



Instructions for use

In Cytotox GLU-XTT-CVDE

3 - Parameter Cytotoxicity Kit

Glucose Consumption GLU	-	Metabolic Activity
Tetrazolium XTT	-	Mitochondrial Activity
Crystal Violet Dye Elution CVDE	-	Nuclear Stain

Art. No. AKGXCV 96.300
Art. No. AKGXCV 96.310
Art. No. AKGXCV 96.1200
Art. No. AKGXCV 96.1210

For research use only

Version 2.0 12/2008



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Kit contents AKGXCV 96.300/310

GLU I	GOD/POD Reagent	1 x 39.2 ml (after reconstitution)
GLU II	Substrate	1 x 1 ml (after reconstitution)
XTT I	Substrate	1 x 16.5 ml
XTT II	Buffer	1 x 0.165 ml
CVDE II	Labeling solution	1 x 33 ml
CVDE III	Solubilization solution	1 x 33 ml

1 Instruction manual

20 sterile reagent reservoirs (AKGXCV 96.310 only)

8 96-well microplates (AKGXCV 96.310 only)

Kit contents AKGXCV 96.1200/1210

XTT I	Substrate	1 x 66 ml
XTT II	Buffer	1 x 0.66 ml
CVDE II	Labeling solution	1 x 132 ml
CVDE III	Solubilization solution	1 x 132 ml

1 Instruction manual

80 sterile reagent reservoirs (AKGXCV 96.1210 only)

32 96-well microplates (AKGXCV 96.1210 only)

Material required but not provided:

Test cells

Culture medium

Sterile water

Phosphate-buffered saline PBS

12N H₂SO₄ or 37% HCl

96-well microplates (AKGXCV 96.300/1200 only)

Sterile reagent reservoirs (AKGXCV 96.300/1200 only)

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.

GLU:

All GLU reagents must be stored at 2-8°C. After reconstitution, the Glucose Working Solution is stable when protected from light at 2-8°C for up to 1 month. Discard if turbidity develops or color forms.

XTT:

XTT I and XTT II are shipped at ambient temperature, but 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.

CVDE:

All reagents must be stored at 2-8°C.

Limitation of use and interfering factors

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

- Erroneous results may be obtained upon uneven evaporation of culture fluid.
- Colored compounds and substances inhibiting GOD/POD enzymes may lead to erroneous results in the GLU assay.
- 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.

Additional information on individual kit components:

GLU: GLU I and GLU II may be harmful if swallowed, inhaled, or absorbed through skin. In case of contact of eyes, immediately flush eyes with copious amounts of water and consult a physician.

To protect your multichannel pipettor from corrosion, it is advisable to use filter tips when dispensing H₂SO₄ or HCl.

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

CVDE: CVDE II and CVDE III may be harmful if swallowed, inhaled, or absorbed through skin. In case of contact of eyes with CVDE II or CVDE III 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 Aniara:

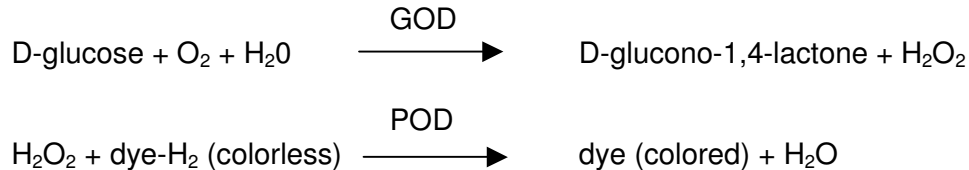
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**Important note:
Please read the complete manual carefully before starting the assay!**

GLU test principle

Many cultured cells continually consume glucose from their culture media for the generation of biosynthetic intermediates. By knowing the initial glucose concentration in the media, its consumption rate and an IC₅₀ value may be assessed by measuring glucose levels in the culture medium after incubation in the absence and presence of a test compound. Differences of glucose consumption in treated and untreated cells reflect changes in their metabolic state after drug exposure.

