For life science research only. Not for use in diagnostic procedures.
FOR *IN VITRO* USE ONLY.

Cytotoxicity Detection Kit (LDH)

A non-radioactive alternative to the [^{3}H]-thymidine release assay and the [^{51}Cr]-release assay.

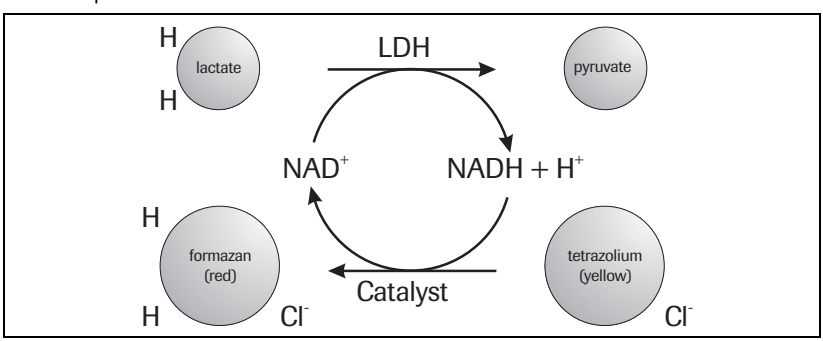
Colorimetric assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant

Cat. No. 1 644 793 Store at –15 to –25°C
1 Kit (2000 tests)

Instruction Manual

Version 6, May 2004

Test Principle



Roche

Contents

Kit contents

Bottle 1, blue cap, Catalyst, Diaphorase/NAD^+ mixture, lyophilizate, stabilized, (5 ×).
Bottle 2, red cap, Dye solution, containing iodotetrazolium chloride (INT) and sodium lactate, 45 ml (5 ×)

Stability The kit should be stored at −15 to −25°C. The lyophilizate (bottle 1) is stable at 2-8°C. The reconstituted catalyst solution is stable for several weeks when stored at 2-8°C. Once thawed, the dye solution (bottle 2) is stable for several weeks when stored at 2-8°C.

Advantages of the Cytotoxicity Detection Kit (LDH)

- **Safe** · no radioactive isotopes are used
- **Accurate** · results obtained strongly correlate to lysed cell number (see fig. 3)
- **Sensitive** · low cell numbers are detected (see fig. 3)
- **Fast** · the use of a multiwell-ELISA reader allows a large number of samples to be processed simultaneously
- **Convenient** · no prelabeling and washing steps are required
  · no disposal and radiation safety paperwork
- **Function tested** · every lot is function-tested in comparison to a master lot

1. Introduction

Cell death is classically evaluated by the quantification of plasma membrane damage. The need for sensitive, quantitative, reliable and automated methods for the precise determination of cell death led to the development of several standard assays for the quantification of cellular viability.

Widely used standard methods are based on the uptake or exclusion of vital dyes like trypan blue, eosin Y, nigrosine, propidium iodide or ethidium bromide (1-4). Dead and viable cells are discriminated by differential staining and counted using a light or fluor-escence microscope. These methods are troublesome, do not allow the processing of large sample numbers and do not account for dead cells which may have lysed. Thus, the actual rate of cell death in long term cultures can be underestimated.

A second group of standard assays is based on the release of radioactive isotopes like [^{51}Cr], [^{3}H]-thymidine, [^{3}H]-proline, [^{75}Se]-methionine, [^{125}J]-5-iodo-2-deoxyuridine or fluorescence dyes like bis-carboxyethyl-carboxyfluorescein (BCECF) or calcein-AM from prelabeled target cells (5-8). The disadvantages of these assays are (i) the use of radioactive isotopes in most of them, (ii) the necessity for prelabeling of the target cells and (iii) the high spontaneous release of most labels from the prelabeled target cells.

A third type of assay is based on the measurement of cytoplasmic enzyme activity released by damaged cells. The amount of enzyme activity detected in the culture supernatant correlates to the proportion of lysed cells (9-12). Enzyme release assays have been described for alkaline and acid phosphatase, for glutamate-oxalacetate transaminase, for glutamate pyruvate transaminase and for arginosuccinate lyase. However, their use has been hampered by the low amount of those enzymes present in many cells and by the elaborate kinetic assays required to quantitate most enzyme activities.

