ANIARA

Manufactured By: Xenometrix AG

Instructions for use

In Cytotox - MTT

1- Parameter Cytotoxicity Kit

Diphenyltetrazolium Bromide MTT - Mitochondrial metabolism
Respiratory toxicity

Art. No. AKMT96.300
Art. No. AKMT96.310
Art. No. AKMT96.1200
Art. No. AKMT96.1210

For research use only

Version 2.1 07/2009

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Kit contents AKMT96.300/310

MTT I	Substrate solution	1 x 3.3 ml
MTT II	Solubilization solution	1 x 33 ml

1 Instruction manual

10 sterile reagent reservoirs (AKMT96.310 only)
4 96-well microplates (AKMT96.310 only)

Kit contents AKMT96.1200/1210

MTT I	Substrate solution	4 x 3.3 ml
MTT II	Solubilization solution	4 x 33 ml

1 Instruction manual

40 sterile reagent reservoirs (AKMT96.1210 only)
16 96-well microplates (AKMT96.1210 only)

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Material required but not provided:

Test cells
Culture medium
Sterile water

96-well microplates (AKMT96.300/1200 only)
Sterile reagent reservoirs (AKMT96.300/1200 only)

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Storage conditions

- The reagents are shipped at ambient temperature.
- Upon arrival MTT I and MTT II should be stored frozen at -20°C. They can be thawed and frozen repeatedly without noticeable loss of activity.
- Before use warm the solutions in a water bath (37°C) until completely dissolved.
- MTT I can form an insoluble precipitate upon storage. If this happens it should be filtered prior to use (0.22 µm).

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.

Caution ! Substrate solution MTT I may be harmful if swallowed, inhaled or absorbed through skin. MTT is able to damage genetic material. Solubilization solution MTT II is corrosive. In case of contact of eyes with MTT solutions, immediately flush eyes with copious amounts of water and consult a physician.

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Limitations of use and interfering factors

- Media and salt solutions with phenol red will contribute to higher background and thus may decrease the sensitivity of the assay. Use media without phenol red if possible.
- When using colored test compounds or chemicals that interfere with MTT, it is strongly advised to wash the cells with PBS before starting the assay. Do not forget to add 100 µl/well of culture medium before addition of MTT I solution.
- Erroneous results may be obtained by microbial contamination of the cultures or reagents, which contributes to the cleavage of MTT and formation of MTT formazan. Cultures containing microorganisms may not be tested with this method
- Any bubbles have to be eliminated before OD reading.

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

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Technical information, questions

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

Phone : 513-770-1991
Fax : 513-573-9241
Email : info@aniara.com

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

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MTT 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.

MTT 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 an water-insoluble purple formazan.

After solubilization of the formazan the amount of dye can be quantified with a microplate reader at 540 nm.

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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.

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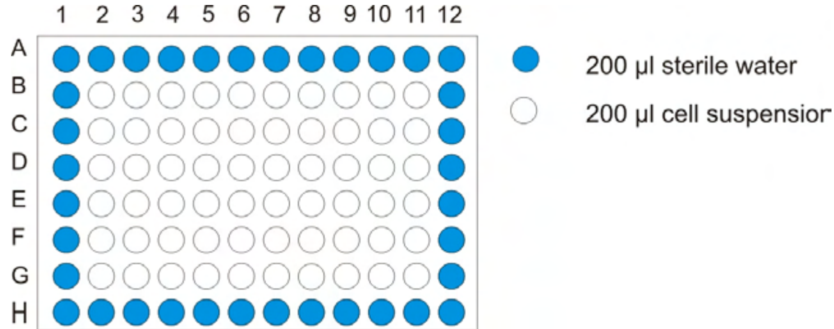
- Dispense 200 µl of the sterile water in each well of rows A and H, and in each well of columns 1 and 12 (see figure), to reduce culture medium evaporation during incubation. (Erroneous results may be obtained upon uneven evaporation of culture fluid.)
- Pour the cell suspension into the same sterile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

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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).

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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.

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- 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.