The assay allows to determine the physiological state of cultured cells by measuring glucose consumption. This procedure utilizes the coupled activities of glucose oxidase (GOD) and peroxidase (POD):



The amount of oxidized dye in the supernatant is measured spectrophotometrically at 540 nm.

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.

CVDE test principle

This is a simple assay useful for obtaining quantitative information about the relative density of adherent cells in 96-well plates. Crystal Violet is a dye that stains DNA. After elimination of excess dye and solubilization of the fixed dye, the amount taken up by the cells can be measured spectrophotometrically at 540 nm and can be correlated to cell number.

Protocol

Subculture of cells to 96-well plates

Note:

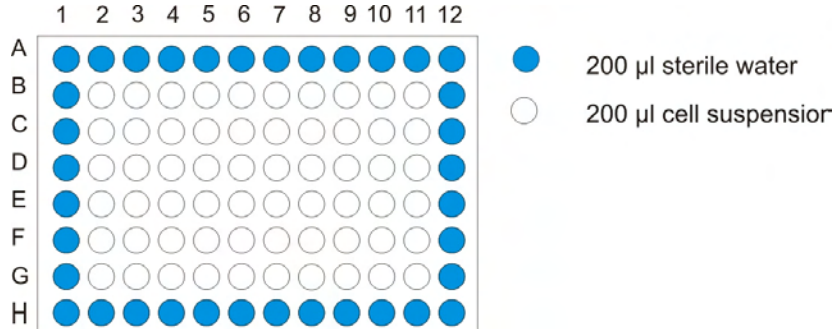
The cell seeding step can easily result in uneven cell densities in the wells of the microplates. Make sure your cell suspension is homogeneous and devoid of large cell aggregates!

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 μ l 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 μ l of cell suspension into the remaining wells of the 96-well plates.



- Incubate the plate in a humidified incubator at 37°C, 5% of CO₂ 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 μ l 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

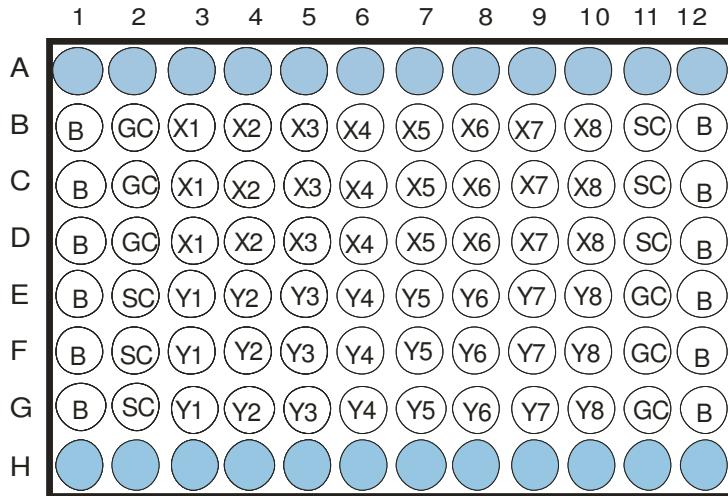
- Inspect the cells with a microscope. Cells should be distributed evenly between wells and look healthy.
- Remove the culture medium sterily from each well either with a multi-channel pipette (recommended) or by using a sterile pipette attached to a vacuum source. Avoid scratching the bottom of the 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 13) 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 μ l of the lowest test compound concentrations (Y1) to wells E, F, G of column 3, 200 μ l of test compound concentration Y2 to wells E, F, G of column 4 and proceed through column 10 by adding 200 μ l 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.