In contrast to the above mentioned cytoplasmic enzymes, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. With the use of the Cytotoxicity Detection Kit, LDH activity can easily be measured in culture supernatants by a single measurement at one time point. The use of a spectrophotometric microplate reader (ELISA reader) allows the simultaneous measurement of multiple probes and thereby guarantees the easy processing of a large number of samples.

2. Applications

The Cytotoxicity Detection Kit is designed as a precise, fast and simple colorimetric alternative to quantitate cytotoxicity/cytolysis based on the measurement of LDH activity released from damaged cells. Thus, the Cytotoxicity Detection Kit can be used in many different in vitro cell systems when damage to the plasma membrane occurs.

Examples are:

- Detection and quantification of cell mediated cytotoxicity induced by cytotoxic T-lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer (LAK) cells or monocytes (12, 13)
- Determination of mediator-induced cytolysis (12)
- Measurement of antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis
- Determination of the cytotoxic potential of compounds in environmental and medical research and in the food, cosmetic and pharmaceutical industries (14-21)
- Determination of cell death in bioreactors (22-24)

It has been shown that a precise evaluation of cell death during fermentation in bioreactors could be performed by the measurement of the release of cytoplasmic LDH enzyme activity to the culture medium. In addition, there is a good correlation between the LDH release assay and the [^{51}Cr] release assay as shown for cell-mediated cytotoxicity using a variety of murine and human effector-target cell systems, including NK cells, CTL and macrophages as effector cells.

3. Test principle

The culture supernatant is collected cell-free and incubated with the reaction mixture from the kit. The LDH activity is determined in an enzymatic test: In the first step NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan (fig.1).

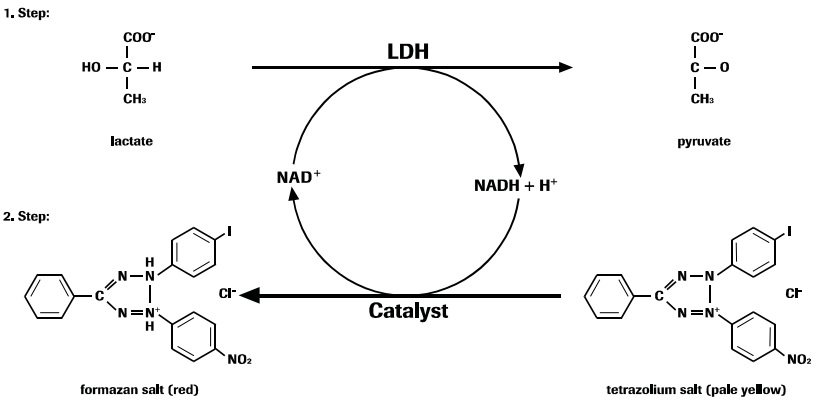


Fig. 1: In the first step, released lactate dehydrogenase (LDH) reduces NAD^+ to $NADH + H^+$ by oxidation of lactate to pyruvate. In the second enzymatic reaction 2 H are transferred from $NADH + H^+$ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) by a catalyst.

An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant directly correlates to the amount of formazan formed during a limited time period. Therefore, the amount of color formed in the assay is proportional to the number of lysed cells. The formazan dye formed is water-soluble and shows a broad absorption maximum at about 500 nm, whereas the tetra-zolium salt INT shows no significant absorption at these wavelengths (fig. 2).

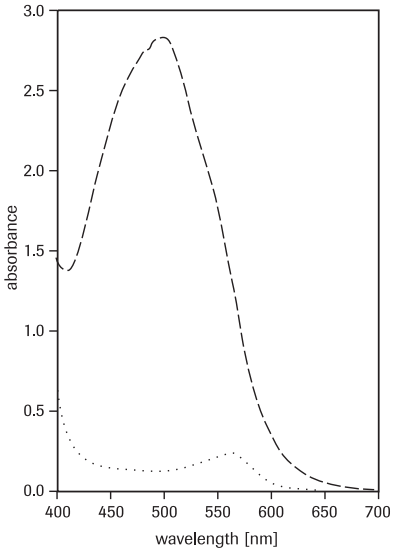


Fig. 2: Absorbance spectra of the working solution of the Cytotoxicity Detection Kit (LDH). The reaction mixture of the Cytotoxicity Detection Kit (LDH) was added to RPMI 1640 with 1% BSA and the absorbance spectra was measured in the absence (.....) and presence (– – –) of LDH.

