

PEPCY PRACTICAL GUIDELINES

TITLE: Cytotoxicity test with intestinal and/ or hepatic cell lines.

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1. PURPOSE

Description of the procedure for planning, preparing and execution of a combined cytotoxicity test system using intestinal and/or hepatic cell lines examining the endpoints lactate dehydrogenase (LDH) release and 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction.

2. INTRODUCTION

The first endpoint (LDH) detects the release of LDH from damaged cells into the medium by measuring the LDH activity present in the supernatant. Thus, potential damage to the plasma membrane can be examined as an indicator for cytotoxicity. LDH catalyses the NAD^+ - dependent reaction from lactate to pyruvate. In a second step, the enzyme diaphorase transfers H/H^+ from NADH/H^+ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride), which is reduced to formazan (red) and can be measured photometrically.

In the second end point, MTT enters cells via diffusion and is reduced to an alcohol soluble dark blue formazan¹, for which cell membranes are largely impermeable. This reaction is catalysed by NADH-dependent enzymes of the endoplasmatic reticulum and - to a lesser extent - by mitochondrial succinate dehydrogenase. Solubilisation of cells by addition of a detergent results in the release of the solubilised formazan. The number of surviving cells is directly proportional to the concentration of formazan.

3. REQUIREMENTS

Materials

Cytotoxicity Detection Kit (LDH) (Cat. No. 1644793, Roche), 2000 tests
Cell lines HEP-G2 (DSM ACC 180) and CACO2 (DSM ACC 169), e.g. provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany)
RPMI 1640 with L- Glutamine (PAA Laboratories, E15-840)
Fetal bovine serum, gold quality (PAA Laboratories, A15-649)
Trypsin/EDTA (PAA Laboratories, L11-004)
Trypan-blue (Sigma, T-6146)
MTT 5 mg/ml PBS (Sigma-Aldrich, Art.-Nr.: M-2128).
Solubilisation buffer: formic acid 5% (v/v), isopropanol 95% (v/v)
96-well plates, cell culture quality with flat bottom for adherent cells and optically clear flat-bottomed plates for colour development.

0.22 μM filter (e.g. Millex-GV, Millipore) or ultra free centrifugal filter units (e.g. Millipore UFC30GVOS)
PBS: NaCl 137 mM, KCl 2.7 mM, $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$ 8.1 mM, KH_2PO_4 1.5 mM;
pH: 7.4

Equipment

37° Celsius humidified incubator with standard atmosphere (95% air, 5% CO_2)
Microscope
Hemocytometer/Coulter counter
Clean bench
Plate centrifuge
Spectrophotometric microtiter plate reader
Multichannel pipette (100 μl) and associated tips (sterile)
Liquid nitrogen container for storage of cells

4. PROCEDURE

Culture medium

90% RPMI + 10% Fetal bovine serum (FBS), without antibiotics

Seeding/culturing of cells

If cells are delivered frozen, thaw them as fast as possible and initiate a culture with 10 ml RPMI (20% **FBS**) in 75 cm^2 cell culture flasks. After 3 days, wash cells with PBS and change medium from **20% to 10% FBS** with a washing step (PBS) in between. After 2 days, split the confluent culture 1:4 to 1:6 using trypsin/EDTA for detachment of cells.

Splitting:

After washing with PBS, add 3 ml trypsin/EDTA, incubate at 37° (CACO 2 for ~ 4 min, HEP-G2 for ~ 7 min), remove trypsin/EDTA until ~ 0.5 ml are left. As soon as the cells begin to peel away from the flask bottom, gently tap the flask until all cells are floating freely. Add 5 ml 10% FBS medium and make a single cell suspension by trituration with a 10 ml pipette. Put 1.5 ml of this suspension in new 75 cm^2 flasks and add 7.5 ml of RPMI (**10% FBS**). Distribute cells uniformly by moving flasks (follow an 8 during moving). The splitting ratio (in this example 1:6) depends on the cell density, which is determined by Hemacytometer and/or coulter counter.

