XENOMETRIX
by Endotell

ANIARA
Manufactured By: Xenometrix AG

Instructions for use

In Cytotox - PAN I

4 - Parameter Cytotoxicity Kit

Extracellular Lactate Dehydrogenase LDHe	-	Membrane Integrity
Tetrazolium XTT	-	Mitochondrial Activity
Neutral Red NR	-	Lysosomal Activity
Sulforhodamine B SRB	-	Total Protein Content

Art. No. APAN I 96.300
Art. No. APAN I 96.310
Art. No. APAN I 96.1200
Art. No. APAN I 96.1210

For research use only

Version 2.11 10/2011

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Phone: 513.770.1991 Toll Free: 866.783.3797 Fax: 513.573.9241 Email: info@aniara.com
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Kit contents APAN I 96.300/310

LDH I	Reconstitution Solution	1 x 90 ml
LDH II	NADH	1 x 65 ml (after reconstitution)
LDH III	Pyruvate	1 x 15 ml (after reconstitution)
XTT I	Substrate	1 x 16.5 ml
XTT II	Buffer	1 x 0.165 ml
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
SRB I	Wash solution	1 x 100 ml
SRB II	Fixing solution	1 x 33 ml
SRB III	Labeling solution	1 x 16.5 ml
SRB IV	Rinsing solution	1 x 250 ml
SRB V	Solubilization solution	1 x 75 ml

1 Instruction manual

30 sterile reagent reservoirs (APAN I 96.310 only)
8 96-well microplates (APAN I 96.310 only)

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Kit contents APAN I 96.1200/1210

LDH I	Reconstitution Solution	2 x 180 ml
LDH II	NADH	2 x 130 ml (after reconstitution)
LDH III	Pyruvate	1 x 60 ml (after reconstitution)
XTT I	Substrate	1 x 66 ml
XTT II	Buffer	1 x 0.66 ml
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
SRB I	Wash solution	1 x 400 ml
SRB II	Fixing solution	1 x 132 ml
SRB III	Labeling solution	1 x 66 ml
SRB IV	Rinsing solution	2 x 500 ml
SRB V	Solubilization solution	1 x 300 ml

1 Instruction manual

120 sterile reagent reservoirs (APAN I 96.1210 only)
32 96-well microplates (APAN I 96.1210 only)

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Material required but not provided:

Test cells
Culture medium
Sterile water
Phosphate-buffered saline PBS
Triton X-100

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

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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.

LDH:

Upon arrival

LDH I and LDH III should be stored at 4°C
LDH II should be stored at -20°C until use.

LDH II (NADH) should be white to lightly yellow. It should not be used if it has turned into an intense yellow color. NADH is subject to photodegradation. Excessive exposure to light may reduce accuracy and sensitivity.

Storage after reconstitution

Reconstituted LDH II (NADH) solution may be stored refrigerated (4°C) and protected from light for not more than 1 day. Storage for several months is possible in a ultra-low freezer (< -40°C). Solutions of pyruvate (LDH III) may be stored at –20°C or below for up to 2 months.

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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.

NR:

Upon arrival, all NR reagents should be stored at 4°C.

SRB:

SRB I, SRB IV and SRB V can be stored at RT or at 4°C. SRB II and SRB III should be stored at 4°C.

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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 LDHe and XTT assays.
- We recommend to use medium with serum (≤5%) for this assay. If this is not feasible, we recommend to add of serum (1% final) or BSA (0.1% final) to the wells just prior to the transfer of the supernatant for the LDHe assay.
- High concentrations of FCS should be avoided as it can contain LDH and contribute to elevated background activity.
- 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
- In the NR assay, 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.

Additional information on individual kit components:

LDH: NADH (LDH II) is toxic. In case of contact of eyes with NADH solution, immediately flush eyes with copious amounts of water and consult a physician.

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

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.

