ANIARA

Manufactured By: Xenometrix AG

Instructions for use

In Cytotox - NR-CVDE

2 - Parameter Cytotoxicity Kit

Neutral Red NR - Lysosomal Activity
Crystal Violet Dye Elution CVDE - Nuclear Stain

Art. No. AKRCV 96.300
Art. No. AKRCV 96.310
Art. No. AKRCV 96.1200
Art. No. AKRCV 96.1210

For research use only

Version 2.31 10/2011

ANIARA
7768 Service Center Drive • West Chester OH 45069
Phone: 513.770.1991 Toll Free: 866.783.3797 Fax: 513.573.9241 Email: info@aniara.com
www.aniara.com

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Kit contents AKRCV 96.300/310

NR I	Wash solution	1 x 100 ml
NR II	Labeling solution (stock)	1 x 1.3 ml
NR III	Fixing solution	1 x 33 ml
NR IV	Solubilization solution	1 x 66 ml
CVDE I	Wash solution	1 x 125 ml
CVDE II	Labeling solution	1 x 33 ml
CVDE III	Solubilization solution	1 x 33 ml

1 Instruction manual

18 sterile reagent reservoirs (AKRCV 96.310 only)
4 96-well microplates (AKRCV 96.310 only)

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Kit contents AKRCV 96.1200/1210

NR I	Wash solution	1 x 400 ml
NR II	Labeling solution (stock)	1 x 5 ml
NR III	Fixing solution	1 x 132 ml
NR IV	Solubilization solution	1 x 264 ml
CVDE I	Wash solution	1 x 500 ml
CVDE II	Labeling solution	1 x 132 ml
CVDE III	Solubilization solution	1 x 132 ml

1 Instruction manual

72 sterile reagent reservoirs (AKRCV 96.1210 only)
16 96-well microplates (AKRCV 96.1210 only)

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Material required but not provided:

Test cells
Culture medium
Sterile water
Phosphate-buffered saline PBS

96-well microplates (AKRCV 96.300/1200 only)
Sterile reagent reservoirs (AKRCV 96.300/1200 only)

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Storage conditions

All reagents should be stored at 4°C.

Limitation of use and interfering factors

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

- Compounds which induce lysosome formation may cause a non-proportional cell number counting compared to the positive control. Moreover, the neutral red seems to have affinity to collagen and other extra-cellular proteins which limits its use with organ culture tests.
- Media and salt solutions with phenol red may contribute to a higher background and thus may decrease slightly the sensitivity of the assay. For most applications this should not pose a problem.

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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.

NR:

Labeling solution NR II and fixing solution NR III may be harmful if swallowed, inhaled, or absorbed through skin. In case of contact of eyes with NR II, NR III or NR IV solutions, immediately flush eyes with copious amounts of water and consult a physician. Fixing solution NR IV is flammable.

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.

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Technical information, questions

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

Phone : 866-783-3797
Fax : 513-573-9241
Email : info@aniara.com

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

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NR test principle

The neutral red (NR) assay procedure is a cell survival/viability assay based on the ability of viable cells to incorporate and bind neutral red within lysosomes. It is best performed on adherent cells.

NR is a weak cationic dye that readily penetrates the cell membrane and accumulates intracellularly in lysosomes (lysosomal pH < cytoplasmic pH), where it binds to anionic sites of the lysosomal matrix. Changes of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such alterations brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells.

The quantity of dye incorporated into cells is measured by spectrometry at 540 nm, and is directly proportional to the number of cells with an intact membrane.

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.

