XENOMETRIX

Swiss Commitment for Bioassays

Instructions for use

In Cytotox - XTT

1 - Parameter Cytotoxicity Kit

Tetrazolium XTT - Mitochondrial Activity

Art. No. KXT 96.1200
Art. No. KXT 96.2400

For research use only

Version 2.11 06/2015

Table of contents

Kit contents KXT 96.1200

XTT I	Substrate	1 x 66 ml
XTT II	Buffer	1 x 0.66 ml

1 Instruction manual

Kit contents KXT 96.2400

XTT I	Substrate	2 x 66 ml
XTT II	Buffer	2 x 0.66 ml

1 Instruction manual

Material required but not provided:

Test cells
Culture medium
Sterile water
Phosphate-buffered saline PBS

96-well microplates
Sterile reagent reservoirs

Storage conditions

All reagents are shipped at ambient temperature, but need different handling and storage upon arrival for optimal performance and shelf life as indicated on the vials.

XTT I and XTT II need to be frozen at -20°C upon arrival for optimal performance and shelf life as indicated on the vials. If only part of the kit is to be used at once we recommend to prepare aliquots of the reagents upon arrival. Avoid repeated (>2x) freezing and thawing.

The XTT solutions are photosensitive and have to be stored protected from light.

Limitation of use and interfering factors

This kit is **for research use only**, and **not for human diagnostic purposes.**

- Colored compounds and substances inhibiting dehydrogenases may lead to erroneous results in the XTT assay.
- Erroneous results may be obtained by microbial contamination of the cultures or reagents, which contributes to the cleavage of XTT and formation of XTT formazan. Cultures containing microorganisms may not be tested with this method

Precautions

Some components of this kit are potentially carcinogenic or corrosive; it is advisable to work in a hood and to wear glasses, gloves and a mask. After skin contact with any reagents of this kit wash affected areas with water and soap.

In case of contact of eyes with XTT solutions, immediately flush eyes with copious amounts of water and consult a physician.

Technical information, questions

For any questions, supplementary information or suggestions, please contact the technical support department of Xenometrix:

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Important note:
Please read the complete manual carefully before starting the assay!

XTT test principle

Viable cells depend on an intact mitochondrial respiratory chain and an intact mitochondrial membrane. Toxic agents can be identified using mitochondrial dehydrogenases from viable cells.

XTT (2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt) is a tetrazolium salt that is cleaved to formazan by the succinate dehydrogenase system which belongs to the mitochondrial respiratory chain, and is only active in viable cells. The mitochondrial succinate dehydrogenase reduces the yellow tetrazolium salt into soluble orange formazan in the presence of an electron coupling reagent.

In contrast to the insoluble formazan salt crystals of MTT, XTT is converted to a water-soluble formazan product without the need for a solubilization step prior to spectrophotometric quantification. The enzyme activity is measured at 480 nm (optimum) or at 450 nm.

Protocol

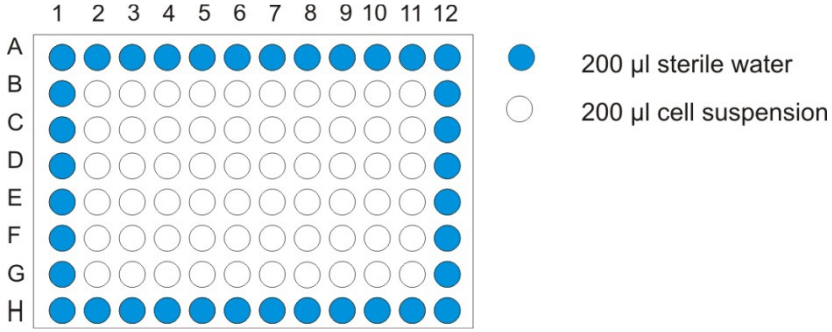
Subculture of cells to 96-well plates

Note:
The cell seeding step can easily result in uneven cell densities in the wells of the microplates. Make sure your cell suspension is homogeneous and devoid of large cell aggregates!

Cell density:
Optimal sensitivity is obtained with near-confluent cells at the time of the assay. The actual cell number to be used depends on the size of the cells, the culture doubling time and the duration of the culture phase before the assay is performed. For average sized adherent cells such as mouse fibroblasts and a total culture phase of 48 hrs we recommend to seed 20'000 cells into each well. Fewer cells may be necessary for longer exposure times and larger cells.

- Trypsinize the cells according to the standard operating procedures of your laboratory. Optimally, cells in the log phase of growth should be used.
- Resuspend the trypsinized cells in complete culture medium such that the desired cell number per well is present in 200 µl of medium.
- Pour sterile water into a sterile reagent reservoir.

