnover of XTT. The use of XTT greatly simplifies the procedure of measuring proliferation, and is, therefore, an excellent solution to the quantitating of cells and their viability without using radioactive isotopes. This kit was developed to assay cell proliferation in reaction to different growth factors, cytokines and nutrient components. In addition, it is suitable for assaying cytotoxicity of materials such as TNF or other growth inhibitors. XTT can be used as a non-radioactive substitute for cytotoxic tests based on the release of ^{51}Cr from cells with no less sensitivity.

XTT Assay Greatly Simplifies Measuring of Cell Proliferation and Viability

The test procedure includes cultivation of cells in a 96-well plate, adding the XTT reagent and incubating for 2 – 24 hours. During the incubation time (usually within 2 – 5 hours) an orange color is formed, the intensity of which can be measured with a spectrophotometer, in this instance, an ELISA reader. The intensity of the dye is proportional to the number of metabolically active cells, i.e. the greater the number of metabolically active cells in the well, the greater the activity of mitochondrial enzymes, and the higher the concentration of the dye formed. The dye formed is water soluble and the dye intensity can be read without further treatments. The use of multiwell plates and an ELISA reader enables testing a large number of samples and obtaining rapid results.

Advantages of AppliChem Cell Proliferation Kit XTT at a glance

- Save: without using radioactive isotopes
- Accurate: dye absorbance is proportional to the number of cells in each well
- Easy-to-use: 1-step process, results within 2 – 5 hours.
- Includes XTT reagent and activation reagent. Additional reagents or cell washing procedures are not required.
- For use in plate readers

Procedure

1 The cells should be cultivated in a flat 96-well plate. To each well add 100 µl of growth medium. The cells should be incubated in a CO_2 incubator at 37°C. In most cases cells can be used to assay proliferation after 24 – 96 hours. Each test should include a blank containing complete medium without cells (see 4.7 background control)

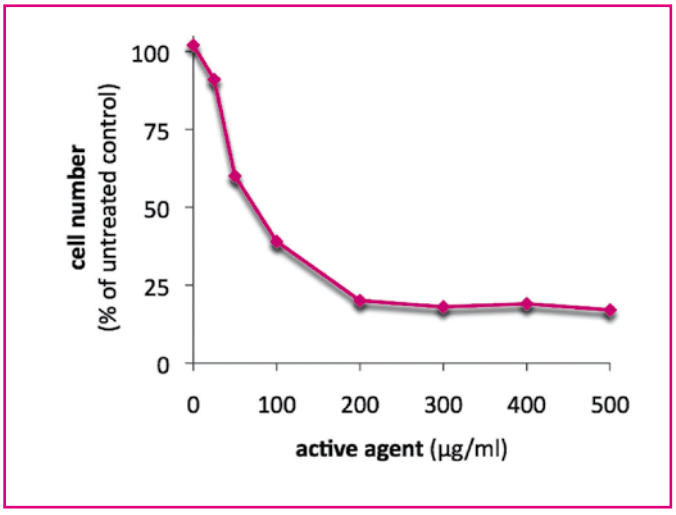
2 Defrost the XTT reagent solution and the activation solution immediately prior to use in a 37°C bath. Swirl gently until clear solutions are obtained.

3 To prepare a reaction solution sufficient for one plate (96 wells), add 0.1 ml activation solution to 5 ml of XTT reagent.

4 Add 50 µl of the reaction solution to each well and incubate the plate in an incubator for 2 – 24 hours depending on cell density and the characteristics of the cells being analyzed (usually, 2 – 5 hours are sufficient).

5 Shake the plate gently to evenly distribute the dye in the wells.

6 Measure the absorbance of the samples against a background control as a blank with a spectrophotometer (ELISA reader) at a wavelength of 450 – 500 nm. In order to measure reference absorbance (to measure non-specific readings), use a wavelength of 630 – 690 nm and subtract from the 450 – 500 nm measurement.



A typical experiment using Cell Proliferation Kit XTT.
Primary cultures of human epithelial cells were seeded in a 96-well plate in medium containing increasing concentrations of an NMDA receptor antagonist. At 48 hours cell numbers were evaluated by XTT assay. Relative cell number is represented by percentage of untreated cells (according to Lewis *et al.* 2001)

7 Background control (blank): Slight spontaneous absorbance around 450 – 500 nanometer occurs in the culture medium incubated with the XTT reagent. This background absorbance depends on the culture medium, pH, incubation time and length of exposure to light. Prepare one or more blank control wells without cells by adding the same volume of culture medium and XTT reagent solution as used in the experiment. Subtract the average absorbance of the blank control wells from that of the other wells.