4. Assay characteristics

4.1 Sensitivity

Depending on the individual cell type used, 0.2-2 × 10^4 cells/well are sufficient for most experimental setups (fig. 3).

4.2 Test interference

- Inherent LDH activity may be found in serum or test substances (see 5.2)
- In cell mediated cytotoxicity assays, the amount of LDH released from damaged effector cells may influence the assay results (see 6.1 and 6.5)
- Substances which inhibit the LDH or diaphorase enzyme activity influence the assay. Appropriate controls should be included in the assay (see 6.1).

4.3 Sample material

Cell-free culture supernatant: Cells have to be removed from the culture medium prior to the determination of LDH activity by centrifugation at about 250 × g.
The cell-free culture supernatant can be stored at 2-8°C without loss of LDH activity for a few days.

4.4 Assay time

0.5 - 1 h, including harvesting of the supernatants and substrate reaction.

5. Preparation of solutions

5.1 Additionally required equipment

- 37°C incubator
- Centrifuge with rotor for microplates
- Microplate (ELISA) reader with 490-492 nm filter
  (if a reference wavelength should be substracted, a filter over 600 nm is recommended)
- Microscope
- Hemacytometer
- Multichannel pipettor (100 µl)
- Sterile pipette tips
- 96-well microplates (MP):
  or the measurement of cell mediated lysis and for the analysis of cytotoxic compounds:
  sterile, cell culture quality with round or V-bottom for suspension cells, with flat bottom for adherent cells
  For color development in all assays: optically clear flat-bottomed.

5.2 Additionally required reagents

- Assay medium (e.g. medium containing 1% serum or 1% bovine serum albumin) Both human and animal sera contain various amounts of LDH, which may increase background absorbance in the assay. Therefore, it is recommended to perform the assay in the presence of low serum concentrations (e.g. 1%) or to replace serum by 1% bovine serum albumin (BSA) (w/v).
- Triton[1)]X-100 solution (2% Triton X-100 in assay medium)
  The maximum amount of releasable LDH enzyme activity is determined by lysing the cells with Triton X-100 (final concentration: 1% Triton X-100). At this concentration Triton X-100 does not affect the LDH activity.
- HCL stop solution (1 N)
  The reaction product can be measured without addition of a stop solution. Alternatively, the enzyme reaction can be stopped by the addition of 50 µl/well 1N HCl (final concentration: 0.2 N HCl).
- LDH standard preparation
  If the released LDH-activity has to be calculated in U/ml instead of relative cytotoxicity in percent or absorbance, it is recommended to use an appropriate LDH preparation as standard.

Assay medium, lysing and stopping solutions as well as LDH standard are not included in the kit; all other reagents necessary to perform 2000 tests are included.

5.3 Preparation of the working solutions

Solution 1: Catalyst (bottle 1, blue cap)
Reconstitute the lyophilisate in 1 ml redist. water for 10 min and mix thoroughly. The reconstituted solution is stable for several weeks at 2-8°C.
Solution 2: Dye solution (bottle 2, red cap)
The INT dye solution is stable for several weeks if stored at 2-8°C,
Reaction mixture
For 100 tests: Shortly before use, mix 250 µl of bottle 1 with 11.25 ml of bottle 2.
For 400 tests: Shortly before use, add the total volume of bottle 1 (1 ml) to the total volume of bottle 2 (45 ml) and mix well.
The reaction mixure should not be stored; prepare immediately before use.

6. Working procedures

6.1 Controls

To calculate percent cytotoxicity the following three controls have to be performed in each experimental setup:

- **Background control:** Provides information about the LDH activity contained in the assay medium. The absorbance value obtained in this control has to be substracted from all other values.
- **Low control**: Provides information about the LDH activity released from the untreated normal cells (= spontaneous LDH release).
- **High control**: Provides information about the maximum releasable LDH activity in the cells (= maximum LDH release).