Change the medium every third day and split again on day 7 (e.g. seeding Monday, medium change Wednesday and Friday, splitting on following Monday). Important: cells must not be post-confluent, which is an adverse condition for making a single cell suspension.

Exposure

Example: Testing cytotoxicity of substances with an initial concentration of 30 μM .

Substances: prepare solutions with concentrations of 900 μM in 10% MeOH, mix 405 μl RPMI (**1% FBS**) with 45 μl of the test substances (1:10) followed by sterile filtration either through 0.22 μM filters or through ultra free centrifugal filter units (sterile). The loss by filtration should be distinctly below

50µl by using those filters, so enough volume of the 90µM substances should remain for the assay. Alternatively, the peptides and extracts could be filtered before mixing with the sterile medium. However, to avoid loss of material, the sterile ultra free centrifugal filter units should be used. In case of extracts it is recommended to centrifuge the samples in a common vial and transfer only the supernatant to the sterile filter unit (to avoid clogging of the filter).

Trypsinise adherent cells in the exponential growth phase with trypsin/EDTA as outlined above and determine cell density and viability, e.g. with trypan blue (>95% viability). Seed cells with a density of $2-3 \times 10^5$ vital cells/ml in RPMI (10% FBS) in 96 well plates (200µl/well), but only in rows 2-12. After 3h (cells should be adherent now), decant medium and add 200 µl RPMI with 1% FBS per well in rows 2-11.

Add 100µl of the test substance in RPMI (1% FBS) to wells 3 A-D, 3 E-H, 8 A-D (8 E-H with 100µl RPMI (1% FBS) and the solvent control, see scheme 1), mix carefully, dilute the substance serially in 1:3 steps (e.g. 90µM to 30µM) by taking 100µl from row 3 and 8 to the following rows (4-6 and 9-11, respectively), after mixing remove 100µl from row 6 and 11. Add RPMI (1%FBS) with 1% Triton X100 to row 12 (positive control for LDH and MTT assay, 0.1 ml Triton X-100 / 10 ml RPMI + 1% FBS), cover plates with a lid and incubate them for the desired time period.

Scheme 1: example for planning a 96 well cytotoxicity test. Blank: no cells, CTRL1: cells with medium (negative control), CTRL2: cells with solvents (solvent control), CTRL3: cells with 1% Triton (positive control).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	CTRL1	S1 I	II	III	IV	CTRL1	S2 I	II	III	IV	CRTL3
B	Blank	CTRL1	S1 I	II	III	IV	CTRL1	S2 I	II	III	IV	CRTL3
C	Blank	CTRL1	S1 I	II	III	IV	CTRL1	S2 I	II	III	IV	CRTL3
D	Blank	CTRL1	S1 I	II	III	IV	CTRL1	S2 I	II	III	IV	CRTL3
E	Blank	CTRL1	S3 I	II	III	IV	CTRL1	CTRL2 I	II	III	IV	CRTL3
F	Blank	CTRL1	S3 I	II	III	IV	CTRL1	CTRL2 I	II	III	IV	CRTL3
G	Blank	CTRL1	S3 I	II	III	IV	CTRL1	CTRL2 I	II	III	IV	CRTL3
H	Blank	CTRL1	S3 I	II	III	IV	CTRL1	CTRL2 I	II	III	IV	CRTL3

Cytotoxicity tests

Procedure

LDH

After 20, 48 or 72h in the incubator transfer 150 µl of the medium into a rounded bottom 96-well plate and centrifuge for 10 min at 250 x g.

Mix bottle 1 and bottle 2 of the LDH kit 1:45. A final volume of 9.6 ml/ plate is needed, but preparation of at least 1 ml extra (e.g. for 1 plate 10.5 ml, for 3 plates 30 ml) is recommended.

Transfer 100µl from the centrifuged round bottom plates to flat-bottom 96-well plates. Add 100µl of LDH kit mix to each well, vortex carefully and store plate in a dark place. Measure plate at app. 490 nm against a reference wavelength of >600 nm after 9 min. If absorption is too low, repeat measurement after 5-10 minutes.

