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Not for use in diagnostic procedures.

Print Protocol

Print Changes to Previous Version

Cytotoxicity Detection Kit^{PLUS} (LDH)

Version 06

Content version: May 2011

Non-radioactive colorimetric assay suitable for high-throughput quantification of cell death and cell lysis, based on measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells

Cat. No. 04 744 926 001 Kit for 400 tests in 96-well plates or 1600 tests in 384-well plates

Cat. No. 04 744 934 001 Kit for 2000 tests in 96-well plates or 8000 tests in 384-well plates

Store the kit at −15 to −25°C

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1. What This Product Does

Number of Tests

Kit 04 744 926 001 is for 400 tests in 96-well plates (or 1600 tests in 384-well plates)

Kit 04 744 934 001 is for 2000 tests in 96-well plates (or 8000 tests in 384-well plates)

Kit Contents

Bottle	Label	Contents/Function A) Cat. No. 04 744 926 001 (400 tests) B) Cat. No. 04 744 934 001 (2000 tests)
1 blue cap	Catalyst	• A) 1 bottle lyophilizate, stabilized B) 5 bottles lyophilizate, stabilized • Diaphorase/NAD^+ mixture • Catalyst for reaction mix
2 red cap	Dye solution	• A) 1 bottle, 45 ml B) 5 bottles, 45 ml each • Iodotetrazolium chloride (INT) and sodium lactate • to dye reaction mix
3 white cap	Lysis solution	• A) 1 bottle, 3 ml B) 5 bottles, 3 m each • for lysis of cells, ready to use
4 green cap	Stop solution	• A) 1 bottle, 25 ml B) 5 bottles, 25 ml each • for stopping the LDH reaction, ready to use

Storage and Stability

- If stored at −15 to −25°C, the kit is stable until the expiration date printed on the label.

Stability of the kit components:

The lyophilizate (bottle 1) is stable at +2 to +8°C.

The reconstituted catalyst solution, the dye solution (bottle 2), the lysis solution (bottle 3) and the stop solution (bottle 4) are stable for

- 4 weeks when stored at +2 to +8°C
- 2 days at +15 to +25°C.
- For long-term storage (up to 3 months), store the bottles frozen.
- Freezing and thawing the solutions up to 3 times will not significantly reduce their performance. However, if you observe crystals in bottle 2 “Dye solution”, shake the bottle at least 1 hour at 37°C. Remaining precipitates will NOT influence the performance. As the precipitates are formed during the freezing of the product, repeated freezing and thawing should be avoided.

The kit is shipped on dry ice

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform reactions include:

- 37°C incubator
- Microplate (ELISA) reader with 490 – 492 nm filter

⚠ If using a reference wavelength for subtraction purposes, choose a reference filter with a wavelength over 600 nm.

- Microscope
- Hemacytometer
- Multichannel pipettor (100 µl)
- Sterile pipette tips
- For measuring cell-mediated lysis or for analyzing cytotoxic compounds: 96-well or 384-well microplates (MP), sterile, cell culture grade.
- Optional: 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, to increase the sensitivity of the assay, perform it in the presence of low serum concentrations (*e.g.,* 1%) or replace serum with 1% bovine serum albumin (BSA) (w/v).

- LDH standard solution (*e.g.*, 0.05 U LDH/ml; see section 2.1 below)

If you intend to calculate the released LDH activity in U/ml instead of percent relative cytotoxicity or absorbance, use an appropriate LDH preparation as a reference standard.

⚠ Neither the assay medium nor the LDH standard are included in the kit; all other reagents necessary to perform 400 (1600) or 2000 (8000) tests are included.

Application

The Cytotoxicity Detection Kit^{PLUS} (LDH) is a precise, fast and simple colorimetric assay for quantitating cytotoxicity/cytolysis by measuring LDH activity released from damaged cells. This colorimetric assay is suitable for high-throughput quantification using 96-well or 384-well format. Thus, the Cytotoxicity Detection Kit^{PLUS} (LDH) can be used to monitor many different *in vitro* cell systems where damage to the plasma membrane occurs.

The assay can also be used to determine the total numbers of cells present at the end of a proliferation assay.

Examples of applications include:

- Determination of the cytotoxic potential of compounds in environmental and medical research and in the food, cosmetic and pharmaceutical industries (3 – 10)
- Determination of mediator-induced cytolysis (1)
- Detection and quantification of cell-mediated cytotoxicity induced by cytotoxic T-lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer (LAK) cells or monocytes (1, 2)

The LDH release assay and the [^{51}Cr] release assay show good correlation when used to monitor cell-mediated cytotoxicity in a variety of murine and human effector-target cell systems, including NK cells, CTL and macrophages.

- Measurement of antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis
- Determination of cell death in bioreactors (11 - 13)

Experiments have shown that measurement of the release of cytoplasmic LDH enzyme activity to the culture medium can provide a precise evaluation of cell death during fermentation in bioreactors.