- Dispense 200 µl of the sterile water in each well of rows A and H, and in each well of columns 1 and 12 (see figure), to reduce culture medium evaporation during incubation. (Erroneous results may be obtained upon uneven evaporation of culture fluid.)
- Pour the cell suspension into the same sterile reagent reservoir.
- Dispense 200 µl of cell suspension into the remaining wells of the 96-well plates.



- Incubate the plate in a humidified incubator at 37°C, 5% of CO_2 overnight or for 24 hours.

Preparation of test compound stock solutions

We recommend to use the plate layout described below. This allows to evaluate 2 compounds per plate in triplicate, 8 serial dilutions, with negative and positive controls.

- Prepare 8 sterile concentrations of each test compound that are 10X more concentrated than the concentrations to be used in the assay: Prepare the highest desired concentration and then the seven lower concentrations by serial dilutions with appropriate solvent. Use culture medium as solvent if possible.
- Dilute each 10X concentration 1:10 with culture medium

> *Note that it may be necessary to make more concentrated stock solutions in order to avoid final solvent concentrations that may interfere with cell growth. If DMSO, ethanol or methanol are used as solvents, the final solvent concentration should not exceed 2%. Equal solvent concentrations should be used for all compound solutions.*

Preparation of the negative control

For 1 plate:

- Mix 500 µl of solvent with 4.5 ml of culture medium (if 10X concentration of test compounds is used) (SC).

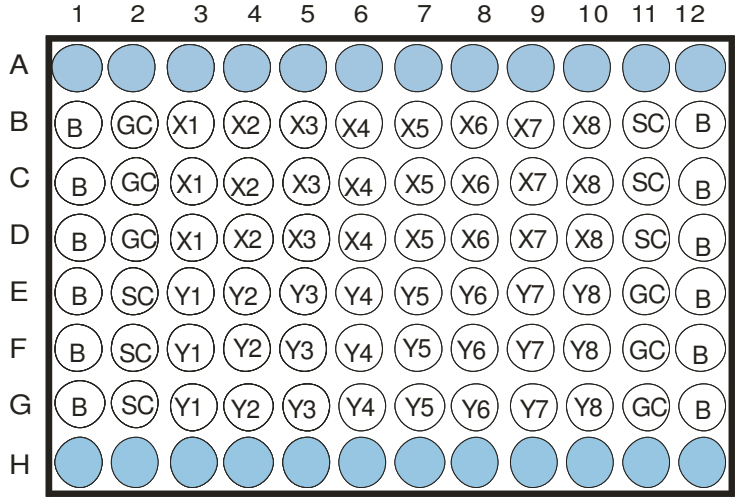
Exposure of the cells with test compounds

- Inspect the cells with a microscope. Cells should be distributed evenly between wells and look healthy.
- Remove the culture medium sterilly from each well either with a multi-channel pipette (recommended) or by using a sterile pipette attached to a vacuum source. Avoid scratching the bottom of the wells with the cells. The removed medium should be replaced rapidly with the test compounds in medium to avoid stress and cell death due to drying cell layers.

Recommended dosing configuration (see figure below):

- Add 200 µl of culture medium to wells B2, C2 , D2, E11, F11, and G11 (cell growth control = GC).
- Add 200 µl of culture medium – solvent mixture (page 11) to wells E2, F2, G2, B11, C11 and D11 (solvent control = SC) and to the wells of columns 1 and 12 (blank = B).
- Add 200 µl of the lowest test compound concentrations (X1) to wells B, C, D of column 3, 200 µl of test compound concentration X2 to wells B, C, D of column 4 and proceed through column 10 by adding 200 µl of test compound concentrations X3-X8.
- Add 200 µl of the lowest test compound concentrations (Y1) to wells E, F, G of column 3, 200 µl of test compound concentration Y2 to wells E, F, G of column 4 and proceed through column 10 by adding 200 µl of test compound concentrations Y3-Y8.
- Incubate the plate for the desired length of time.