SRB: Fixing Solution SRB II may be harmful if swallowed, inhaled or absorbed through skin. Fixing Solution SRB II can cause severe chemical burns. In case of contact of eyes with SRB II, SRB III, SRB IV or SRB V 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:

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

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

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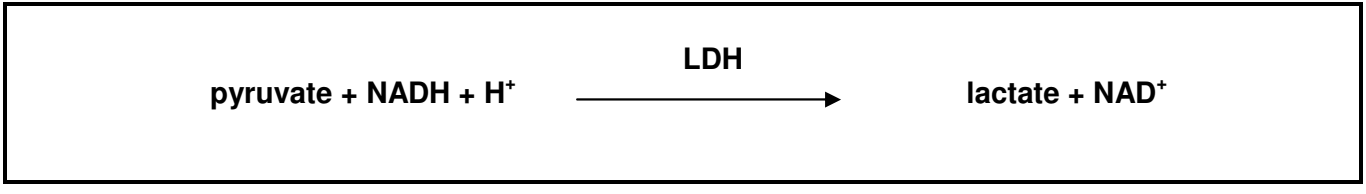
Extracellular LDH test principle

Cell death or cytotoxicity is classically evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells, and is rapidly released into the cell culture supernatant upon membrane damage or cell lysis. This assay is a fast and simple method to determine changes in the plasma membrane upon incubation with a test compound. The assay is carried out with an aliquot of the supernatant and leaves the cells undisturbed and they may therefore be used for further testing.

This version of the LDH test is unaffected by pyruvate present in the medium.

LDH reduces pyruvate to lactate by oxidizing NADH to NAD^+:



The consumption of NADH is measured spectrophotometrically by a decrease in the OD at 340 nm.

Determinations of LDHe can be performed with the same cells at several time points of test compound exposure.

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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.

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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.

SRB test principle

Cell proliferation, measured as total protein synthesis, is a very sensitive toxicology marker. Sulforhodamine B (SRB, Acid Red 52) is an anionic dye that binds electrostatically to cellular proteins. The fixed dye is solubilized and is measured photometrically at OD 540 nm with a reference filter of 690 nm. The OD values correlate with total protein content and therefore with cell number.

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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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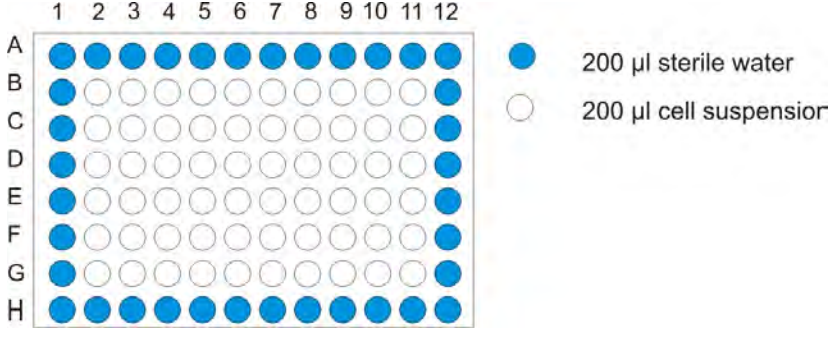
- Dispense 200 µl of the sterile water in each well of rows A and H, and in each well of columns 1 and 12 (see figure), to reduce culture medium evaporation during incubation. (Erroneous results may be obtained upon uneven evaporation of culture fluid.)
- Pour the cell suspension into the same sterile 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 and the total LDH 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).
- Prepare 700 µl of culture medium containing 1% Triton X-100 for the total LDH control (TL)

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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 and D2 (cell growth control = GC).
- Add 200 µl of culture medium with 1% Triton X-100 to wells E11, F11 and G11 (total LDH control =TL)
- Add 200 µl of culture medium – solvent mixture (page 16) 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. Several LDHe measurements can be performed at different time points of compound exposure.