Assay Time

Standard assay time: 15 minutes.

Maximum assay time: up to 30 minutes for low cell numbers (less than 100 cells/well).

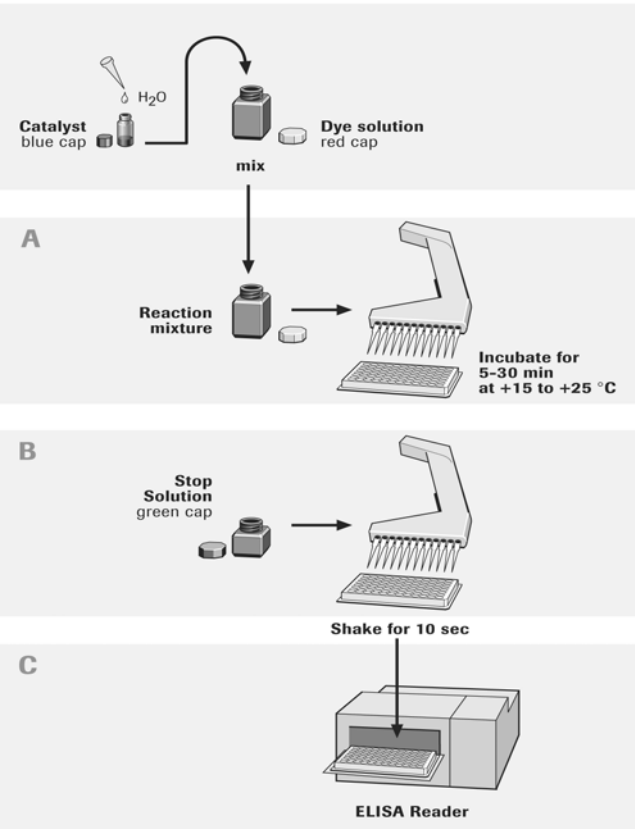

Fig. 1: Overview of procedure

2. How To Use this Product

2.1 Before You Begin

Sample Material Cell culture or cell-free culture supernatant. The assay reagent is not harmful to the cells and can be added directly to the cell culture plate.

Alternatively, when the samples are not tested directly, remove the cells from the culture medium prior to the determination of LDH activity by centrifuging them at about 250 × *g*.

⚠ After centrifugation, the cell-free culture supernatant can be stored at +2 to +8°C for a few days without loss of LDH activity.

Preparation of Working Solutions

Content	Reconstitution/ Preparation	Storage and Stability
Catalyst (bottle 1, blue cap)	Let the lyophilizate dissolve in 1 ml double dist. water for 10 min, then mix thoroughly.	• 4 weeks at +2 to +8°C. • 2 days at +15 to +25°C • Stable when stored frozen at −15 to −25°C for up to 3 months.
Reaction mixture	For 100 tests: Shortly before use, mix 250 µl of reconstituted bottle 1 with 11.25 ml of bottle 2. For 400 tests: Shortly before use, add the total volume of reconstituted bottle 1 (1 ml) to the total volume of bottle 2 (45 ml) and mix well. ⚠ In case you observe crystals in bottle 2 “Dye solution”, shake the bottle at least 1 hour at 37°C. Remaining precipitates will NOT influence the performance. ⚠ The precipitates were formed during the freezing of the product. Repeated freezing and thawing should be avoided.	⚠ Prepare immediately before use. Do not store the Reaction mixture.

Controls To calculate percent cytotoxicity, you must include the following three controls in each experimental setup:

- Background control: Determines the LDH activity contained in the assay medium.

⚠ The absorbance value obtained from this control must be subtracted from all other absorbance values.

- Low control: Determines the LDH activity released from the untreated normal cells (= spontaneous LDH release).
- High control: Determines the maximum releasable LDH activity in the cells (= maximum LDH release).

⚠ When performing this control, you must add the Lysis reagent to the samples at the correct time to get an accurate estimate of maximum releasable LDH. Since the control cells grow during the period of exposure to the cytotoxic compounds, the total LDH may be underestimated if you add the Lysis solution at the beginning of the exposure. Also, since the half-life of LDH at 37°C is about 9 hours, the activity of LDH in the High control may drop significantly if Lysis reagent is added at the beginning of the exposure. Therefore, always add the Lysis reagent to the High control at the end of the exposure period.

⚠ The detergent in the Lysis reagent can slightly enhance the High control LDH activity for some cell lines. To correct for that cell line specific factor add 5 µl of Lysis buffer to the supernatant of an experimental sample (to be set up in parallel).