The following two controls are facultative:

- **Substance control I:** Provides information about the LDH activity contained in the test substance. If cell mediated cytotoxicity is measured, this control pro-vides information about the LDH activity released from the effector cells (= effector cell control, see 6.5).
- **Substance control II:** Provides information whether the test substance itself interferes with LDH activity.
  To perform this control proceed as follows:
  Add 50 µl/well test substance solution (diluted in assay medium) in triplicate in an optically clear 96-well flat bottom plate.
  Add 50 µl/well LDH solution (0.05 U/ml).
  Add 100 µl/well reaction mixture (5.3) and measure absorbance using an ELISA reader as described below.
  Compare the measured absorbance values with those absorbance values obtained with the control sample containing 50 µl/well LDH solution (0.05 U/ml), 50 µl/well assay medium and 100 µl/well reaction mixture.

Tab. 1: Overview of the controls

Contents of the well	Back-ground control	Low control	High control	Sub-stance control I	Sub-stance control II	Experi-mental setup
Assay medium	200 µl	100 µl	–	100 µl	–	–
Cells	–	100 µl	100 µl	–	–	100 µl
Triton X-100 solution (2% in assay medium)	–	–	100 µl	–	–	–
Test sub-stance or effector cells	–	–	–	100 µl	50 µl	100 µl
LDH-standard	–	–	–	–	50 µl	–

Note: The background, low and high controls have to be determined in each experimental setup.

6.2 Calculation

To determine the percentage cytotoxicity, calculate the average absorbance values of the triplicates and substract from each of these the absorbance value obtained in the background control.
The resulting values are substituted in the following equation:

$$\textbf{Cytotoxicity (\%)} = \frac{\text{exp. value - low control}}{\text{high control} - \text{low control}} \times 100$$

To determine the percentage cell mediated cytotoxicity calculate the average absorbance of the triplicates and substract the background. These values are substituted into the following equation:

$$\textbf{Cytotoxicity (\%)} = \frac{(\text{effector}-\text{target cell mix}-\text{effector cell control})-\text{low control}}{\text{high control} - \text{low control}} \times 100$$

6.3 Determination of the optimal cell concentration for the assay

Different cell types may contain different amounts of LDH. Therefore, the optimum cell concentration for a specific cell type should be determined in a preliminary experiment. In general, this cell concentration, in which the difference between the low and high control is at a maximum, should be used for the subsequent assay. With most cell lines the optimal cell concentration is between $0.5 - 2 \times 10^4$ cells/well in 200 µl (= $0.25 - 1 \times 10^5$ cells/ml).

6.3.1 Assay procedure

- Fill the entire 96-well tissue culture plate with 100 µl/well assay medium.
- Wash cells with assay medium.
- Adjust cell suspension to a concentration of 2×10^6 cells/ml and titrate the cells by two-fold serial dilutions across the plate using a multichannel pipette (experimental setup see 6.3.2).
- Determine the background control: Fill 200 µl assay medium into triplicate wells (see also 6.1).
- Determine the low control (= spontaneous LDH release): Add 100 µl/well assay medium to triplicate wells containing 100 µl/well cells (see also 6.1).
- Determine the high control (= maximum LDH release): Add 100 µl/well Triton X-100 solution to triplicate wells containing 100 µl/well cells (see also 6.1).
- Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity) for the time used in the final assay.
- Centrifuge the microplate at 250 × g for 10 min.
- Remove 100 µl/well supernatant carefully (do not disturb the cell pellet) and transfer into corresponding wells of an optically clear 96-well flat bottom microplate (MP).
- To determine the LDH activity in these supernatants, add 100 µl reaction mixture (5.3) to each well and incubate for up to 30 min at 15-25°C. During this incubation period the MP should be protected from light.
- Measure the absorbance of the samples at 490 or 492 nm according to the filters available using an ELISA reader. The reference wavelength should be more than 600 nm.

6.3.2 Experimental setup

Background Control			
Spontaneous Release Dilution 1	Spontaneous Release Dilution 8	Maximal Release Dilution 1	Maximal Release Dilution 8
Spontaneous Release Dilution 2	Spontaneous Release Dilution 9	Maximal Release Dilution 2	Maximal Release Dilution 9
Spontaneous Release Dilution 3	Spontaneous Release Dilution 10	Maximal Release Dilution 3	Maximal Release Dilution 10
Spontaneous Release Dilution 4	Spontaneous Release Dilution 11	Maximal Release Dilution 4	Maximal Release Dilution 11
Spontaneous Release Dilution 5	Spontaneous Release Dilution 12	Maximal Release Dilution 5	Maximal Release Dilution 12
Spontaneous Release Dilution 6	Spontaneous Release Dilution 13	Maximal Release Dilution 6	Maximal Release Dilution 13
Spontaneous Release Dilution 7	Spontaneous Release Dilution 14	Maximal Release Dilution 7	Maximal Release Dilution 14

Note: All tests should be performed in triplicate.