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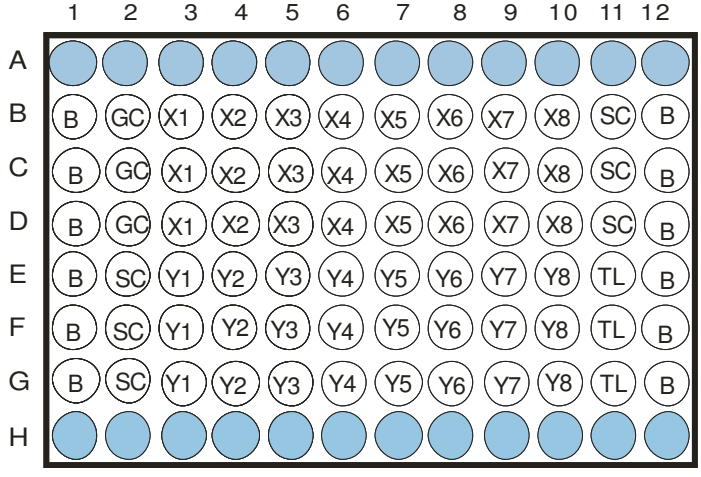
Recommended 96-well plate configuration



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)
TL: culture medium + cells + 1% Triton X-100 (total LDH content)
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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Extracellular LDH Test Procedure

Caution: the contents of LDH II and LDH III may stick to the walls of the bottles. Add the required volume of LDH I and invert the bottles until the contents are completely dissolved. Immediately before use, reconstitute LDH II and LDH III as follows:

Small kit (APAN I 96.300 and APAN I 96.310) with 65 ml and 15 ml, respectively, of LDH I reconstitution solution.

This provides enough solution for 4 plates.

Large kit: (APAN I 96.1200 and APAN I 96.1210) with 130 ml and 60 ml, respectively, of LDH I reconstitution solution. This provides enough solution for 8 (LDH II) and 16 (LDH III) plates. Reconstitute the second LDH II bottle only when needed (more than 8 plates to be tested).

If fewer plates are used at a time, the LDH II (NADH) solution may be stored in a ultra-low freezer (<40°C). Solutions of NADH may be kept refrigerated and protected from light for not more than 1 day. Solutions of pyruvate (LDH III) may be stored at –20°C or below for up to 2 months.

It is recommended to pre-heat the LDH I, II, III solutions at 37°C in a water bath before use

- Remove the 96-well plate from the incubator
- With a multichannel pipettor, transfer 20 µl of the supernatant from each well (rows B to G) to a new 96-well plate duplicating the previous plate configuration. Return the original plate to the incubator for the other tests.

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- Mix 16 ml LDH II with 3.4 ml LDH III (for 1 plate, 72 wells).
- Add 240 µl/well of the LDH II / LDH III mix. This starts the reaction.
- Start immediately to read kinetically at 340 nm for 25 min at 37°C. Readings can also be done at RT, with an extended incubation time of 1 hr.

Air bubbles interfere with the measurement and must be removed prior to reading, e.g. by quickly moving with a gas flame over the wells; (CAUTION!). A hair dryer can be used for the same purpose.