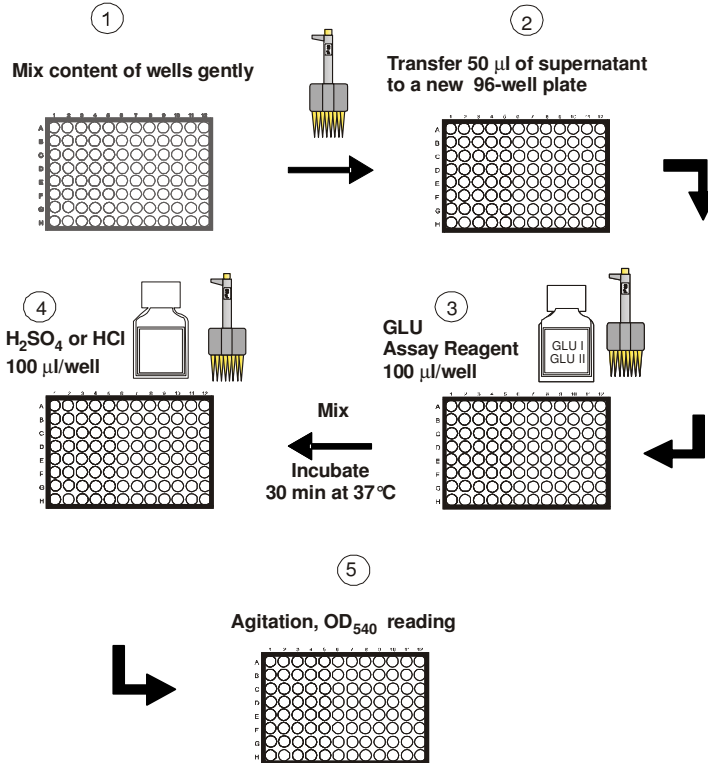
GLU Test Procedure

Before the assay, reconstitute the following bottle contents and prepare the Assay Reagent:

- Reconstitute the contents of the capsule in GLU I with 39.2 ml sterile water. Pipette up and down until completely dissolved. If necessary warm to 37°C. Avoid the formation of foam!
- Reconstitute GLU II with 1.0 ml of sterile water. Invert bottle several times until contents are completely dissolved.
- Prepare the Assay Reagent: Add 0.8 ml of GLU II to the amber bottle GLU I. Invert bottle several times to mix. Solution is stable for up to 1 month at 2-8°C protected from light. Discard if turbidity develops.
- Remove the plate from the incubator
- Mix the content of the wells by a gentle orbital agitation. Be careful not to disrupt the cellular layer.
- Note: If you use culture medium with a high glucose content such as Ham F-12 or RPMI 1640, you may have to dilute the supernatant. We recommend a 1:20 dilution with water for media containing 2 g/l of glucose. Media with other glucose content should be diluted accordingly. This kit measures glucose from about 1 – 100 µg/ml.
- With a multichannel pipettor, transfer 50 µl of the (diluted) supernatant from each well to new 96-well plate duplicating the previous plate configuration. Return the plate with the cells to the incubator.

- Add 100 µl/well of Assay Reagent to the wells with the transferred supernatant.
- Incubate for 30 minutes at 37 °C.
- Stop the reaction of each well by adding 100 µl/well 12 N H₂SO₄ or 37% HCl (not provided)
- Mix and read the OD of each well at 540 nm. (Air bubbles may interfere with the measurement and should be removed, e.g. by quickly moving with a gas flame over the wells; CAUTION!)