MTT

Add 50µl RPMI (1% FBS) to the incubated cells, add 10µl of the MTT - solution and incubate plate for 1.5h at 37°.

Remove (decant) supernatant, empty plate carefully on tissue paper and add 100µl of the solubilisation solution, vortex carefully for 5-10 minutes and measure at 550 nm without reference filter.

Calculation of viability

LDH (see also Scheme 2)

1. All values minus blank
2. Values of samples 1-3, CTRL2, CTRL3 minus CTRL1 (see **Scheme 1**)
3. Values of samples I-IV minus the respective solvent control I-IV (CTRL2) (e.g. sample 2 III minus CTRL2 III)
4. CTRL3 = 100% LDH release, calculation of % LDH release
5. 100% minus calculated LDH release to show viability

Scheme 2: LDH test results after exposure with 30,10, 3.3 and 1.1 µM of substances I-III (example); the values are an example for one of the four values per substance and dilution (see scheme 1). Standard deviation is calculated from the four values of % vitality (only one value is shown in the scheme); the calculation shown in this scheme must be carried out for each of the four wells to allow the calculation of the standard deviation. Blank: no cells, CTRL1: cells with medium (neg. control), CTRL2: cells with solvents (solvent control), CTRL3: cells with 1% Triton (pos. control), S1-S3: three test substances, each in four dilutions I-IV.

toxin [μM]		30	10	3.3	1.1	30	10	3.3	1.1	
raw data	Blank CTRL1	S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
	0.1 0.3	1.4	0.8	0.5	0.4	0.8	0.4	0.4	0.3	1.5
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		1.5	1.4	1.4	0.5	0.35	0.3	0.3	0.3	
minus blank	CTRL1	S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
	0.2	1.3	0.7	0.4	0.3	0.7	0.3	0.3	0.2	1.4
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		1.4	1.3	1.3	0.4	0.25	0.2	0.2	0.2	
minus CTRL1		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
		1.1	0.5	0.2	0.1	0.5	0.1	0.1	0	1.2
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		1.2	1.1	1.1	0.2	0.05	0	0	0	
minus CTRL2		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
		1.05	0.5	0.2	0.1	0.45	0.1	0.1	0	1.2
		S3 I	S3 II	S3 III	S3 IV					
		1.15	1.1	1.1	0.2					
% CTRL3		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
		87.5	41.7	16.7	8.3	37.5	8.3	8.3	0	100
		S3 I	S3 II	S3 III	S3 IV					
		95.8	91.7	91.7	16.7					
% viability		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
		12.5	58.3	83.3	91.7	62.5	91.7	91.7	100.0	100
		S3 I	S3 II	S3 III	S3 IV					
		4.2	8.3	8.3	83.3					

MTT-assay (see also Scheme 3)

1. All values minus blank
2. Values of samples 1-3, CTRL1, CTRL2 minus CTRL3 (see scheme 1)
3. The respective solvent control I-IV (CTRL2) =100% MTT reduction (100% viability)

Attention: viability of solvent control and medium control has to be in the same range. If the viability of solvent control is distinctly lower compared to the medium control, the test must be repeated with lower solvent concentrations.

Scheme 3: MTT test results after exposure with 30,10, 3.3 and 1.1 μM of substances I-III (example). Standard deviation is calculated from the four values of % vitality, the calculation (here shown for one value only) must be carried out for each of the four wells per substance and dilution.

Blank: no cells, CTRL1: cells with medium (neg. control), CTRL2: cells with solvents (solvent control), CTRL3: cells with 1% Triton (pos. control), S1-S3: three substances, I-IV