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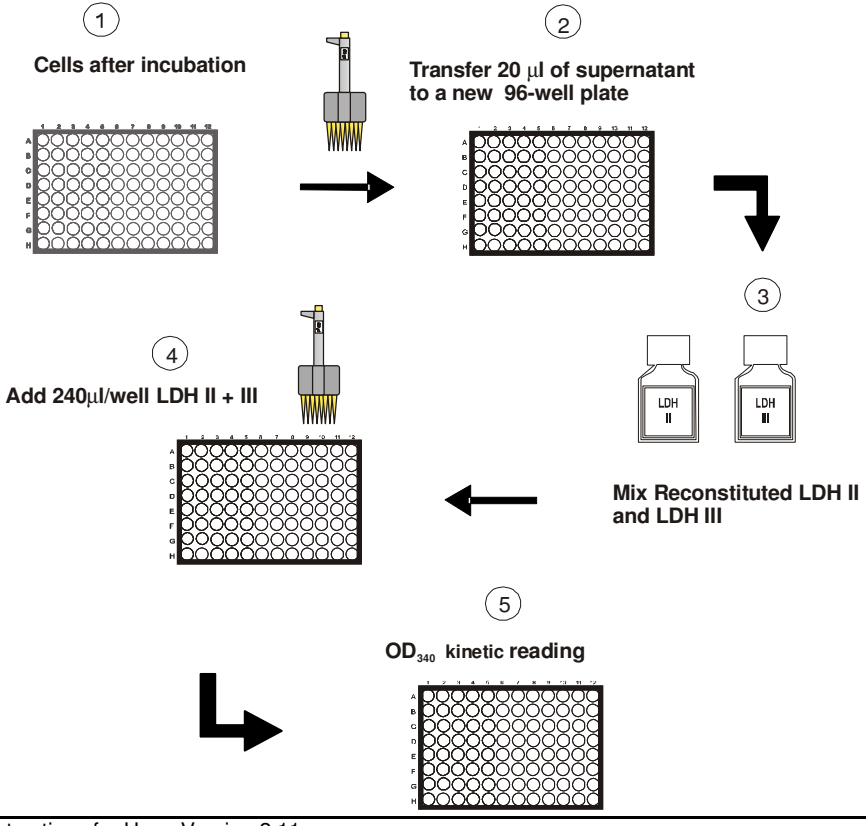
LDHE assay



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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.

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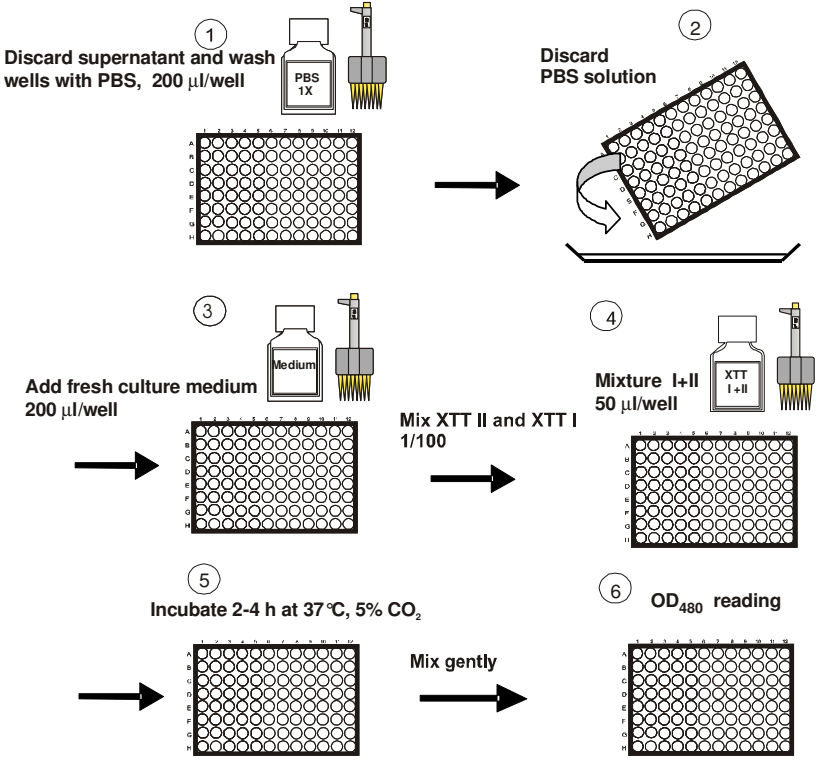
XTT assay



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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 20) 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.