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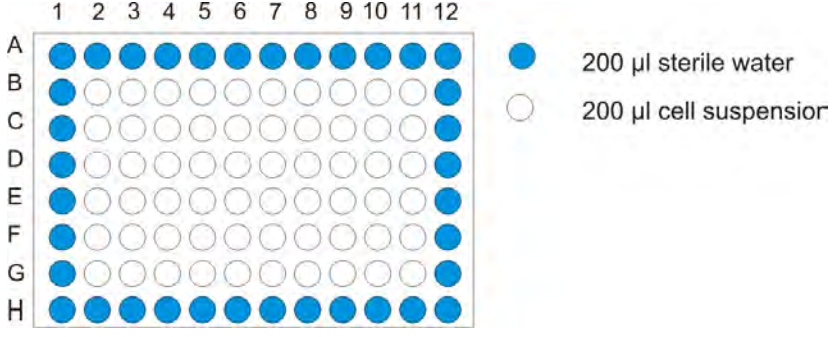
- Dispense 200 µl of the sterile water in each well of rows A and H, and in each well of columns 1 and 12 (see figure), to reduce culture medium evaporation during incubation. (Erroneous results may be obtained upon uneven evaporation of culture fluid.)
- Pour the cell suspension into the same sterile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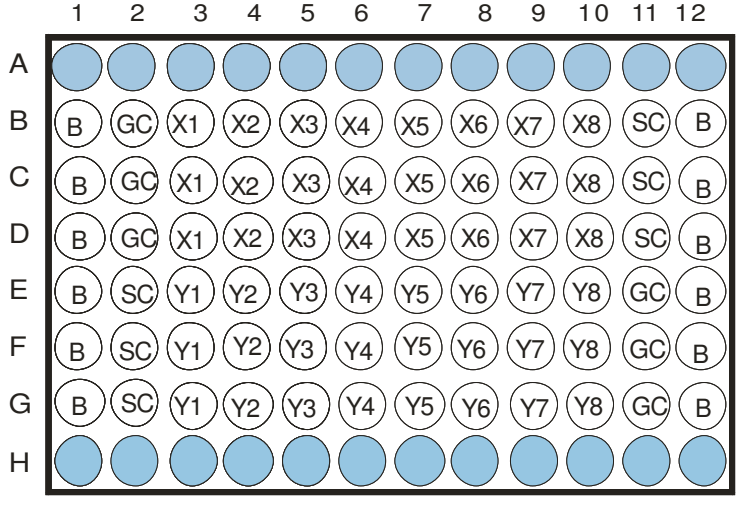
Recommended 96-well plate configuration



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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Preparation and testing of 1x Neutral Red labeling solution

We recommend to determine the optimal NR II dilution for your culture system in advance by preparing NR II dilutions 1:50, 1:100, 1:150, 1:200 in culture medium and incubate 200 µl thereof at 37°C in a microtiter plate. Choose the highest dye concentration that gives no or almost no crystals after 2 hrs of incubation.

We have found a 1:100 dilution appropriate in a culture medium using DMEM/Ham's F-12 with 10% FCS.

Pre-incubation of the staining solution and centrifugation or filtration prior to addition to the cells has been recommended in some protocols. We found this to be of little value as the crystals are easily re-suspended by handling after centrifugation, and filtration can remove a substantial amount of dye from the staining solution.

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NR Test Procedure

NR easily precipitates in culture medium which can lead to elevated background levels and increased variation within replica values. We recommend to add NR II stock solution to **pre-warmed media** at a predetermined ratio (see page 16) and distribute this staining solution **without delay** into the test wells. Place the plate back into the incubator as soon as the staining solution has been added. Observe the plate with an inverted microscope prior to fixation to check for crystal formation. If excessive crystals are observed (sparse small crystals are acceptable), the medium control values might be elevated and replica values may fluctuate too much. In such a case you should try to use a more diluted staining solution for the next experiment.

- Remove the culture medium by aspiration, with the cells remaining at the bottom of the wells.
- Wash each well with 300 µl of wash solution NR I.
- Add 200 µl labeling solution NR II 1X to each well (for preparation, handling and determination of optimal dilution see above).
- Incubate 3 hours (2 - 4 hours depending on cell line, cell density) at 37°C, 5% CO_2.
- Inspect the wells for crystal formation.
- Discard the labeling solution.
- Add 100 µl fixing solution NR III to each well.
- Discard the fixing solution NR III after 1 min.
- Add 200 µl solubilization solution NR IV to each well.

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- Incubate for 15 minutes at room temperature. Mix gently using a multichannel pipette.
- If any bubbles are observed, remove them prior to reading.
- Read the OD of each well at 540 nm with a reference wavelength at 690 nm.