6.3.3 Results

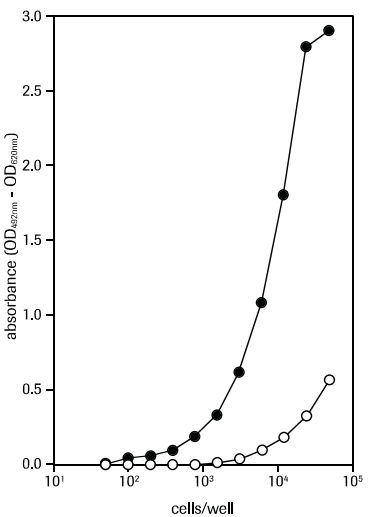


Fig. 3: Determination of the optimal target cell concentration for K562 cells. K562 cells were titrated in microplates as described in 6.3 at cell concentrations indicated in the figure. Culture medium (❍) was added for the determination of the spontaneous release of LDH activity and Triton X-100 (●) was added to a final concentration of 1% for the determination of maximal release of LDH activity. Optimal target cell concentration in this experiment is at about 1×10^4 cells/well.

6.4 Example 1: Measurement of the cytotoxic potential of soluble substances

6.4.1 Assay procedure for suspension cells

- Titrate test substances (mediators, cytolytic or cytotoxic agents) in the appropriate assay medium in sterile 96-well tissue culture plates by serial dilutions (final volume of 100 µl/well).
- After washing the cells in assay medium, dilute to the concentration determined in the preliminary experiment (see 6.3).
- Add 100 µl/well cell suspension to the dilutions of the test substances (experimental setup see 6.4.3),
- Determine the background control: Fill 200 µl assay medium into triplicate wells (see also 6.1).
- Determine the low control: Add 100 µl/well cell suspension to triplicate wells containing 100 µl/well assay medium (see also 6.1).
- Determine the high control: Add 100 µl/well cell suspension to triplicate wells containing 100 µl/well Triton X-100 solution (see also 6.1).
- Determine the substance control I: Add 100 µl/well test substance (in the maximum concentration used in the experiment) to triplicate wells containing 100 µl/well assay medium (see also 6.1).
- Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity). Dependent on the experimental set up incubation times between 2 and 24 h are recommended.
- Centrifuge the cells at 250 g for 10 min.
- Remove 100 µl/well supernatant carefully (do not disturb the cell pellet) and transfer into corresponding wells of an optically clear 96-well flat bottom MP.
- To determine the LDH activity in these supernatants, add 100 µl reaction mixture (5.3) to each well and incubate for up to 30 min at 15-25°C. During this incubation period the MP should be protected from light.
- Measure the absorbance of the samples at 490 or 492 nm according to the filters available using a microplate (ELISA) reader. The reference wavelength should be more than 600 nm.

6.4.2 Assay procedure for adherent cells

- After washing the cells in assay medium, dilute to the concentration determined in the preliminary experiment (see 6.3).
- Add 100 µl/well cell suspension per well in a sterile 96-well tissue culture plate. No cells should be added to wells for background control and substance control I.
- Incubate the cells overnight in an incubator (37°C, 5% CO_2, 90% humidity) to allow the cells to adhere tightly.
- Immediatly before use, titrate test substances (mediators, cytolytic or cytotoxic agents) in the appropriate assay medium in a separate MP by serial dilutions (final volume of 200 µl/well).
- Remove the assay medium from the adherent cells (to remove LDH activity released from the cells during the overnight incubation step) and add 100 µl fresh assay medium to each well.
- Transfer 100 µl of the test substance dilutions into corresponding wells containing the adherent cells (experimental setup see 6.4.3).
- Determine the background control: Fill 200 µl assay medium into triplicate wells (see also 6.1).