- Discard the XTT staining solution.
- 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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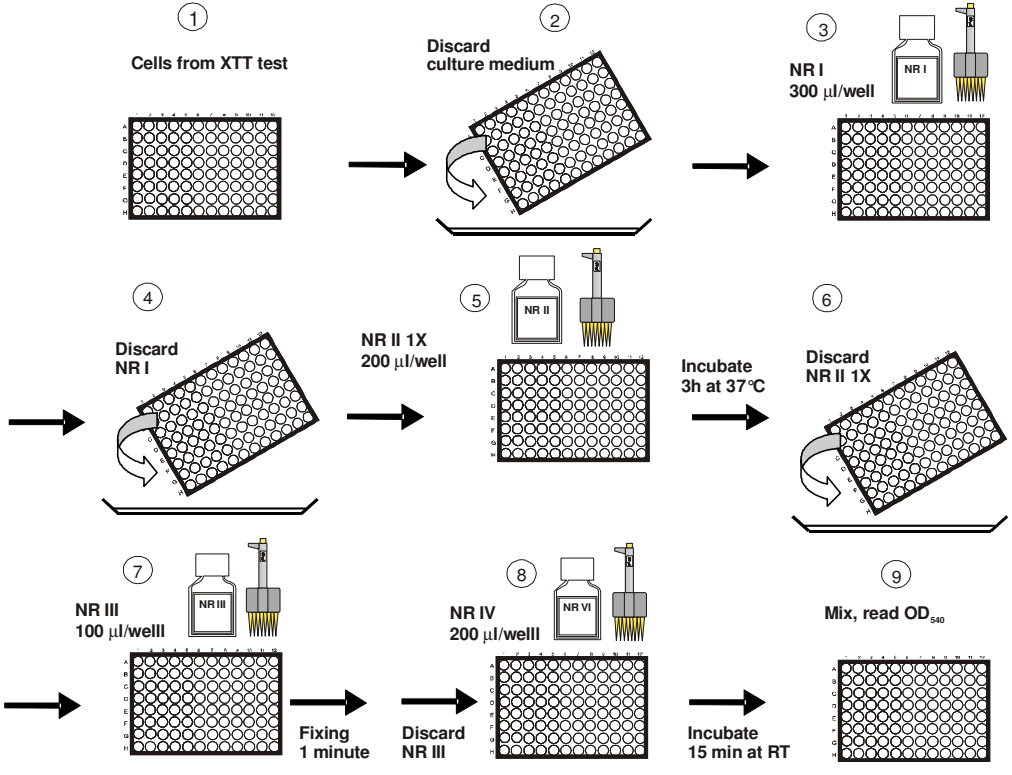
NR assay



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SRB Test Procedure

- Remove the NR IV solution carefully, with the cells remaining attached to the bottom of the wells.
- Wash the cells with 300 µl/well wash solution SRB I.
- Add 100 µl fixing solution SRB II to each well.
- Incubate the plate for 1hour at 4°C.
- Wash the cells 3 times with 200 µl/well distilled or de-ionized water (not provided).
- Add 50 µl/well of labeling solution SRB III. Make sure bottom of wells is completely covered with dye.
- Incubate the plate for 15 minutes at room temperature.
- Wash 2 times with 400 µl/well rinsing solution SRB IV. Note: The wells should be completely filled with rinsing solution. Proceed quickly to avoid leaching of the fixed dye into the rinsing solution.
- After being washed, the cultures are air dried until no moisture is visible. At this stage the plate may be stored for many weeks.
- Dissolve the cells with 200 µl/well solubilization solution SRB V.
- Incubate for 15 to 60 minutes at room temperature.
- Mix gently using a multichannel pipette.

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- Read the OD at 540 nm. If intense color (> 1.8) is observed, a suboptimal wavelength (490-530nm) may be used instead. Measure the background absorbance with a reference filter at 690 nm.