The following two (optional) controls may also be useful:

- Substance control I: Determines the LDH activity contained in the test substance. In an assay of cell-mediated cytotoxicity, this control provides information about the LDH activity released from the effector cells (= effector cell control). (See section 3.3.)
- Substance control II: Determines whether the test substance itself interferes with LDH activity.
  To perform this control: To each control sample (assayed in triplicate) in an optically clear 96-well flat bottom plate, add 50 µl assay medium containing the test substance. Next, add 50 µl/well LDH standard solution (0.05 U/ml). Finally, add 100 µl/well Reaction mixture. Measure absorbance with an ELISA reader as described in the procedures below. Compare the absorbance values in these controls with absorbance values obtained in separate (triplicate) LDH control samples that contains only 50 µl/well assay medium, 50 µl/well LDH standard solution (0.05 U/ml), and 100 µl/well Reaction mixture.

Contents of the Well	Back-groun d control	Low control	High control	Subs-tanc e control I	Subs-tanc e control II	Experimental sample
Cell Cell-free culture medium	100 µl	50 µl	50 µl	–	–	–
Cells	–	50 µl	50µl	–	–	50 µl
Lysis buffer [1)]	–	–	5 µl	–	–	–
Test substance or Effector cells diluted in culture medium	–	–	–	100 µl	50 µl	50 µl
LDH standard solution	–	–	–	–	50 µl	–

[1)] Added at the end of the exposure to cytotoxic compounds.

⚠ The background, low and high controls must be included in each experiment.

Calculations with the Controls

A. To determine the percentage cytotoxicity, calculate the average absorbance values of the triplicate samples and controls, then substract from each of these the absorbance value obtained in the background control. Then substitute the resulting values in the following equation:

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

B. To determine the percentage cell-mediated cytotoxicity, calculate the average absorbance of the triplicate samples and controls, subtract the background from each, then substitute the resulting values in the following equation:

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

2.2 Determination of the Optimal Cell Concentration for the Assay

⚠ Different cell types may contain different amounts of LDH. Therefore, for each specific cell type, always perform a preliminary experiment to determine the optimum cell concentration. In general, this optimal cell concentration is the one that produces the greatest difference between the low and high control; use this concentration for the subsequent assay.

For most cell lines the optimal cell concentration is (0.25 – 1) × 10^4 cells/100 µl assay [= (0.25 – 1) × 10^5 cells/ml].

Procedure (for a 96-Well Plate)

⚠ To adapt this procedure to a 384-well plate, use 25 µl/well instead of 100 µl/well in each of steps 2-4 (*e.g.*, in step 2, add 25 µl assay medium to each well of a 384-well plate)

For an overview of the procedure see figure 1

❶	• Wash cells with assay medium. • Adjust cell suspension to a concentration of 2 × 10^6 cells/ml in assay medium.
❷	Add 100 µl assay medium to each well of an entire 96-well tissue culture plate.

❸ • Use a multichannel pipette to prepare two-fold serial dilutions of the cells across the plate. Prepare 6 wells of each dilution.

⚠ After dilution, the final volume in each well should be 100 µl. Be sure to leave at least 3 wells cell-free to be used as a Background control.

• For each cell dilution, designate:
  – 3 wells as a Low control (= spontaneous LDH release).
  – 3 wells as a High control (= maximum LDH release).

For an overview of the purpose of each control, see section 2.1.

❹ Incubate the plate in an incubator (37°C, 5% CO_2, 90% humidity).

⚠ The incubation time here should be the same as the incubation time to be used in the final assay.

❺ • To the cell dilutions designated as High controls in step 3, add 5 µl/well Lysis solution. (For a 384-well plate, use 1.5 µl/well Lysis solution)
• Incubate the plate for an additional 15 minutes.

⚠ Shaking the plates during lysis speeds up the process especially for adherent and clumpy cells.

❻ To determine the LDH activity, add 100 µl Reaction mixture (freshly prepared) to each well on the 96-well plate and incubate 5 – 10 minutes for high cell numbers or up to 30 minutes for low cell numbers (under 100 cells/well) at +15 to +25°C. (For a 384-well plate, use 25 µl/well Reaction mixture.)

⚠ During this incubation, you should protect the plate from light. (See (A), fig.1.)

❼ • Add 50 µl Stop Solution to each well on the 96-well plate. (For a 384-well plate, use 12.5 µl/well Stop Solution.)
• Shake the plate for 10 seconds. (See (B), fig.1.)

❽ Use an ELISA reader to measure the absorbance of the samples at 490 or 492 nm (depending on the filters available in the reader).

⚠ The reference wavelength should be more than 600 nm. (See (C), fig.1.)

❾ Determine the optimal cell concentration for the assay (*i.e.*, the concentration with the greatest difference between the High and the Low control values.)

Results

Figure 2: Linear relationship between cell number and absorbance at 492 nm obtained with the Cytotoxicity Detection Kit^{PLUS} (LDH) in 384- and 96-well plates.