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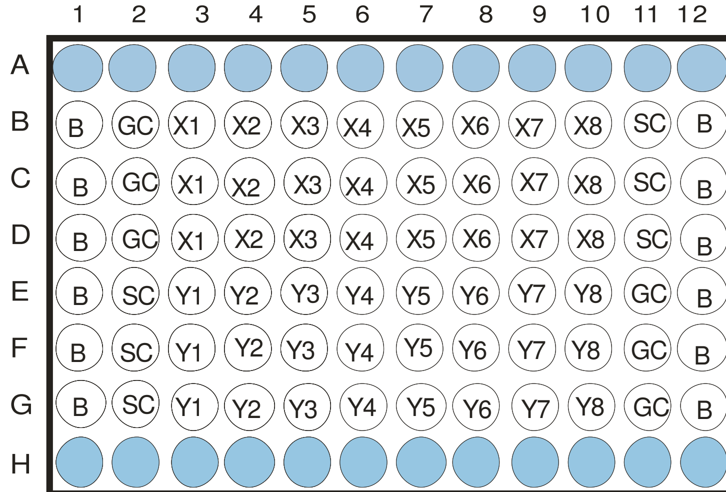
Recommended 96-well plate configuration



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.

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MTT Test Procedure

We recommend to pre-heat the MTT solutions at 37℃ in a water bath before use.

- Remove the 96-well plate from the incubator and place it in the laminar flow bench.
- Wash the cells carefully with PBS 1X and add 100 µl/well of fresh culture medium in order to avoid any interaction of the test compound with the dye. Make sure the cell layer is not disturbed by the washing procedure.
- Add 10 µl of MTT I to all wells, including the blanks
- Incubate the plate for 3 hours in a 37℃, 5% CO2 incubator. Incubation time may be varied from 2 – 4 hours depending on cell type, maximum cell density and metabolic activity of cells.
- Add 100 µl of MTT II solubilization reagent to all wells, including the blanks and incubate at 37℃, 5% CO2 overnight
- When necessary, mix very carefully. Avoid air bubble formation. Remove any bubbles prior to reading.
- Read the OD at 540 nm. Measure the background absorbance with a reference filter of 690 nm.

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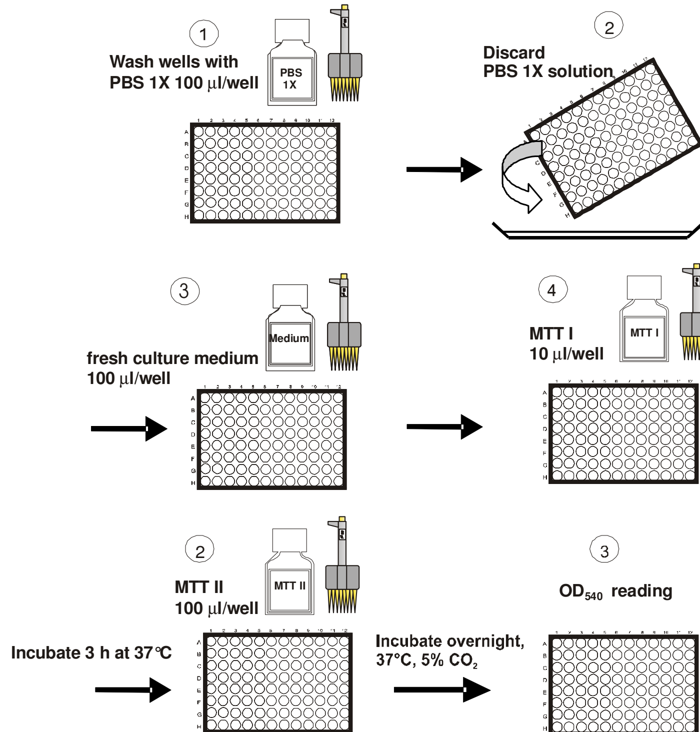
MTT assay



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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.

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Data Analysis

For each well subtract the OD_{690} values from the OD_{540} 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 toxic compound on L929 cells measured with the MTT test. IC_{50} is around 3 µM.