GLU assay

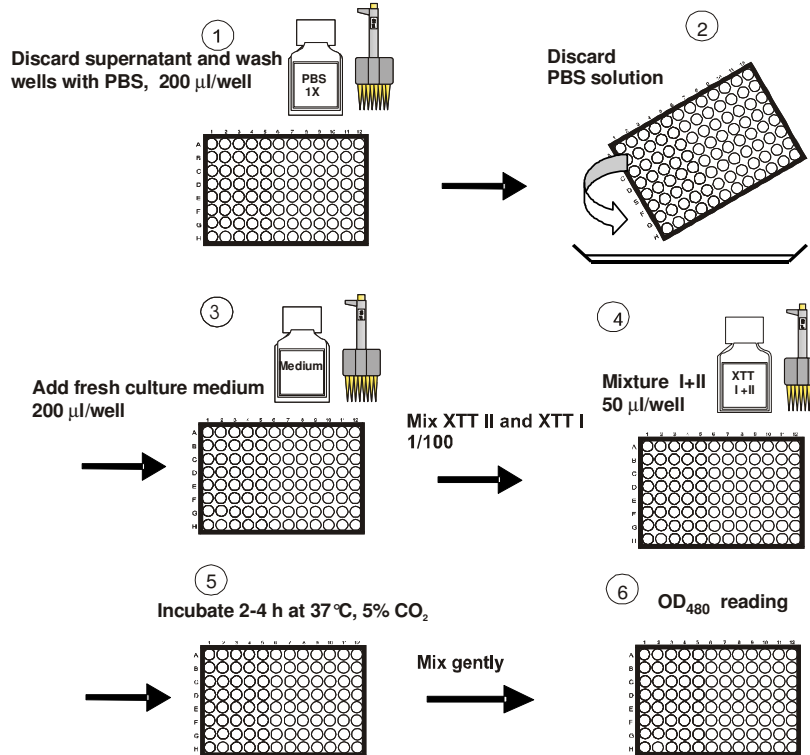


XTT Test Procedure

- 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% CO₂ . 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.