U 937 cells were diluted in microplates as described above to obtains the cell concentrations indicated in the figure. Culture medium was added to determine the spontaneous release of LDH activity (control) and Lysis reagent was added to determine the maximal release of LDH activity (lysed). The LDH reaction was allowed to continue for 15 min.

Graph A shows the values obtained from a 384-well plate and graph B, the values from a 96-well plate. The assay can determine LDH release from less than 100 cells/well on 384-well plates and from less than 500 cells/well on 96-well plates. A longer incubation would increase the sensitivity even further.

The assay shows a linear relationship between cell number and LDH signal intensity (maximum LDH released from lysed cells) in the high control.

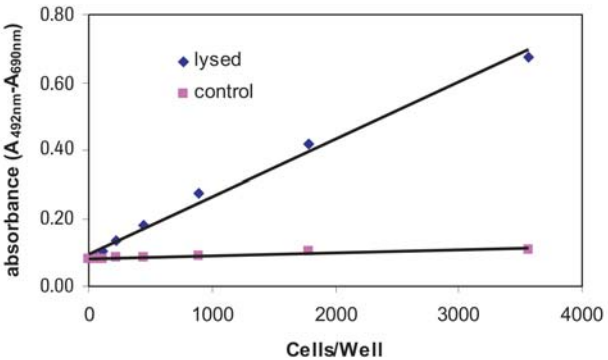

Fig. 2A: 384-well plate

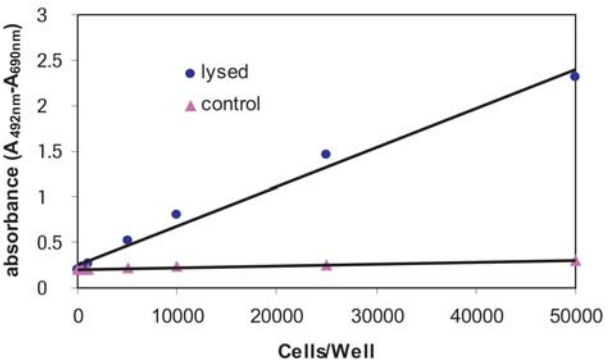

Fig. 2B: 96-well plate

2.3 Measurement of the Cytotoxic Potential of Soluble Substances

Procedure (for a 96-Well Plate)

⚠ To adapt this procedure to a 384-well plate, use only ¼ the amount of cells and assay medium in steps 1–3 (*e.g.*, in step 1, start with 25 µl cells /well and resuspend in 12.5 µl medium).

❶
- Grow cells to the concentration determined in the preliminary experiment (section 2.2).
- Pipette 50 µl of cell suspension into wells of a 96-well plate.

⚠ Prepare enough cell-containing wells to test each of the test substances (to be prepared in step 3 below) in triplicate and prepare the two cell-containing controls listed in step 4.

❷ Wash cells with assay medium.

❸
- Immediately before the experiment, use a separate microplate to prepare serial dilutions of the test substances (mediators, cytolytic or cytotoxic agents) in assay medium.
- Transfer 50 µl of each dilution of test substance into wells that contain 50 µl cells (in the microplate prepared in step 1).

⚠ Prepare all test samples in triplicate.

❹ On the same plate, prepare the following controls in triplicate:

Control	Add to each well
Background control	100 µl assay medium only
Low control	50 µl cell suspension plus 50 µl assay medium
High control	50 µl cell suspension plus 50 µl assay medium
Substance control I	50 µl test substance (at the maximum concentration used in the experiment) plus 50 µl assay medium
Substance control II	50 µl test substance (at the maximum concentration used in the experiment) plus 50 µl LDH standard solution

🔍 For an overview of the purpose of each control, see section 2.1.

❺ Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity).

⚠ Depending on the experiment, appropriate incubation time will fall between 2 and 24 h.

❻
- To each of the wells that contain High control samples (from step 4), add 5 µl Lysis solution. (For a 384-well plate, use 1.5 µl/well Lysis Solution.)
- Incubate the plate for an additional 15 min.

⚠ Shaking the plates during lysis speeds up the process.

❼	To determine the LDH activity, add 100 µl Reaction mixture (freshly prepared) to each well on the 96-well plate and incubate for up to 30 min at +15 to +25°C [see fig.1, (A)]. (For a 384-well plate, use 25 µl/well Reaction mixture.) ⚠ During this incubation, you should protect the plate from light.
❽	• Add 50 µl Stop Solution to each well on the 96-well plate [see fig.1, (B)]. (For a 384-well plate, use 12.5 µl/well Stop Solution.) • Shake the plate for 10 seconds.
❾	Use an ELISA reader to measure the absorbance of the samples at 490 or 492 nm (depending on the filters available in the reader) [see fig.1, (C)]. ⚠ The reference wavelength should be more than 600 nm.
❿	Calculate the percent cytotoxicity for each sample (as described in section 2.1)

Results

The following results (fig. 3 and 4) show experiments with a suspension cell line (U 937) and an adherent cell line (WEHI 164).