Additional Notes

1 Defrost and prepare the reaction mixture only immediately prior to use.

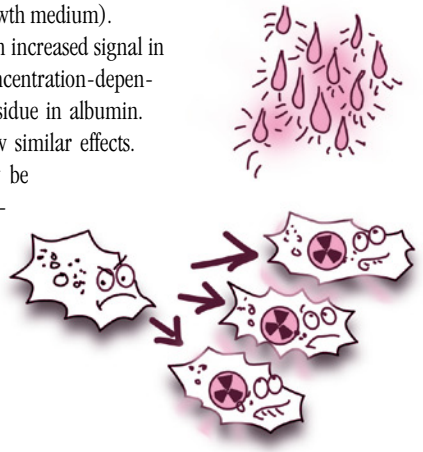
2 Since the test is extremely sensitive, it is possible to use a low concentration of cells in the wells (approximately 5000 cells per well). Since there are cell types which show low metabolic activity, such as lymphocytes, keratinocytes and melanocytes, it is recommended to increase the concentration of cells to 2.5×10^5 cells per well, in order to obtain development of formazan color within a reasonable period of time.

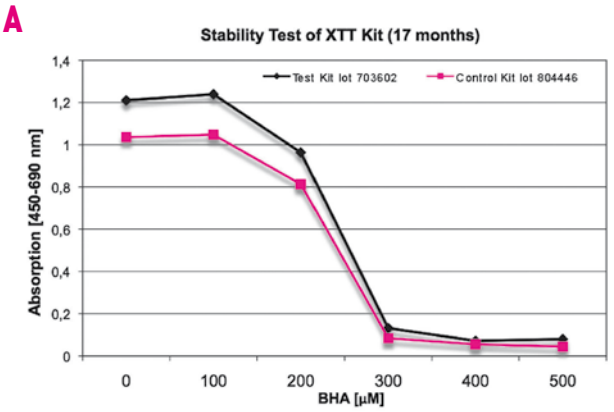
3 Incubation time with the reaction mixture varies according to the type and concentration of the cells. Therefore, it is advisable to perform an initial test by reading the absorbance at various time lapses, i.e. after 4, 6, 8, 12 hours using the same plate.

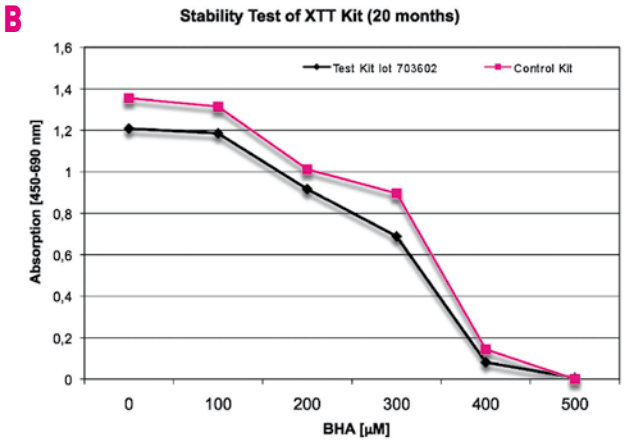
4 Prior to reading the absorbance with a spectrophotometer, the plate should be gently shaken in order to evenly distribute the dye in the wells.

5 If the volume of the medium in each well is larger than 100 µl, add a larger amount of reaction mixture by the same increment (i.e. 100 µl reaction mixture to 200 µl growth medium).

6 Serum albumin may lead to an increased signal in the XTT assay. This effect is concentration-dependent due to a free cysteine residue in albumin. Glutathione and cysteine show similar effects. Interference by albumin may be reduced by addition of N-ethylmaleimide. Therefore, include the background control in all assays!







Stability Test
To evaluate the stability of the Cell Proliferation Kit XTT reagents, tests were performed with kits up to 20 months after date of production, using the same lot 703602 in comparison to new standard kits. Graphs show samples tested at points of 17 and 20 months, respectively. Vero cells were cultured in 96 well plates for 24 h. Then, cells were exposed to increasing concentrations of BHA for 24 h, then viability was measured, using the colorimetric Cell Proliferation Kit XTT based method.