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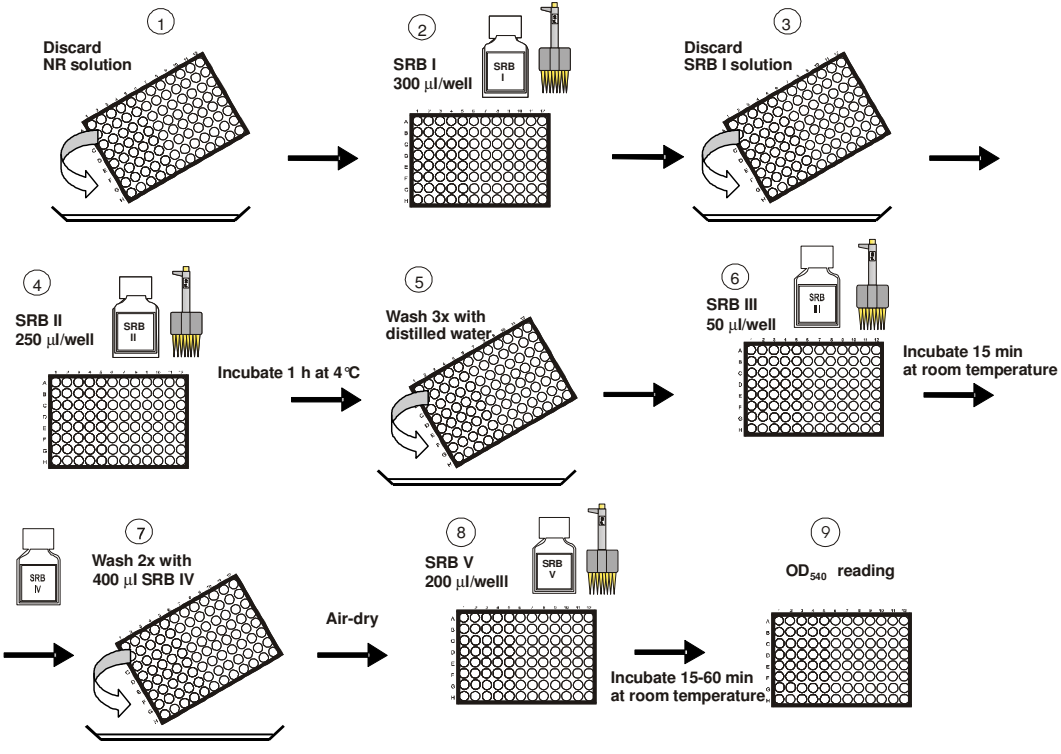
SRB assay



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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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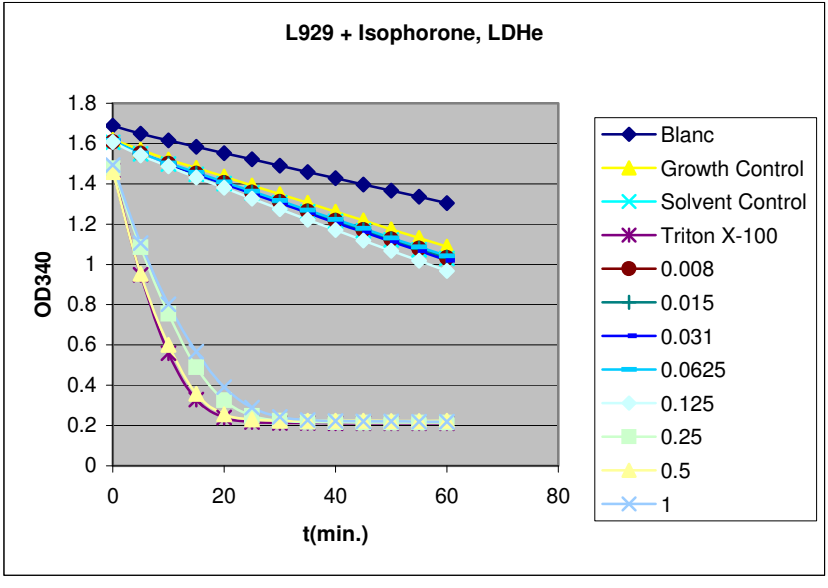
Data analysis of the LDH assay

After the kinetic reading, plot OD_{340} vs. time (min.). This allows you to see the activities of test compounds relative to the B, SC, GC, and TL controls.