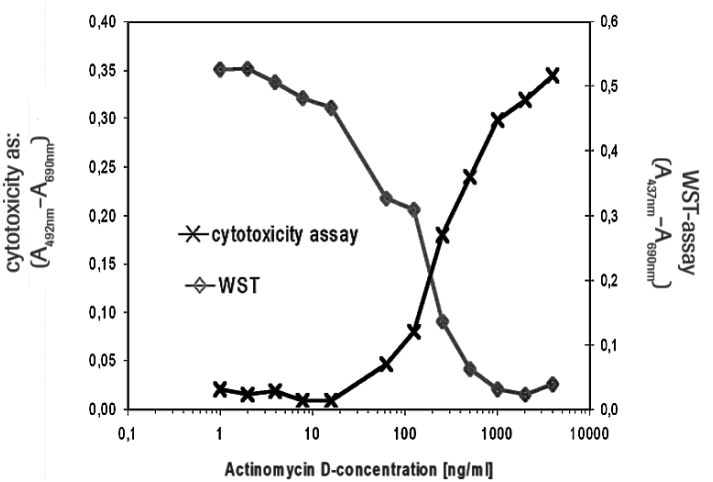

Fig. 3: Determination of cytotoxic activity of Actinomycin D on U 937 suspension cells. U 937 cells were seeded into fresh medium at a density of 10000 cells/well in a 96-well plate. Different concentrations of Actinomycin D were then added to the cultures and incubated for 16 hours. Cytotoxicity was then measured with the Cytotoxicity Detection Kit^{PLUS} (LDH). In a separate set of control wells, which were treated in the same way, cell proliferation was measured with the Cell Proliferation Reagent, WST-1*. The results shows that both types of assay give similar IC_{50} values.

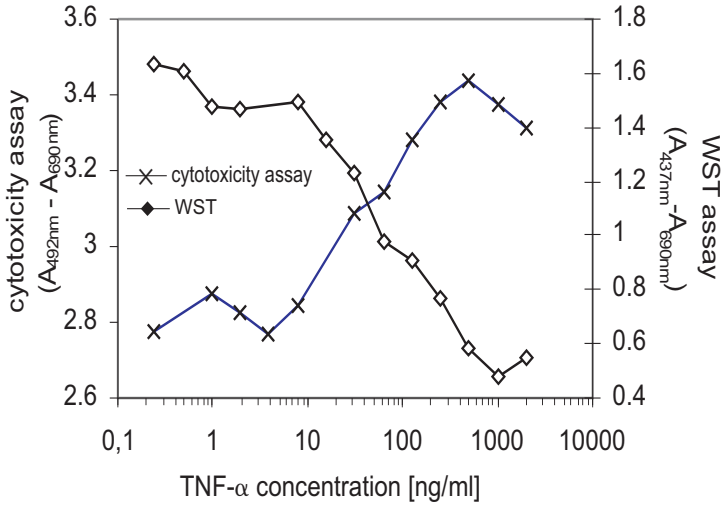

Fig. 4: Determination of cytotoxic activity of TNF-α on adherent WEHI 164 cells. Cell cultures containing 50,000 WEHI 164 cells/well in a 96-well plate were washed with new medium and pre-incubated for 3 hours with 1 μg/ml of Actinomycin D. Then different amounts of TNF-α were added to the wells and the plates were incubated for 16 hours. Cytotoxicity was then measured in the culture with the Cytotoxicity Detection Kit^{PLUS} (LDH). In a separate set of control wells, which were treated in the same way, cell proliferation was measured with the Cell Proliferation Reagent, WST-1. The results shows that both types of assay give similar IC_{50} values.

2.4 Measurement of Cell Proliferation

⚠ You can use the Cytotoxicity Detection Kit^{PLUS} (LDH) to perform a proliferation assay. To do this, let the cells proliferate and, at the end of an experiment, lyse the cells by adding the lysis reagent supplied with the kit. Measure the total released LDH with the Reaction Mix from the kit. You need no Low control nor a High control in that case.

Procedure (for a 96-Well Plate)

⚠ For information on adapting this procedure to a 384-well plate, see sections 2.2 and 2.3 above.

❶ • Into the wells of a 96-well tissue culture plate, pipette samples (100 µl/well) of a cell suspension in assay medium.
• Grow cells to the desired density.

❷ Wash cells with assay medium.

❸ To each cell-containing well, add 50 µl assay medium containing a substance that you want to test for its effect on cell proliferation.

⚠ Prepare all test samples in triplicate.

❹ • On the same plate, prepare the following controls in triplicate:

🔍 For an overview of the purpose of each control, see section 2.1.