Recommended 96-well plate configuration



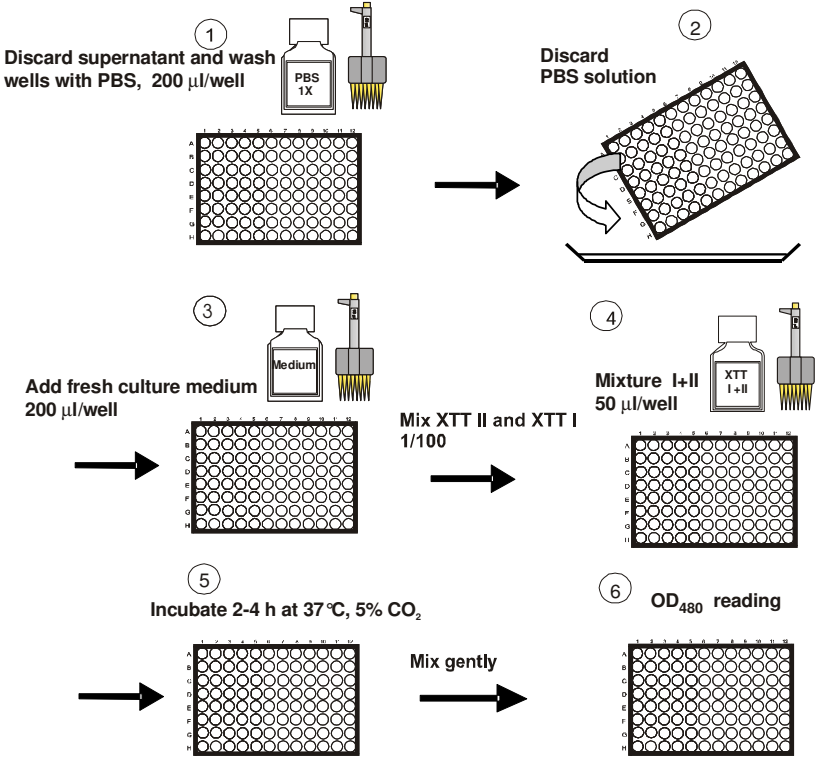
Rows A + H: sterile water
B: culture medium + solvent without cells (negative control)
GC: culture medium + cells (cell growth control)
SC: culture medium + cells + solvent (solvent control)
X1, X2,…, X8 and Y1, Y2,…, Y8: culture medium + cells + different concentrations of two compounds X, Y.

XTT Test Procedure

- Warm the XTT solutions to 37°C in a water bath until a clear solution is obtained.
- Remove the 96-well plate from the incubator
- Wash the cells with PBS (not provided) and add 200 µl/well of fresh culture medium in order to avoid any interaction of the test compound with the dye
- Immediately before use, mix the XTT II and XTT I solutions at a 1 : 100 ratio. For 1 plate (72 wells) mix 4 ml XTT I and 40 µl XTT II.
- Add 50 µl of this mixture to all wells, including the blanks
- Incubate the plate for 2-3 hours at 37°C, 5% CO_2 . The incubation time may be varied between 1 – 4 hours depending on cell type and maximum cell density. [1)]
- Mix the content of the well very carefully by pipetting up and down or by orbital agitation to enhance dispersion. Pipetting up and down may be required in dense cultures to completely disperse the XTT formazan.
- Remove any air bubbles if present.
- Read the OD at 480 nm (or 450 nm) with a reference wave length at 690 nm

[1)] If visual inspection during the incubation shows a clear difference between "Blank" and "Solvent Control" wells a longer incubation is usually not necessary.

XTT assay


1
Discard supernatant and wash wells with PBS, 200 µl/well
PBS 1X
2
Discard PBS solution
3
Add fresh culture medium 200 µl/well
Medium
4
Mixture I+II 50 µl/well
XTT I+II
Mix XTT II and XTT I 1/100
5
Incubate 2-4 h at 37°C, 5% CO_2
6
OD_{480} reading
Mix gently

Quality control of the assay

The mean OD values in the wells without test sample (solvent control, SC) correspond to a viability of 100% (wells E2, F2, G2, B11, C11 and D11).
The solvent controls (SC) are placed at the left and right side of the 96-well microplate to detect systematic errors. The assay is acceptable, if the left and right sided mean values do not differ more than 15% from the mean of all solvent controls (+/- 15%)

The cell growth controls (GC) allow to detect solvent effects. If the solvent control values differ significantly from the growth control values, inhibition values of test compounds are to be interpreted with caution. If possible chose a different solvent.

Data Analysis

For each well subtract the OD_{690} values from the OD_{480} values.

Calculate the mean OD values for every test sample concentration.