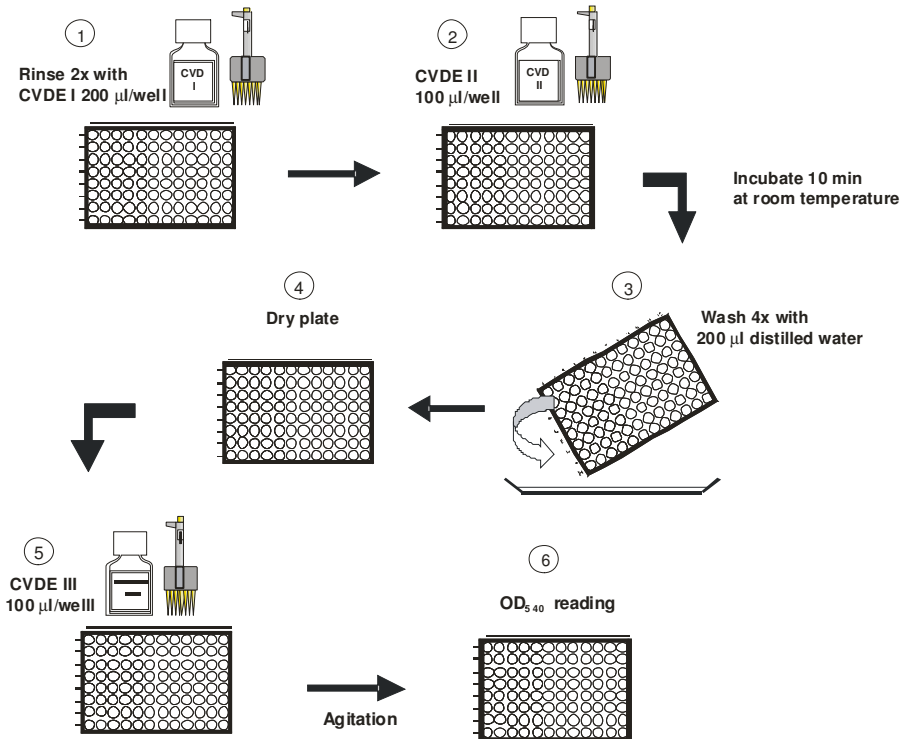
XTT assay



CVDE Test Procedure

- After reading the plate for the XTT test, remove the liquid by aspiration or by gently dumping the plate, with the cells remaining at the bottom of the wells
- Wash the cells 2 times with 200 μ l/well of wash solution CVDE I
- Add 100 μ l/well of labeling solution CVDE II
- Incubate the plate for 10 minutes at room temperature
- Discard the dye solution CVDE II by aspiration or by gently dumping the plate. Be careful not to disturb the cell layer.
- Wash the cells at least 4 times with 200 ml/well of distilled or de-ionized water (not provided) until the supernatant is clear
- Dry the plates in air or by using a hair dryer.
- Dissolve the cell layer with 100 ml/well of solubilization solution CVDE III
- Mix by pipetting up and down or by gentle swinging on a microplate shaker to enhance mixing of the solubilized dye. Read the OD at 540 nm with a reference wavelength at 690 nm.

CVDE assay



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

Glucose data analysis:

Read the plate at 540 nm. The rate of glucose consumption is calculated for every test compound concentration. The mean OD values of the blanks (wells without cells, columns 1 and 12) correspond to an consumption rate of 0%.

Calculate the normal (uninhibited) rate (Rn) of consumption:

$$\text{Rn} = \text{Mean of blank OD minus mean OD of solvent controls (SC)}$$

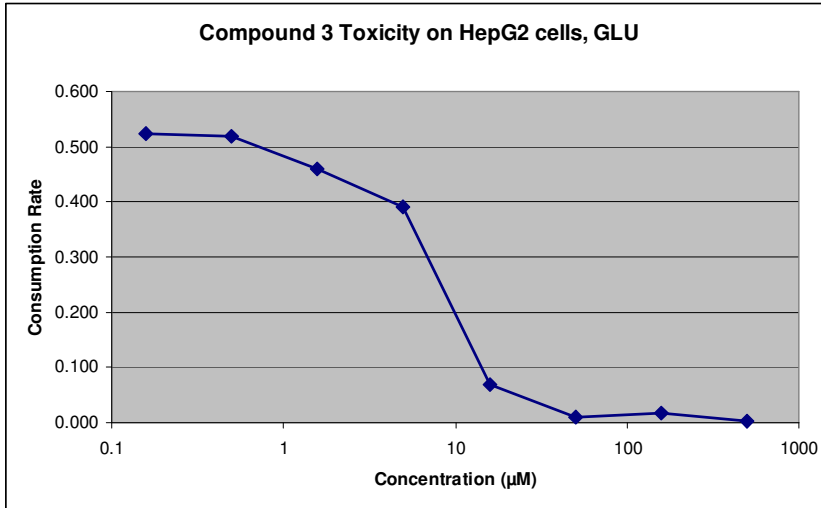
Similarly, the consumption rate (Rt) for every concentration of the test compound is calculated:

$$\text{Rt} = \text{Mean of blank OD minus mean OD of concentration (c) of test compound}$$

Shown here is an example how results may look:

	Blank	GC	SC	0.158	0.500	1.58	5	15.8	50	158	500
Consumption rate	1.353	0.842	0.830	0.828	0.835	0.892	0.962	1.284	1.342	1.336	1.350
(Rn-Rt)/Rt		0.511	0.523	0.525	0.518	0.461	0.391	0.069	0.011	0.017	0.003
log (Rn-Rt)/Rt			0.000	-0.003	0.010	0.135	0.339	6.613	47.879	30.317	192.704
log conc.			#ZAHL!	#ZAHL!	-1.990	-0.869	-0.470	0.820	1.680	1.482	2.285
				-0.801	-0.301	0.199	0.699	1.199	1.699	2.199	2.699