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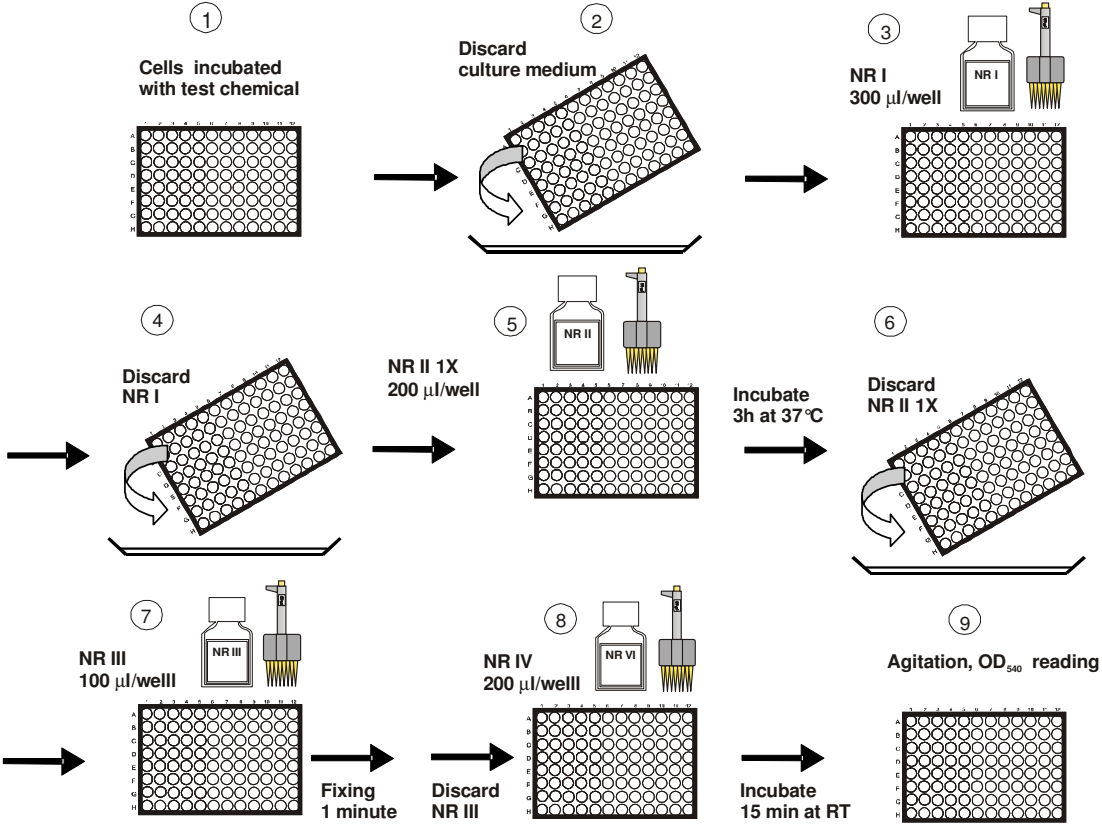
NR assay



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CVDE Test Procedure

- Remove the dissolved NR dye by aspiration or by gently dumping the plate, with the cells remaining at the bottom of the wells
- Wash the cells 2 x with 200 µl of wash solution CVDE I
- Add 100 µl/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 µl/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 µl/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.

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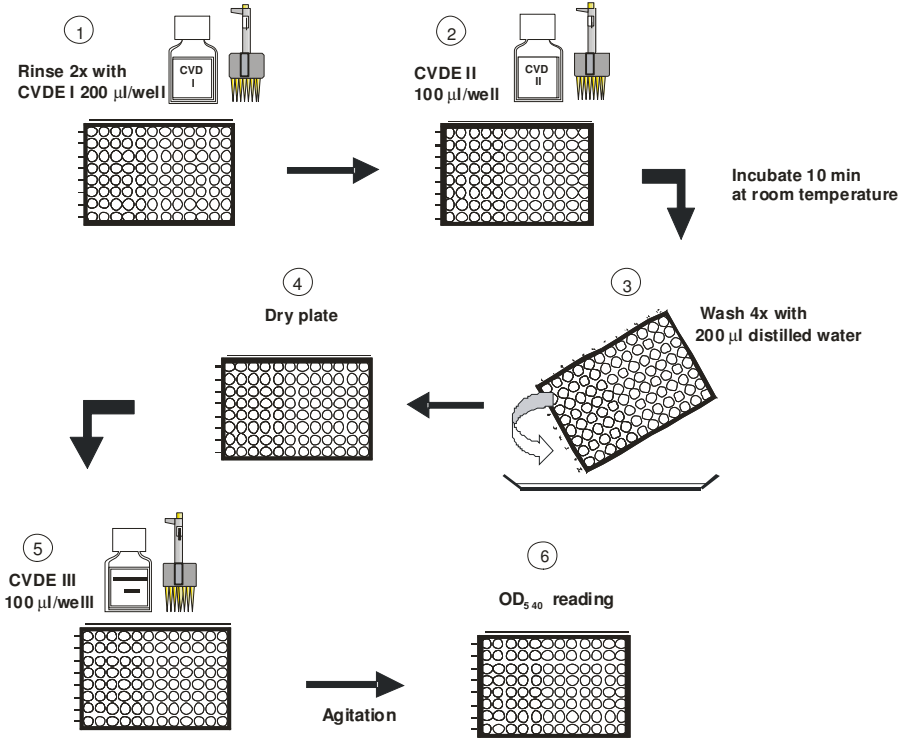
CVDE assay



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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 Isophorone on HepG2 cells measured with the NR test. IC_{50} is about 0.05 %.