Calculate the mean OD values of the blanks (columns 1 and 12). Correct the sample and solvent control OD's:

Mean OD of samples/controls – mean OD of blanks

Relative inhibition activity is then expressed as percent of solvent control:

% inhibition = 100 - (corrected mean OD sample x 100 / corrected mean OD solvent controls)

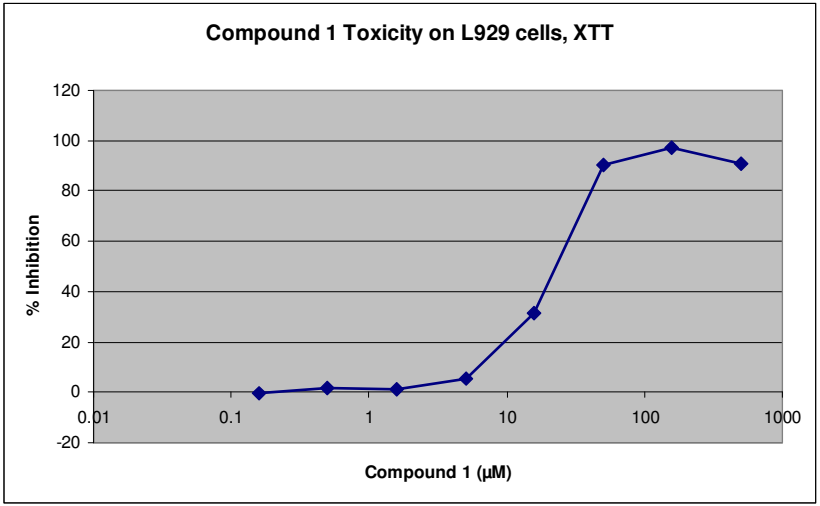
Plot the % inhibition of activity against the test compound concentration. For serial dilutions plot the concentrations on a log scale.

The 50% inhibiting concentration IC_{50} can be determined graphically.

The example below shows the toxicity of a test compound on L929 cells measured with the XTT test. IC_{50} is about 20 µM.

Example:

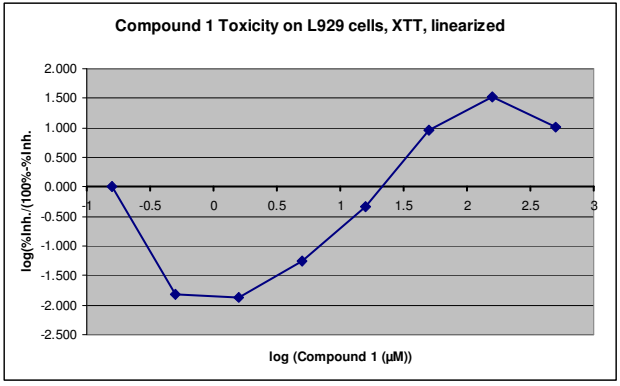
	Blank	GC	SC	0.158	0.500	1.58	5	15.8	50	158	500
	0.202	1.472	1.455	1.461	1.436	1.438	1.388	1.063	0.326	0.239	0.315
Corrected	0.000	1.270	1.253	1.259	1.234	1.236	1.186	0.861	0.124	0.037	0.113
% Inhibition		-1.357	0.000	-0.479	1.516	1.357	5.347	31.285	90.104	97.047	90.982
%inh/(100%-%inh)			0.000	-0.005	0.015	0.014	0.056	0.455	9.105	32.865	10.088
log (%inh/(100%-%inh)			#ZAHL!	#ZAHL!	-1.813	-1.862	-1.248	-0.342	0.959	1.517	1.004
log conc.				-0.80134291	-0.30103	0.19865709	0.69897	1.19865709	1.69897	2.19865709	2.69897
log (%inh/(100%-%inh)				#ZAHL!	-1.813	-1.862	-1.248	-0.342	0.959	1.517	1.004

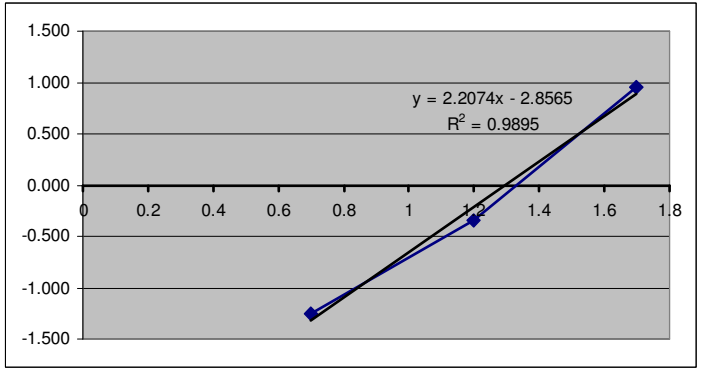


Alternatively you can plot:

Log (% inhibition / (100 -% inhibition)) vs. log (concentration)

The IC_{50} is obtained from the intercept on the x-axis. Note that values ≥ 100% and ≤ 0% can not be used with this analysis! A regression line using the linear part of the curve can be used to obtain a more precise estimation of the IC_{50}. In this case (y=0): (log IC_{50}) = 2.8565 / 2.2074) = 1.294, giving an IC_{50} of 19.7 µM





IC_{50} values can also be calculated using the CelTox software available from Xenometrix.

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