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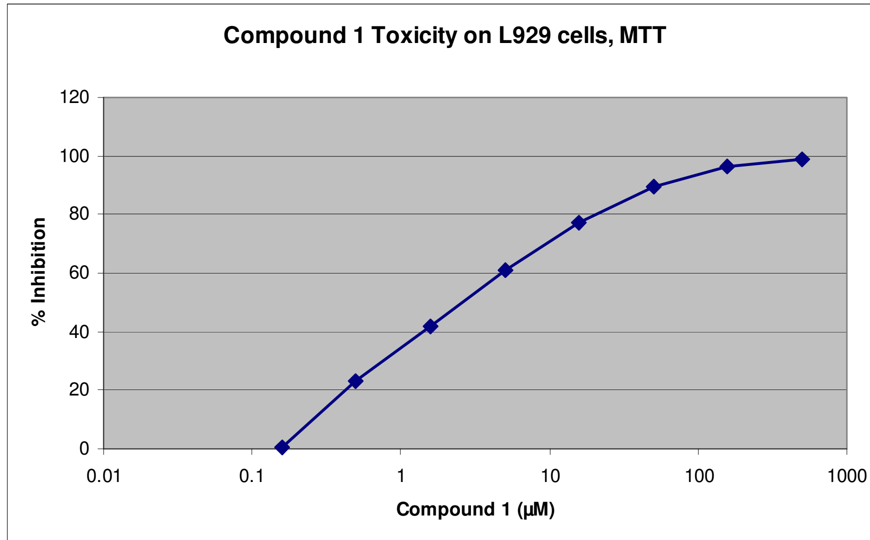
Example:

	Blank	GC	SC	0.158	0.500	1.58	5	15.8	50	158	500
	0.044	0.660	0.659	0.657	0.517	0.4023	0.285	0.1854	0.1091	0.0652	0.051
Corrected	0.000	0.616	0.615	0.613	0.473	0.358	0.241	0.141	0.065	0.021	0.007
% Inhibition		-0.163	0.000	0.341	23.171	41.805	60.878	77.073	89.480	96.618	98.927
%inh/(100%-%inh)			0.000	0.003	0.302	0.718	1.556	3.362	8.505	28.567	92.182
log (%inh/(100%-%inh)			#ZAHL!	-2.465	-0.521	-0.144	0.192	0.527	0.930	1.456	1.965
log conc.				-0.80134291	-0.30103	0.19865709	0.69897	1.19865709	1.69897	2.19865709	2.69897
log (%inh/(100%-%inh)				-2.465	-0.521	-0.144	0.192	0.527	0.930	1.456	1.965



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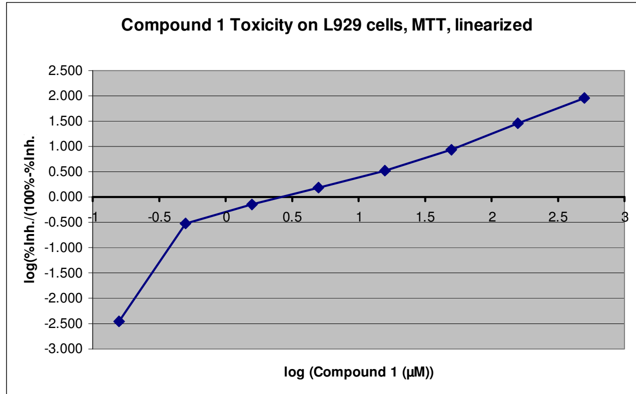
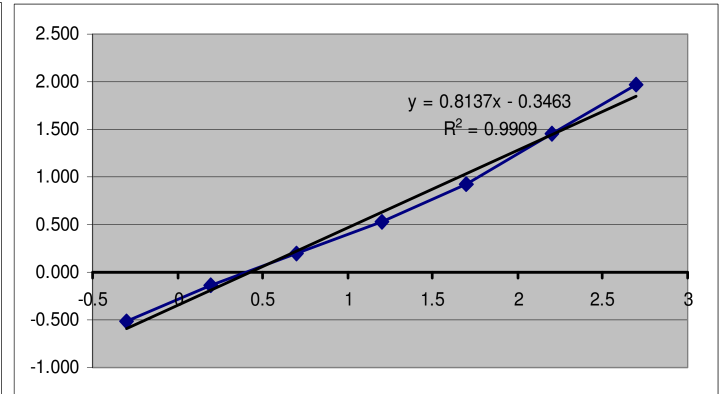
Alternatively you can plot:

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 crossing the y-axis at 0, can be used to obtain a more precise estimation of the IC_{50}. In this case (y=0): (log IC_{50}) = (0.3463 / 0.8137) = 0.4256, giving an IC_{50} of 2.7 µM.





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

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