toxin [μM]		30	10	3.3	1.1	30	10	3.3	1.1	
raw data	Blank CTRL1	S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
	0.1 1	0.1456	0.6	0.9	0.9	0.413	0.85	0.9	0.8	0.11
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		0.123	0.114	0.35	0.96	0.9	0.91	0.89	0.98	
minus blank	CTRL1	S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	CTRL3
	0.9	0.0456	0.5	0.8	0.8	0.313	0.75	0.8	0.7	0.01
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		0.023	0.014	0.25	0.86	0.8	0.81	0.79	0.88	
minus CTRL3		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	
		0.0356	0.49	0.79	0.79	0.303	0.74	0.79	0.69	
		S3 I	S3 II	S3 III	S3 IV	CTRL2 I	CTRL2 II	CTRL2 III	CTRL2 IV	
		0.013	0.004	0.24	0.85	0.79	0.8	0.78	0.87	
viability		S1 I	S1 II	S1 III	S1 IV	S2 I	S2 II	S2 III	S2 IV	
		4.5	61.3	101.3	90.8	38.4	92.5	101.3	79.3	
		S3 I	S3 II	S3 III	S3 IV					
		1.6	0.5	30.8	97.7					

Calculation of EC₅₀-value

For calculation of the EC₅₀-value, three independent assays are necessary. In this example, the mean value of each assay is the result of 4 individual values i.e. the final mean value is the result of three mean values i.e. 12 single values. The mean values of the three single assays are the data basis for calculation of

the final mean value (see **scheme 4** for the LDH example and **scheme 5** for the MTT example).

Scheme 4: LDH cytotoxicity test; three replicates for each test substance (S1, S2, S3), the values of assay 1 are the result of the LDH test shown in **Scheme 2**. In **Figure 1** the data of Scheme 4 are shown graphically.

toxin [μM]	30	10	3.3	1.1	30	10	3.3	1.1
	single mean values of the quadruplicates				mean of the three single mean values			
	S1 I	S1 II	S1 III	S1 IV	S1 I	S1 II	S1 III	S1 IV
assay1	12.5	58.3	83.3	91.7	7.8	57.9	85.2	95.3
assay2	2.4	42.0	90.3	90.0	STDEV	STDEV	STDEV	STDEV
assay3	8.4	73.4	82.0	104.2	5.1	15.7	4.5	7.8
	S2 I	S2 II	S2 III	S2 IV	S2 I	S2 II	S2 III	S2 IV
assay1	62.5	91.7	91.7	100.0	48.5	96.7	92.1	100.2
assay2	40.3	97.3	102.4	112.2	STDEV	STDEV	STDEV	STDEV
assay3	42.7	101.0	82.3	88.4	12.2	4.7	10.1	11.9
	S3 I	S3 II	S3 III	S3 IV	S3 I	S3 II	S3 III	S3 IV
assay1	4.2	8.3	8.3	83.3	1.9	9.5	7.0	92.5
assay2	0.2	2.1	5.5	105.3	STDEV	STDEV	STDEV	STDEV
assay3	1.4	18.1	7.2	88.9	2.0	8.1	1.4	11.4

Scheme 5: MTT cytotoxicity test; three replicates for each test substance (S1, S2, S3), the values of assay 1 are the result of the LDH test shown in **Scheme 3**. In **Figure 2** the data of Scheme 4 are shown graphically.

toxin [μM]	30	10	3.3	1.1	30	10	3.3	1.1
	single mean values of the quadruplicates				mean of the three single mean values			
	S1 I	S1 II	S1 III	S1 IV	S1 I	S1 II	S1 III	S1 IV
assay1	4.5	61.3	101.3	90.8	2.6	48.0	105.6	104.9
assay2	2.0	32.4	110.0	120.4	STDEV	STDEV	STDEV	STDEV
assay3	1.2	50.3	105.4	103.4	1.7	14.6	4.4	14.9
	S2 I	S2 II	S2 III	S2 IV	S2 I	S2 II	S2 III	S2 IV
assay1	38.4	92.5	101.3	79.3	44.9	91.4	102.6	97.4
assay2	46	82.3	92.5	110.3	STDEV	STDEV	STDEV	STDEV
assay3	50.4	99.4	113.9	102.5	6.1	8.6	10.8	16.1
	S3 I	S3 II	S3 III	S3 IV	S3 I	S3 II	S3 III	S3 IV
assay1	1.6	0.5	30.8	97.7	2.1	1.6	17.5	98.0
assay2	0.2	1.4	9.2	109	STDEV	STDEV	STDEV	STDEV
assay3	4.6	2.9	12.4	87.4	2.2	1.2	11.6	10.8

ATTENTION

For calculation of a reliable EC_{50} value, more than four concentrations are necessary to confirm the tendency of the dose-response curve resulting from the four dilutions of the investigated substances in the example presented here. However, the EC_{50} value calculated from four dilutions gives an idea, in which concentration range the EC_{50} value is to be expected and thus, allows the planning of further experiments (see **Figure 1 + 2**).