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This graph shows the titration of Isophorone on L929 fibroblasts. It can be seen that there is a decrease of OD_{340} even in the Blank control, due to LDH present in the FCS. Growth and Solvent Controls are a bit more active than the Blank probably due to the presence of LDH from the cultured cells. The Triton X-100 control gives the maximum activity after total lysis.

The highest concentration (1%) of Isophorone shows an apparent decrease of LDHe activity. This is possibly due to interference with the assay such as inhibition of the released LDH.

Inspect your graph and identify a time interval where the curves are almost linear. In the above example we would choose the interval from 0 to 10 min.

Determine the ΔOD/min for each well. Calculate the mean ΔOD/min for each test compound concentration. Extracellular LDH is expressed in Units as " nanomoles NADH consumed / min / ml ". Calculate as follows:

$$\text{NADH consumption} = \frac{\Delta OD/min \times 0.260 \times 1000}{6.2 \times 20}$$

0.260 ml:	reaction volume in ml
1000 µl:	allows to calculate the result in ml
6.2:	millimolar extinction coefficient of NADH at 340 nm
20 µl:	volume taken for the assay

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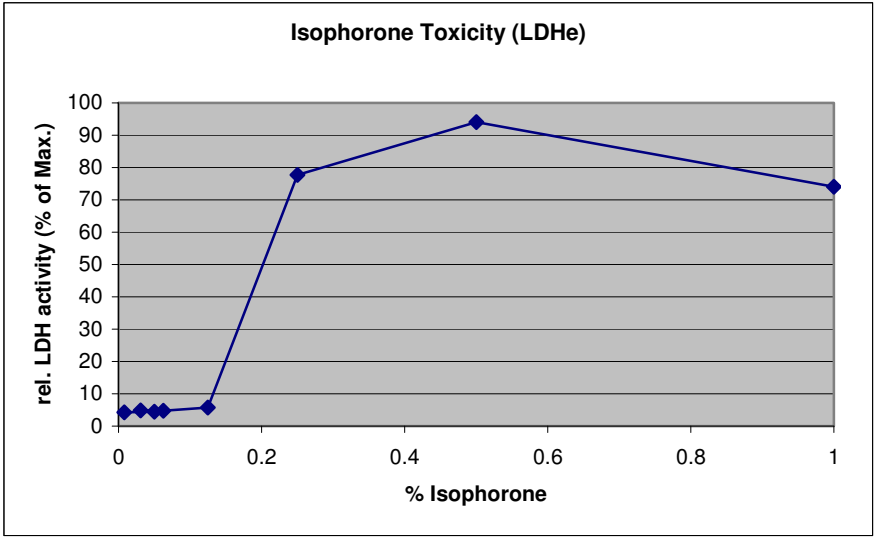
For the above example this gives

	Blank	GC	SC	0.008	0.015	0.031	0.0625	0.125	0.25	0.5	1	Triton X-100
t0 - t10	0.073	0.098	0.109	0.108	0.110	0.113	0.113	0.121	0.722	0.858	0.691	0.907
t0 - t10/min	0.007	0.010	0.011	0.011	0.011	0.011	0.011	0.012	0.072	0.086	0.069	0.091
t0 - t10/min - blank		0.003	0.004	0.004	0.004	0.004	0.004	0.005	0.065	0.079	0.062	0.083
Units NADH consumed (nMol/min/ml)		0.006	0.009	0.008	0.009	0.009	0.009	0.011	0.151	0.183	0.144	0.194
% of Max. (Triton X-100)		3	4	4	4	5	5	6	78	94	74	100

A graphic representation allows to determine the concentration of test compound giving an inhibition of 50% (IC_{50}): For Isophorone on L929 cells this is 0.2%.