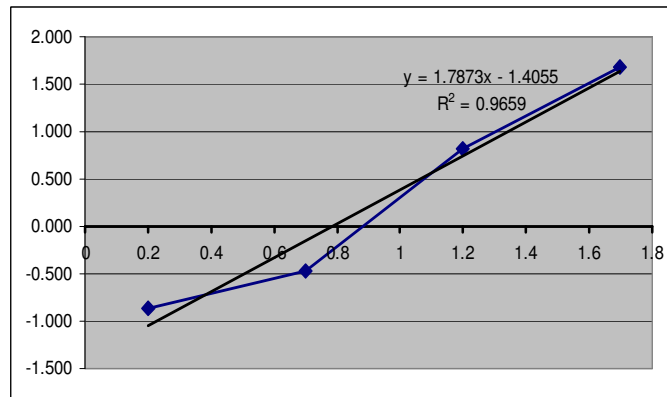
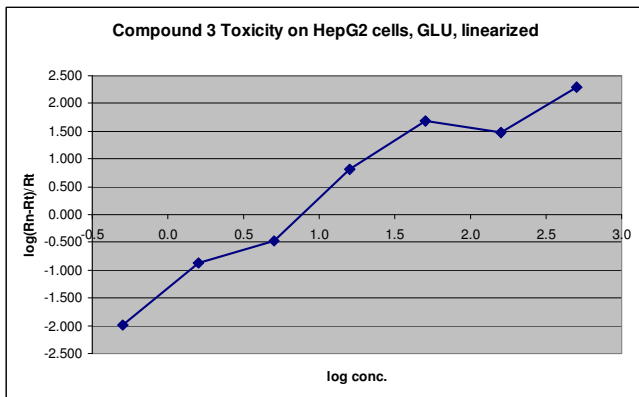
The results may be represented graphically: $R_t = f(c)$:



A linear regression rate may be calculated as follows:

$$\text{Log} \left(\frac{R_n - R_t}{R_t} \right) = f(\text{log } c)$$

With the above example this gives:



The IC_{50} is obtained from the intercept on the x-axis. Note that negative $(Rn-Rt)/Rt$ values can not be used with this analysis! A regression line using the central linear part of the curve can be used to obtain a more precise estimation of the IC_{50} . In this case ($y=0$): $(\log IC_{50}) = (1.4055 / 1.7873) = 0.7864$, giving an IC_{50} of $6.1 \mu M$.

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

Absolute glucose levels:

If needed, a glucose standard solution can be used to calculate the glucose consumption based on absolute values (available separately from Xenometrix). The standard solution contains 1 mg/ml glucose. A standard curve OD₅₄₀ vs. glucose concentration allows then to express the effect of an inhibitor in mg/ml glucose consumption rather than OD₅₄₀ units. The useful range of this kit is 1 – 100 µg/ml glucose. For plain IC₅₀ calculations the absolute values of glucose consumption are not needed, however.

XTT and CVDE Data analysis:

For each well subtract the OD₆₉₀ values from the OD₄₈₀ or OD₅₄₀ 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:

$$\% \text{ inhibition} = 100 - (\text{corrected mean OD sample} \times 100 / \text{corrected mean OD solvent controls})$$

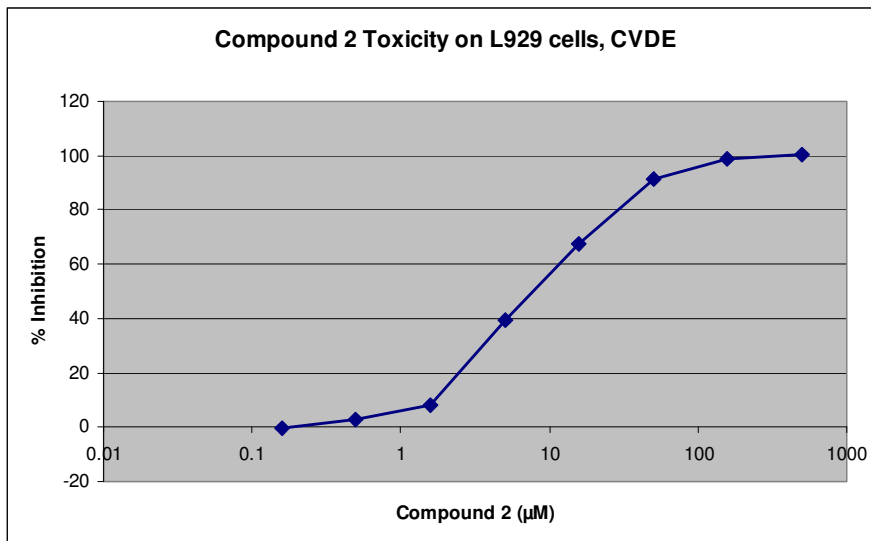
Plot the % inhibition of activity against the test compound concentration. For serial dilutions plot the concentrations on a log scale.