Control	Add to each well
Background control	100 µl assay medium
Substance control I	50 µl test substance (at the maximum concentration used in the experiment) plus 50 µl assay medium
Substance control II	50 µl test substance (at the maximum concentration used in the experiment) plus 50 µl LDH standard solution

❺ Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity) for an appropriate time.

❻ • Add 5 µl Lysis solution to each cell-containing well.
• Incubate the plate for an additional 15 minutes.

⚠ Shaking the plates during lysis speeds up the process, especially for adherent or clumpy cells.

❼ To determine the LDH activity, add 100 µl Reaction mixture (freshly prepared) to each well and incubate for up to 30 min at +15 to +25°C [see fig.1, (A)].

⚠ During this incubation, you should protect the plate from light.

❽ • Add 50 µl Stop Solution to each well.
• Shake the plate for 10 seconds [see fig.1, (B)].

❾ Use an ELISA reader to measure the absorbance of the samples at 490 or 492 nm (depending on the filters available in the reader) [see fig.1, (C)].

⚠ The reference wavelength should be more than 600 nm.

❿ Calculate the percent cytotoxicity for each sample (as described in section 2.1)

Results

Fig 2A and 2B (in section 2.2) show that the number of cells in the lysed cell control is proportional to the LDH signal intensity.

2.5 Measurement of Cell-Mediated Cytotoxicity

Experimental Set-Up (Sample Arrangement on a 96-Well Plate)

⚠ All tests should be performed in triplicate.

Background control	Target cell low control	Target cell high control	Blank
Effector–Target cell mix (ratio 1)	Effector–Target cell mix (ratio 7)	Effector cell control (for mix ratio 1)	Effector cell control (for mix ratio 7)
Effector–Target cell mix (ratio 2)	Effector–Target cell mix (ratio 8)	Effector cell control (for mix ratio 2)	Effector cell control (for mix ratio 8)
Effector–Target cell mix (ratio 3)	Effector–Target cell mix (ratio 9)	Effector cell control (for mix ratio 3)	Effector cell control (for mix ratio 9)
Effector–Target cell mix (ratio 4)	Effector–Target cell mix (ratio 10)	Effector cell control (for mix ratio 4)	Effector cell control (for mix ratio 10)
Effector–Target cell mix (ratio 5)	Effector–Target cell mix (ratio 11)	Effector cell control (for mix ratio 5)	Effector cell control (for mix ratio 11)
Effector–Target cell mix (ratio 6)	Effector–Target cell mix (ratio 12)	Effector cell control (for mix ratio 6)	Effector cell control (for mix ratio 12)

Procedure (for a 96-Well Plate)

⚠ For information on adapting this procedure to a 384-well plate, see sections 2.2 and 2.3 above.

❶ In sterile 96-well tissue culture plates, prepare serial dilutions of effector cells (NK cells, LAK cells, CTLs) in the appropriate assay medium (final volume of each dilution = 50 µl/well).

⚠ Prepare all test samples in triplicate.

❷
- Wash the target cells in assay medium.
- Dilute target cells until they are twice as concentrated as the optimal concentration determined in the preliminary experiment (section 2.2).

❸ To each well containing a dilution of effector cells, add 50 µl of the target cell suspension (= effector-target cell mix).

⚠ For experimental set-up, see diagram above.

❹ On the same plate, prepare the following controls in triplicate:

For an overview of the purpose of each control, see section 2.1.

Control	Add to each well
Background control	100 µl assay medium
Low control	50 µl target cells plus 50 µl assay medium
High control	50 µl target cells plus 50 µl assay medium
Substance control I	Add 50 µl assay medium plus 50 µl effector cells
Substance control II	50 µl test substance (at the maximum concentration used in the experiment) plus 50 µl LDH standard solution

⚠ Always determine the spontaneous LDH release for each effector cell concentration used in the assay. For experimental set-up, see diagram above.

❺ Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity) for the appropriate time period.

❻ • To each of the wells that contain High control samples (from step 4), add 5 µl Lysis solution.
• Incubate the plate for an additional 15 min.

⚠ Shaking the plates during lysis speeds up the process especially for adherent and clumpy cells.

❼ To determine the LDH activity, add 100 µl Reaction mixture (freshly prepared) to each well on the 96-well plate and incubate for up to 30 min at +15 to +25°C [see fig.1, (A)].

⚠ During this incubation, you should protect the plate from light.

❽ • Add 50 µl Stop Solution to each well.
• Shake for 10 seconds [see fig.1, (B)].

❾ Use an ELISA reader to measure the absorbance of the samples at 490 or 492 nm (depending on the filters available in the reader) [see fig.1, (C)].

⚠ The reference wavelength should be more than 600 nm.