- Determine the low control: Add 100 µl/well assay medium to triplicate wells containing 100 µl/well cells (see also 6.1).
- Determine the high control: Add 100 µl/well Triton X-100 solution to triplicate wells containing 100 µl/well cells (see also 6.1).
- Determine the substance control I: Add 100 µl/well test substance (maximum concentration used in the experiment) to triplicate wells containing 100 µl/well assay medium (see also 6.1).
- Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity). Dependent on the experimental set up incubation times between 2 and 24 h are recommended.
- Remove 100 µl/well supernatant carefully and transfer the supernatant into corresponding wells of an optically clear 96 well flat bottom MP.
- To determine the LDH activity contained in the supernatants, add 100 µl reaction mixture (5.3) to each well and incubate for up to 30 min at 15-25°C. During this incubation periode the MP should be protected from light.
- Measure the absorbance of the samples at 490 or 492 nm according to the filters available using an ELISA reader. The reference wavelength should be more than 600 nm.

6.4.3 Experimental setup

Background control	**Test Substance 1 Substance Control I (Dilution 1)**	**Test Substance 2 Substance Control I (Dilution 1)**	
Test Substance 1 Dilution 1	Test Substance 1 Dilution 8	Test Substance 2 Dilution 1	Test Substance 2 Dilution 8
Test Substance 1 Dilution 2	Test Substance 1 Dilution 9	Test Substance 2 Dilution 2	Test Substance 2 Dilution 9
Test Substance 1 Dilution 3	Test Substance 1 Dilution 10	Test Substance 2 Dilution 3	Test Substance 2 Dilution 10
Test Substance 1 Dilution 4	Test Substance 1 Dilution 11	Test Substance 2 Dilution 4	Test Substance 2 Dilution 11
Test Substance 1 Dilution 5	Test Substance 1 Dilution 12	Test Substance 2 Dilution 5	Test Substance 2 Dilution 12
Test Substance 1 Dilution 6	Test Substance 1 Dilution 13	Test Substance 2 Dilution 6	Test Substance 2 Dilution 13
Test Substance 1 Dilution 7	Low Control	Test Substance 2 Dilution 7	High Control

Note: All tests should be performed in triplicate

6.4.4 Results

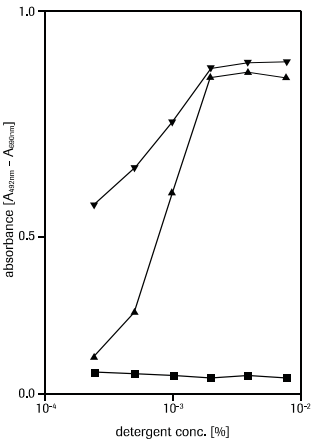


Fig. 4: Measurement of the cytotoxic potential of various detergents

Synperonic®[2] F68 (■), TritonX-100 (▲) and Nonident[3]P40 (▼) were titrated in microplates in culture medium as described in 6.4 to final concentrations indicated in the figure. Subsequently P815 cells were added to a final concentration of 1×10^4 cells/well. The cells were incubated for 18h and LDH release was determined as described in 6.4.

6.5 Example 2: Measurement of cell mediated cytotoxicity

6.5.1 Assay procedure

- Titrate effector cells (NK cells, LAK cells, CTLs) in the appropriate assay medium in sterile 96-well tissue culture plates by serial dilutions (final volume of 100 µl/well).
- After washing the target cells in assay medium, dilute to the concentration determined in the preliminary experiment (see 6.3).
- Add 100 µl/well target cell suspension to the dilutions of effector cells (= effector-target cell mix). For experimental setup see 6.5.2.
- Determine the background control: Fill 200 µl assay medium into triplicate wells (see also 6.1).
- Determine the low control: Add 100 µl/well target cells to triplicate wells con- taining 100 µl/well assay medium (see also 6.1).
- Determine the high control: Add 100 µl/well target cells to triplicate wells containing 100 µl/well Triton X-100 solution (see also 6.1).
- Determine the substance control I (= effector cell control = spontaneous release of LDH by the effector cells): Add 100 µl/well assay medium to triplicate wells containing 100 µl/well effector cells (see also 6.1).
  Note: The spontaneous LDH release has to be determined for each effector cell concentration used in the assay.
- Incubate cells in an incubator (37°C, 5% CO_2, 90% humidity) for the appropriate time periode.
- Centrifuge the cells at 250 × g for 10 min.