Figure 1: Results of the LDH assays including the EC₅₀ values calculated using GraphPad Prism software.

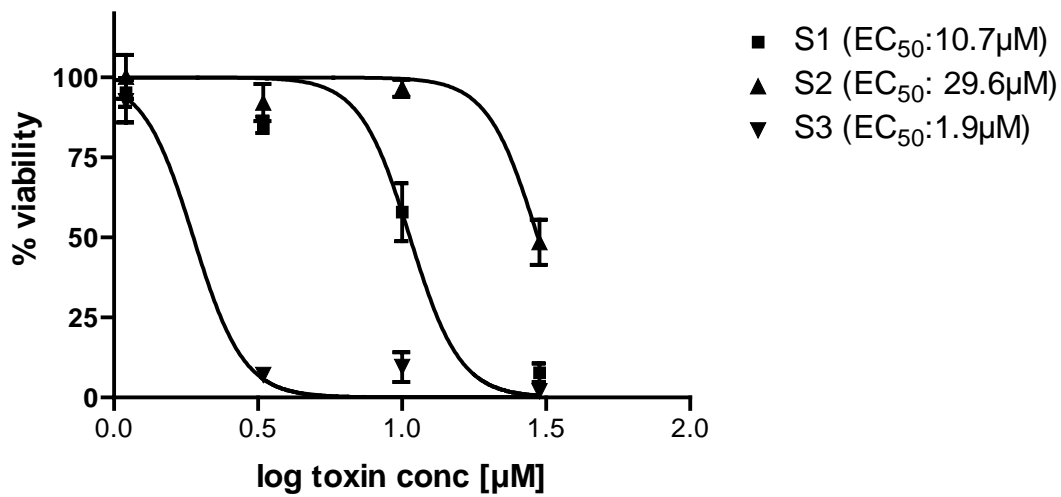
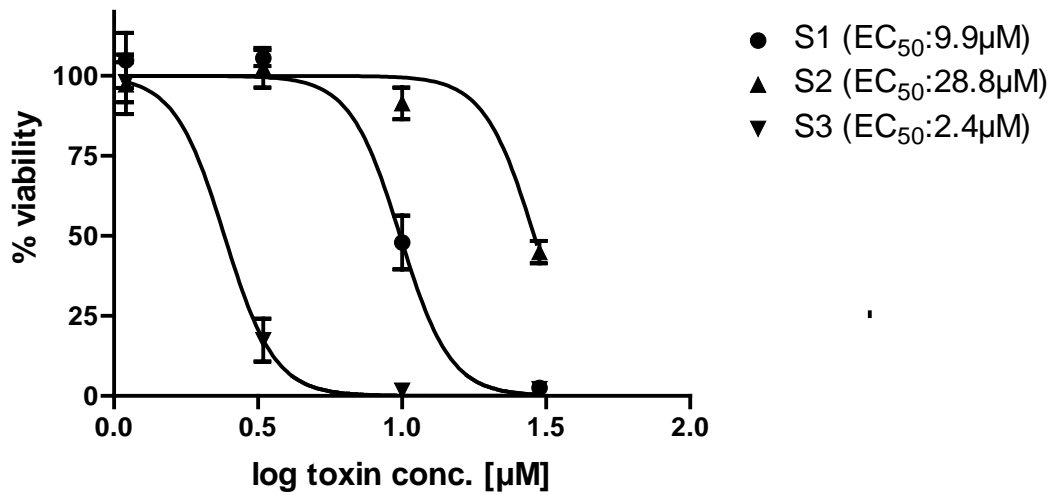


Figure 2: Results of the MTT assays including the EC₅₀ values calculated using GraphPad Prism software.



5. References

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63