❿ Calculate the percent cytotoxicity for each sample (as described in section 2.1)

3. Troubleshooting

Observation	Possible cause	Recommendation
Weak color reaction	Cell concentration is too low.	Titrate cell concentration.
	Substance or assay medium inhibit LDH activity.	• Use substance control II (section 2.1) to test substance and/or assay medium for compounds that inhibit LDH activity. • Do not use culture media that contains pyruvate.
Strong color reaction present in low controls	Cell concentration too high	Titrate cell concentration.
	Substance or assay medium has LDH activity.	Use substance control I (section 2.1) to test substance and/or assay medium for compounds with LDH activity.
	High spontaneous release may be due to poor condition of the cells used in the assay.	Check culture conditions. Some cell lines do not survive in serum-free media, even for short incubation times. Increase serum concentration to about 1 - 5%.
Strong color reaction but low absorbance values	Background values too high.	High background values may lead to low absorbance values if background is automatically subtracted by the plate reader.
	Substance or assay medium has LDH activity.	Use substance control I (section 2.1) to test substance and/or assay medium for compounds with LDH activity.
Strong color reaction in effector cells controls	Poor condition of the effector cells due to inappropriate isolation or culture conditions.	• Improve cell culture conditions. • Separate viable effector cells from dead cells by density gradient centrifugation.

4. Additional Information on this Product

How this Product Works

Cell death is classically evaluated by quantifying plasma membrane damage. The need for sensitive, quantitative, reliable and automated methods for precisely determining cell death led to the development of several standard assays that quantify cellular viability.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. A single measurement with the Cytotoxicity Detection Kit^{PLUS} (LDH) can easily determine LDH activity in culture supernatants or in whole cell cultures. The assay can be used to measure LDH directly in a culture plate even in wells that contain large numbers of cells, since the proprietory Stop Reagent increases the transparancy of the cells. A spectrophotometric microplate reader (ELISA reader) may be used to simultaneously measure multiple wells and thereby makes easy processing of a large number of samples possible. By stopping the color reaction, assay conditions can be clearly defined. The test is safe as no radioactive isotopes are used. Other benefits of the Cytotoxicity Detection Kit^{PLUS} (LDH) are suitability for high throughput because fewer handling steps are necessary and no transfer, centrifugation, or prelabeling steps are required.

Test Principle

The cell-free culture supernatant is collected 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. 5).

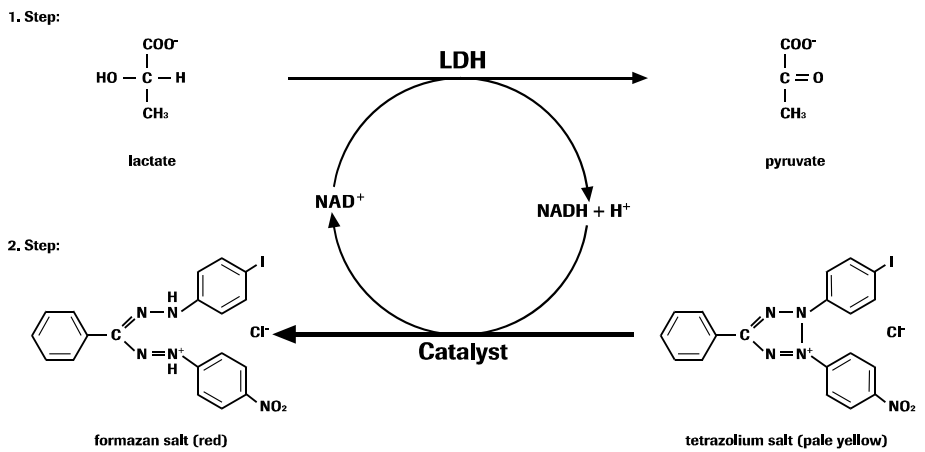

Fig. 5: Assay of released LDH. In the first step, released lactate dehydrogenase (LDH) reduces NAD^+ to $NADH^+/H^+$ by oxidizing lactate to pyruvate. In the second enzymatic reaction 2 hydrogens are transferred from $NADH^+/H^+$ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium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

An increase in the amount of dead or plasma membrane-damaged cells results in an increase of LDH 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 has a broad absorption maximum at about 500 nm, whereas the tetrazolium salt INT has no significant absorption at these wavelengths (fig. 6).

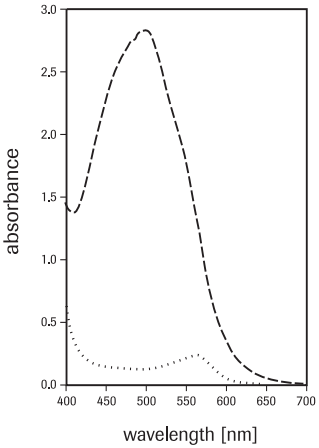

Fig. 6: Absorbance spectra of the working solution of the Cytotoxicity Detection Kit^{PLUS} (LDH). The reaction mixture of the Cytotoxicity Detection Kit^{PLUS} (LDH) was added to RPMI 1640 with 1% BSA and the absorbance spectra was measured in the absence (.....) and presence (–––) of LDH.