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Data Analysis of the XTT, NR, and SRB Assays

For each well subtract the OD_{690} values from the OD_{480} or 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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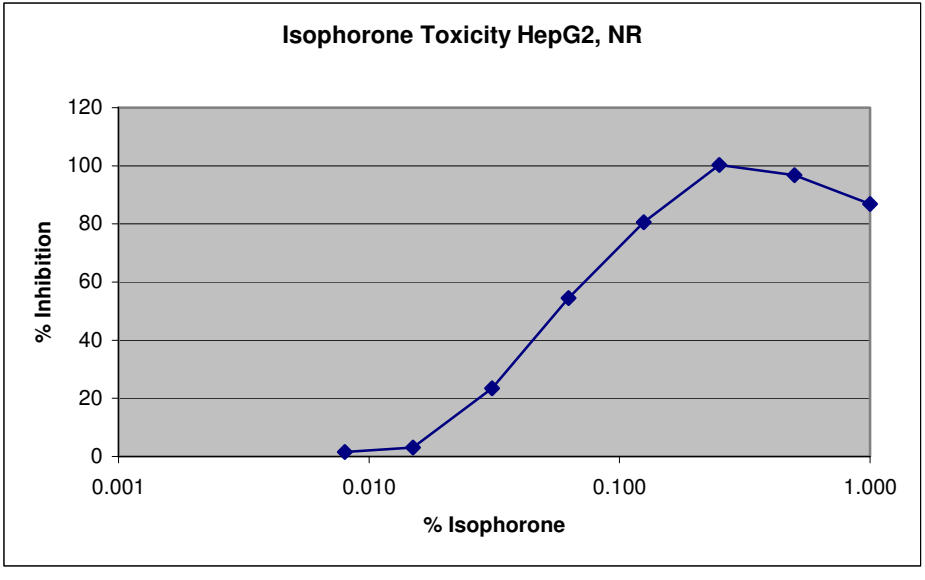
Example:

	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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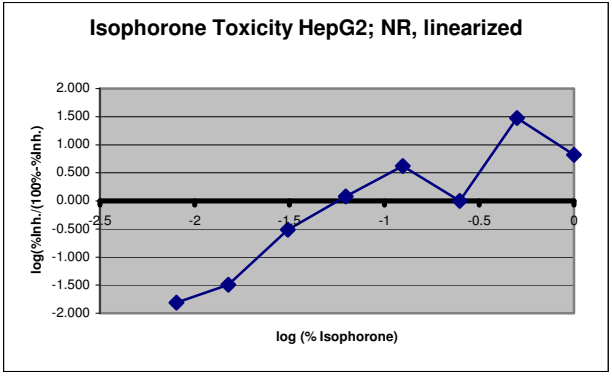
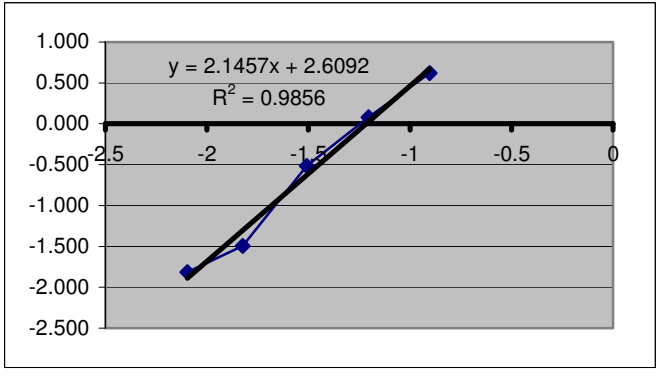
Alternatively you can plot:

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%.





The same approach can be used to calculate IC_{50} values from the LDHe assay by using the "% of Maximum" values in place of the "% Inhibition" values.

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

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