- Remove 100 µl/well supernatant carefully (do not disturb the cell pellet) and transfer into corresponding wells of an optically clear 96-well flat bottom MP.
- Add 100 µl reaction mixture (5.3) to each well and incubate for up to 30 min at room temperature. During this incubation period the MP should be protected from light.
- Measure the absorbance of the samples at 490 or 492 nm using an ELISA reader. The reference wavelength should be more than 600 nm.

6.5.2 Experimental setup

Background control	**Target cell low control**	**Target cell high control**	
effector-target cell mix. ratio 1	effector-target cell mix. ratio 7	effector cell control for ratio 1	effector cell control for ratio 7
effector-target cell mix. ratio 2	effector-target cell mix. ratio 8	effector cell control for ratio 2	effector cell control for ratio 8
effector-target cell mix. ratio 3	effector-target cell mix. ratio 9	effector cell control for ratio 3	effector cell control for ratio 9
effector-target cell mix. ratio 4	effector-target cell mix. ratio 10	effector cell control for ratio 4	effector cell control for ratio 10
effector-target cell mix. ratio 5	effector-target cell mix. ratio 11	effector cell control for ratio 5	effector cell control for ratio 11
effector-target cell mix. ratio 6	effector-target cell mix. ratio 12	effector cell control for ratio 6	effector cell control for ratio 12

Note: All tests should be performed in triplicate

6.5.3 Results

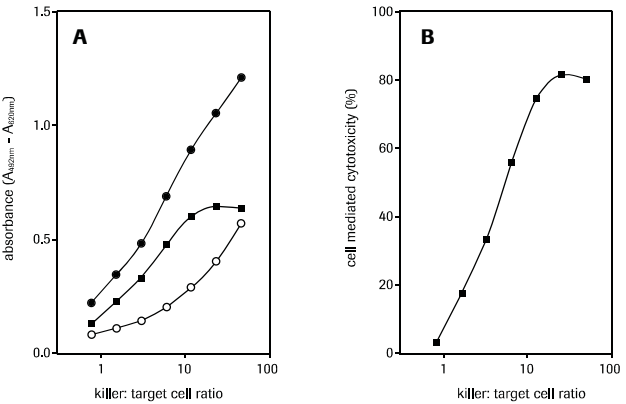


Fig. 5: Determination of the cytolytic activity of allogen-stimulated, cytotoxic T lymphocytes (CTLs). Spleen cells of C57/Bl 6 mice (H-2b) were stimulated *in vitro* with P815 cells (H-2d). Viable CTLs were purified by ficoll density gradient, washed and titrated in the microplate as described in 6.5. 1×10^4 P815 target cells/well were added to the effector cells. The cells were centrifuged and incubated for 4 h. Afterwards, 100 µl of culture supernatant were removed and LDH activity was determined as described in 6.5.
A. Absorbance values. Effector cell control (○), effector-target cell mix (●), effector-target cell mix minus effector cell control (■).
B. Percentage cell mediated cytotoxicity, calculated as described in 6.2

6.6 Example 3: Measurement of cell death in eukaryotic cell fermentation

6.6.1 Assay procedure

- Collect samples (0.5 - 1 ml) at regular intervals of 12 or 24 h from cell culture.
- Spin the samples and remove culture supernatant carefully. The cell-free supernatants can be collected and stored at 2-8°C without loss of enzyme activity for a few days.
- Titrate the culture supernatants in the appropriate culture medium by serial dilutions to obtain a final volume of 100 µl/well
- Add 100 µl reaction mixture (5.3) to each well and incubate for up to 30 min at 15-25°C. During this incubation period the MP should be protected from light.
- Measure the absorbance of the samples at 490 or 492 nm using a microplate reader. The reference wavelength should be more than 600 nm.

6.6.2 Results

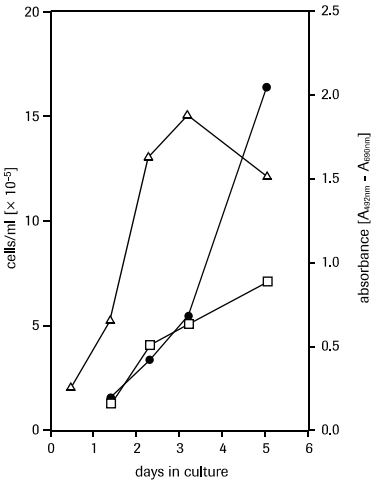


Fig. 6: Correlation of cell death and LDH release in cell culture.
Ag 8 cells were seeded at a concentration of 2 x 10^5 cells/ml and incubated at 37°C, 5% CO_2. At day 1, 2, 3 and 5 of culture aliquots were removed. The amount of viable (△) and dead (●) cells were determined by trypan blue exclusion. LDH activity of cell free culture supernatant (□) was determined as described (see 6.6).