Sensitivity

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

Potential Sources of Test Interference

- Inherent LDH activity may be found in serum or test substances (see section 2.1).
- In cell-mediated cytotoxicity assays, the amount of LDH released from damaged effector cells may influence the assay results (see sections 2.1 and 3.3).
- Substances which inhibit the LDH or diaphorase enzyme activity influence the assay. Appropriate controls should be included in the assay (see section 2.1).
- Pyruvate is an inhibitor of the LDH reaction and is contained in some culture media (*e.g.*, some formulations of DMEM, Ham´s F12, or Iscove´s).

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5. Supplementary Information

5.1 Conventions

Text Conventions To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered instructions labeled ❶, ❷ etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

5.2 Changes to previous version

Additional information for preparation of High control.

5.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page www.roche-applied-science.com and our Apoptosis special interest site:

http://www.roche-applied-science.com/sis/apoptosis

	Detection mode/ Product	Pack Size	Cat. No.
Plasma membrane damage	**Colorimetric assay**		
	Cytotoxicity Detection Kit (LDH)	1 kit (2000 tests)	11 644 793 001
	ELISA		
	Cellular DNA Fragmentation ELISA	1 kit (500 tests)	11 585 045 001
Metabolic activity	**Colorimetric assay**		
	Cell Proliferation Reagent, WST-1	25 ml (2500 tests)	11 644 807 001
	Cell Proliferation Kit I (MTT)	1 kit (2500 tests)	11 465 007 001
	Cell Proliferation Kit II (XTT)	1 kit (2500 tests)	11 465 015 001

	Detection mode/ Product	Pack Size	Cat. No.
DNA Synthesis	**Colorimetric ELISA**		
	Cell Proliferation ELISA, BrdU (colorimetric)	1 kit (1000 tests)	11 647 229 001
	BrdU Labeling and Detection Kit III (POD)	1 kit (1000 tests)	11 444 611 001
	Chemiluminescence ELISA		
	Cell Proliferation ELISA, BrdU (chemiluminescent)	1 kit (1000 tests)	11 669 915 001
	Fluorescence microscopy, or flow cytometry		
	BrdU Labeling and Detection Kit I (Fluorescein)	1 kit (100 tests)	11 296 736 001
	In Situ Cell Proliferation Kit, FLUOS	1 kit (1000 tests)	11 810 740 001
	Light microscopy		
	BrdU Labeling and Detection Kit II (AP)	1 kit (100 tests)	11 299 964 001
Apoptosis-specific physiological change	**Gel Electrophoresis**		
	Apoptotic DNA-Ladder Kit	20 tests	11 835 246 001
DNA fragmentation	*In Situ* Cell Death Detection Kit, TMR red (also usuable for FACS)	1 kit (50 tests)	12 156 792 001
	In Situ Cell Death Detection Kit, Fluorescein (also usuable for FACS)	1 kit (50 tests)	11 684 795 001
	In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 001
	In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	11 684 817 001
	Single reagents for TUNEL and supporting reagents		
	TUNEL AP	70 tests (3.5 ml)	11 772 457 001
	TUNEL POD	70 tests (3.5 ml)	11 772 465 001
	TUNEL Enzyme	2× 50 µl	11 767 305 001
	TUNEL Label	3× 550 µl	11 767 291 001
	TUNEL Dilution Buffer	20 ml	11 966 006 001
	ELISA		
	Cell Death Detection ELISA	1 kit	11 544 675 001
	Cell Death Detection ELISA^{PLUS}	1 kit (96 tests)	11 774 425 001
	Cell Death Detection ELISA^{PLUS}, 10×	1 kit	11 920 685 001
	Cellular DNA Fragmentation ELISA	1 kit (500 tests)	11 585 045 001
Cell membrane alterations	**Microscopy or FACS**		
	Annexin-V-Biotin	250 tests	11 828 690 001
	Annexin-V-FLUOS	250 tests	11 828 681 001

Detection mode/ Product	Pack Size	Cat. No.
Annexin-V-FLUOS Staining Kit	50 tests 250 tests	11 858 777 001 11 988 549 001
Western Blot, FIENA		
Anti-Poly (ADP-Ribose) Polymerase	100 µl	11 835 238 001
Caspase 3 Activity Assay	1 kit	12 012 952 001
Homogenous Caspases Assay, fluorometric	100 tests 1000 tests	03 005 372 001 12 236 869 001
M30 CytoDEATH (formalin grade)	50 tests 250 tests	12 140 322 001 12 140 349 001
M30 CytoDEATH, Fluorescein	250 tests	12 156 857 001

Enzymatic activity

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