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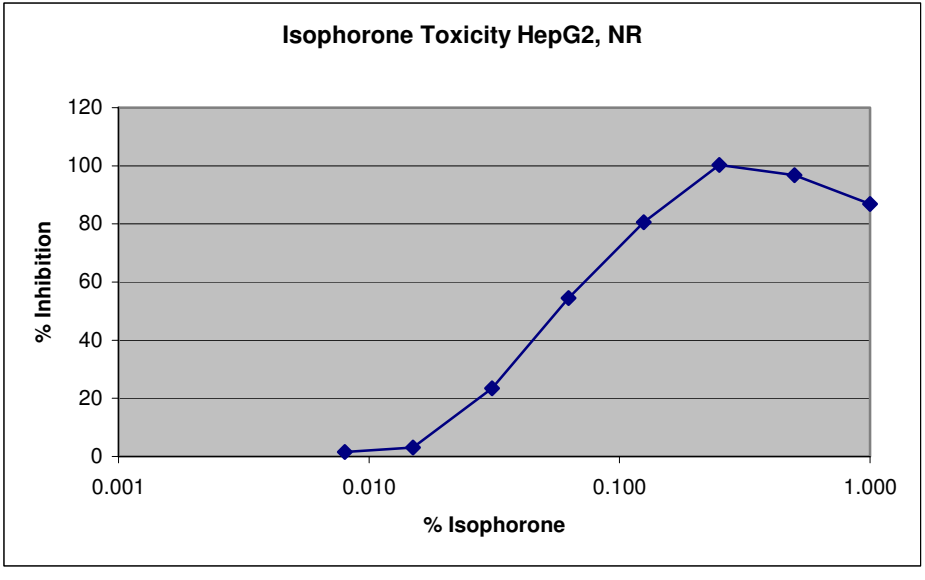
Example:

	Blank	GC	SC	0.008	0.015	0.031	0.063	0.125	0.250	0.500	1.000
	0.358	1.700	1.680	1.660	1.639	1.370	0.960	0.614	0.355	0.401	0.532
Corrected	0.000	1.342	1.322	1.302	1.281	1.012	0.601	0.256	-0.004	0.043	0.174
% Inhibition		-1.490	0.000	1.513	3.096	23.430	54.503	80.641	100.269	96.779	86.816
%inh/(100%-%inh)			0.000	0.015	0.032	0.306	1.198	4.166	-373.380	30.044	6.585
log (%inh/(100%-%inh)			#ZAHL!	-1.814	-1.495	-0.514	0.078	0.620	#ZAHL!	1.478	0.819



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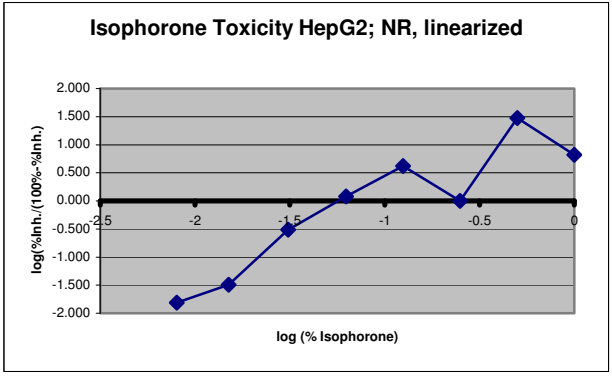
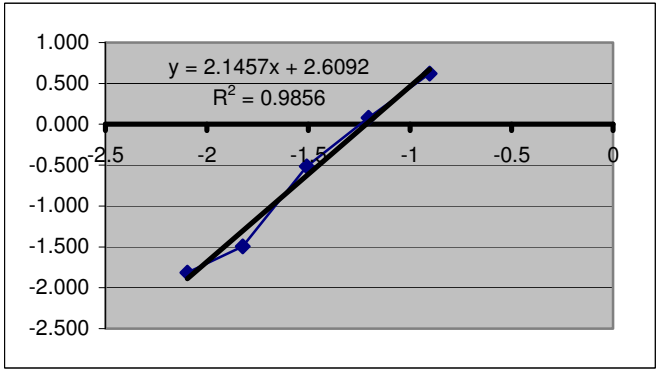
Alternatively you can plot:

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 can be used to obtain a more precise estimation of the IC_{50}. In this case (y=0): (log IC_{50}) = (-2.6092 / 2.1457) = -1.21, giving an IC_{50} of 0.06%.





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

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