7. Trouble shooting guide

Problem	Solution
No color reaction at all	• Check cell concentration: may be too low • Check test substance and/or assay medium for compounds inhibiting LDH activity (substance control II, 6.1)
Strong color reaction also in low controls	• Check cell concentration: may be too high • Check test substance and/or assay medium for compounds with LDH activity (substance control I, 6.1) • High spontaneous release may be due to bad condition of the cells used in the assay. Check culture conditions: some cell lines do not survive in serum free media, even at short incubation times. Increase serum concentration to about 1-5%.
Strong color reaction but low absorbance values	Check background values. High background values may result in too low absorbance values if they are substracted automatically. Check assay medium for compounds with LDH activity (sera, substance control I, 6.1)
Strong color reaction at effector cells controls	Bad conditions of the effector cells due to inappropriate isolation or culture conditions. Improve cell culture. Separate viable from dead effector cells by density gradient centrifugation.

8. References

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9. Related Products

Apoptosis-specific physiological change	Detection mode/ Product	Pack size	Cat. No.
DNA fragmentation	**Gel Electrophoresis**		
	Apoptotic DNA-Ladder Kit	20 tests	1 835 246
	***In situ* assay**		
	In Situ Cell Death Detection Kit, TMR red (also usuable for FACS)	1 kit (50 tests)	2 156 792
	In Situ Cell Death Detection Kit, Fluorescein (also usuable for FACS)	1 kit (50 tests)	1 684 795
	In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	1 684 809
	In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	1 684 817
	Single reagents for TUNEL and supporting reagents		
	TUNEL AP	70 tests (3.5 ml)	1 772 457
	TUNEL POD	70 tests (3.5 ml)	1 772 465
	TUNEL Enzyme	2× 50 µl	1 767 305
	TUNEL Label	3× 550 µl	1 767 291
	TUNEL Dilution Buffer	20 ml	1 966 006
	ELISA		
	Cell Death Detection ELISA	1 kit	1 544 675
	Cell Death Detection ELISA^{PLUS}	1 kit (96 tests)	1 774 425
	Cell Death Detection ELISA^{PLUS}, 10×	1 kit	1 920 685
	Cellular DNA Fragmentation ELISA	1 kit (500 tests)	1 585 045
Cell membrane alterations	**Microscopy or FACS**		
	Annexin-V-Biotin	250 tests	1 828 690
	Annexin-V-FLUOS	250 tests	1 828 681
	Annexin-V-FLUOS Staining Kit	50 tests 250 tests	1 858 777 1 988 549
Enzymatic activity	**Western Blot**		
	Anti-Poly (ADP-Ribose) Polymerase	100 µl	1 835 238
	FIENA		
	Caspase 3 Activity Assay	1 kit	2 012 952
	Fluorimetric microplate Assay		
	Homogenous Caspases Assay, fluorometric	100 tests 1000 tests	3 005 372 2 236 869
	***In situ* Assay**		
	M30 CytoDEATH (formalin grade)	50 tests 250 tests	2 140 322 2 140 349
	M30 CytoDEATH, Fluorescein	250 tests	2 156 857
Expression of apoptosis-related proteins	**ELISA**		
	p53 pan ELISA	1 kit	1 828 789

For further information please access our web-site address at:
http://roche-applied-science.com
or the Apoptosis special interest site:
http:// roche-applied-science.com/apoptosis

10. Quick reference protocols

Step	Procedure	Volume/well	Time/Temperature
1	Incubate target cells with test substance or cytotoxic effector cells	200 µl	4 h–24 h/37°C
2	Centrifuge cells		10 min, 250 × g at 15-25°C
3	Transfer cell free culture supernatant to clear, flat bottom microplate	100 µl	
4	Add reaction mixture and incubate	100 µl	approx. 10–30 min at 15-25°C, proctected from light
5	If the reaction is to be stopped, add 1N HCl to each well	50 µl	
6	Measure absorbance at about 490 nm (reference wavelength 690 nm)		

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