The 50% inhibiting concentration IC₅₀ can be determined graphically.

The example below shows the toxicity of a toxic compound on L929 cells measured with the CVDE test. IC₅₀ is around 10 µM.

Example:

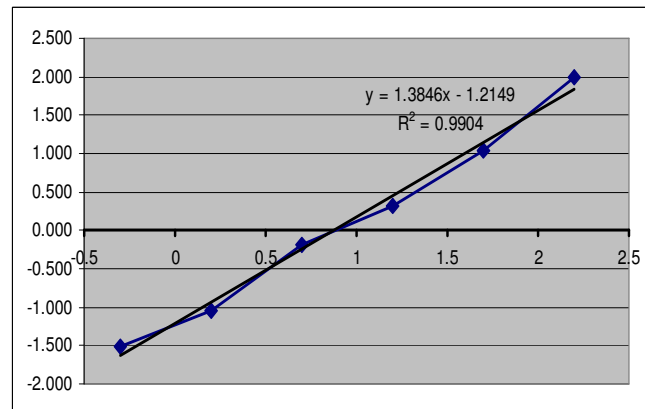
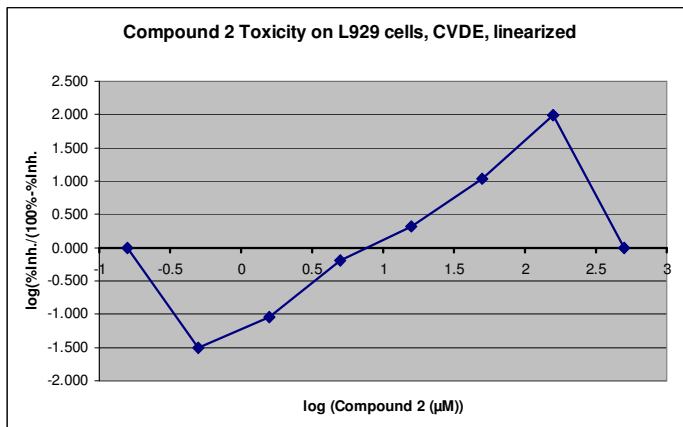
	Blank	GC	SC	0.158	0.500	1.58	5	15.8	50	158	500
Corrected	0.024	0.814	0.829	0.831	0.805	0.762	0.512	0.287	0.092	0.032	0.02
% Inhibition	0.000	0.790	0.805	0.807	0.781	0.738	0.488	0.263	0.068	0.008	-0.004
%inh/(100%-%inh)		1.863	0.000	-0.248	2.981	8.321	39.369	67.312	91.530	98.982	100.472
log (%inh/(100%-%inh))			#ZAH!	#ZAH!	-1.513	-1.042	-0.188	0.314	1.034	1.988	#ZAH!
log conc.				-0.80134291	-0.30103	0.19865709	0.69897	1.19865709	1.69897	2.19865709	2.69897
log (%inh/(100%-%inh))				#ZAH!	-1.513	-1.042	-0.188	0.314	1.034	1.988	#ZAH!



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 $\geq 100\%$ and $\leq 0\%$ 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 ($y=0$): $(\log IC_{50}) = (1.2149 / 1.3846) = 0.8774$, giving an IC_{50} of $7.5 \mu M$.



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