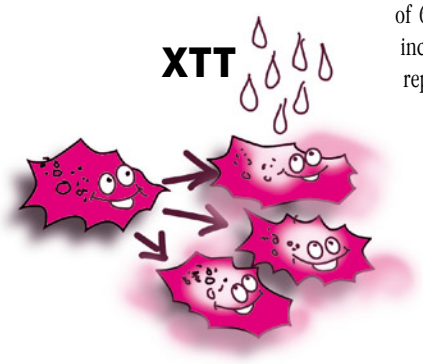
Summary of Proliferation Assay Using XTT Reagent: Flow Chart

- Defrost XTT reagent and activation reagent (37°C)
- Prepare reaction mixture (0.1 ml activation reagent and 5 ml XTT reagent for one plate)
- Add 50 µl reaction mixture for each well (containing 100 µl medium)
- Incubate at 37°C for 2 – 24 hours (in most cases incubation for 2 – 5 hours is sufficient)
- Measure absorbance at a wavelength of 450 – 500 nm (reference absorbance at a wavelength of 630 – 690 nm)

Stability Test of the Cell Proliferation Kit XTT: The Cytotoxicity of Butylated Hydroxyanisole (BHA)

Butylated Hydroxyanisole (BHA) is a synthetic antioxidant used in the food and cosmetic industry. Low doses of BHA exerted a significant cytotoxic effect, associated with loss of mitochondrial function. As the concentration of BHA increases, morphological alterations in critical sub-cellular targets such as lysosomes, mitochondria and actin cytoskeleton, are observed. In parallel, BHA induced an irreversible loss of cell proliferative capacity, preceding apoptosis induction.

BHA Cytotoxicity on VERO cells: Vero cells were cultured (5000 cells per well) in a 96 well plate for 24 h. Then, cells were exposed to increasing concentrations of BHA (0 – 500 µM) for 24 h, then viability was measured, using the colorimetric Cell Proliferation Kit XTT based method. XTT reagent was added and absorbance was measured (wavelength of 450 nm and reference of 690 nm) after a further 5h of incubation. The graphs show two representative experiments.



List of cells which were tested with Cell Proliferation Kit XTT
Pancreatic carcinoma cell line
Monocytes
Human hepatocarcinoma cells – Hep G2
Ehd-1 embryonic fibroblasts
Human embryonic 293 kidney cells
Mouse fibroblasts
Human prostate carcinoma cells – CL1, 22RV1 (subclone of CWR22 xenograft) and LNCaP
Myofibroblasts
Prostate cancer cell lines DU-145 and PC-3
Mammary gland breast cancer cell lines MCF-7alpha and MDA-MB231
Epithelial colorectal adenocarcinoma cell line HT-29
Small cell lung carcinoma cell line AL-780
Mouse myeloid cell line
Primary human umbilical vein endothelial cells
ALL cells of B cell lineage
FDCP cell line
Human monocytic cell lines U937, THP-1 and monomact
Mouse macrophage like cell line RAW 264.7
Freshly isolated human T cells isolated into CD3, CD4 and CD28 populations
293T – (embryonic kidney)
Hela – (cervix carcinoma)
Hep G2 – (heptocellular carcinoma)
D 145 – (prostate cancer)
A375 – (malignant melanoma)
MCF-7 – (breast adenocarcinoma)
BXPC-3 – (pancreatic adenocarcinoma)
Synovial cells
CD3 T-cells from 4–6 week mouse spleens
Human keratinocytes (HaCat) and murine fibroblasts (NIH 3T3)
Human breast cancer cells (T-47D, MDA-MB-468)
Murine C3H10T1/2 progenitor cells
Human embryonic kidney cells (HEK 293)
CHO cells
Early and late murine hematopoietic cells

References

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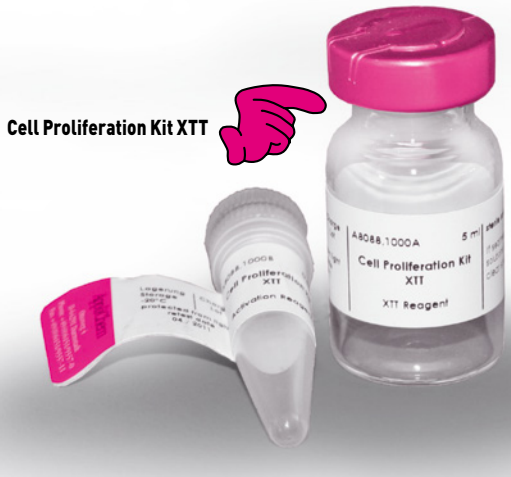
Description	Prod. No.	Quantity
Cell Proliferation Kit XTT	A8088,1000	for 1000 Tests

Related products

XTT sodium salt *BioChemica*	A2240
Dimethyl sulfoxide Cell culture grade	A3672
Dimethyl sulfoxide, sterile filtered (ampules)	A7248
Freez Media – DMSO Cell cryopreservation formulation	A8361
Incubator-Clean™ Disinfectant solution for incubators and sterile benches	A5230



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09/2010

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