For life science research only.
Not for use in diagnostic procedures.

Cytotoxicity Detection Kit^{PLUS} (LDH)

Version: 08
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A nonradioactive alternative to the [^{3}H]-thymidine- and [^{51}Cr]-release assays.
Nonradioactive colorimetric assay suitable for high-throughput quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells.

Cat. No. 04 744 926 001	1 kit 400 tests in 96 wells
Cat. No. 04 744 934 001	1 kit 2,000 tests in 96 wells

Store the kit at −15 to −25°C.

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1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Catalog Number	Content
1	blue	Cytotoxicity Detection Kit^{PLUS} (LDH), Catalyst	▪ Lyophilized, stabilized ▪ Catalyst for reaction mix. ▪ Diaphorase/NAD$^+$ mixture.	04 744 926 001	1 bottle
				04 744 934 001	5 bottles
2	red	Cytotoxicity Detection Kit^{PLUS} (LDH), Dye solution	▪ Ready-to-use solution. ▪ Contains iodotetrazolium chloride (INT) and sodium lactate. ▪ Dyes the reaction mix.	04 744 926 001	1 bottle, 45 ml
				04 744 934 001	5 bottles, 45 ml each
3	white	Cytotoxicity Detection Kit^{PLUS} (LDH), Lysis solution	▪ Ready-to-use solution. ▪ Lyses the cells.	04 744 926 001	1 bottle, 3 ml
				04 744 934 001	5 bottles, 3 ml each
4	green	Cytotoxicity Detection Kit^{PLUS} (LDH), Stop solution	▪ Ready-to-use solution. ▪ Stops the LDH reaction.	04 744 926 001	1 bottle, 25 ml
				04 744 934 001	5 bottles, 25 ml each

1.2. Storage and Stability

Storage Conditions (Product)

The kit is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	blue	Catalyst	Store at −15 to −25°C.
2	red	Dye solution	
3	white	Lysis solution	
4	green	Stop solution	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- +37°C incubator
- ELISA reader for microplates, with 490 to 492 nm filter
  If a reference wavelength will be subtracted, use a filter >600 nm.
- Microscope
- Hemocytometer
- Multichannel pipettor (100 µl)
- Sterile pipette tips
- For the measurement of cell-mediated lysis and for the analysis of cytotoxic compounds, use sterile, cell-culture grade 96- or 384-well microplates.

Standard laboratory reagents

- Double-distilled water
- Assay medium, such as 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, perform the assay in the presence of low serum concentrations, such as 1% or replace serum with 1% bovine serum albumin (BSA) (w/v).

For LDH standard preparation

- LDH standard solution, such as 0.05 U LDH/ml, see section **Controls.**
  If the released LDH activity is calculated in U/ml instead of percent relative cytotoxicity or absorbance, use an appropriate LDH preparation as standard.

⚠ ***The assay medium and the LDH standard are not included in the kit; all other reagents necessary to perform 400 (1,600) or 2,000 (8,000) tests are included.***

1.4. Application

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

- Determination of the cytotoxic potential of compounds in environmental and medical research, and in the food, cosmetic, and pharmaceutical industries.
- Determination of mediator-induced cytolysis.
- Detection and quantification of cell-mediated cytotoxicity induced by cytotoxic T-lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer (LAK) cells, or monocytes.
  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.
  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.
- The assay can also be used to determine the total numbers of cells present at the end of a proliferation assay.

1.5. Preparation Time

Assay Time

Standard assay time: 15 minutes
Maximum assay time: up to 30 minutes for low cell numbers (<100 cells/well).

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The Cytotoxicity Detection Kit^{PLUS} (LDH) is used with cell-free supernatants obtained from cells cultured in 96-well microplates or batch cultures. 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 centrifugation at approximately 250 × *g*.

⚠ ***Store the cell-free culture supernatant at +2 to +8°C for several days without loss of LDH activity.***

Control Reactions

To calculate percent cytotoxicity, use the following three controls in each experimental setup.

Background control

Determines the LDH activity contained in the assay medium.

⚠ ***The absorbance value obtained in this control must be subtracted from all other 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 solution 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 approximately 9 hours, the activity of LDH in the High control may drop significantly if Lysis solution is added at the beginning of the exposure. Therefore, always add the Lysis solution to the High control at the end of the exposure period.***

⚠ ***The detergent in the Lysis solution 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 solution to the supernatant of an experimental sample (to be set up in parallel).***

The following two controls are facultative:

Substance control I

Determines the LDH activity contained in the test substance. If cell-mediated cytotoxicity is measured, this control provides information about the LDH activity released from the effector cells (= effector cell control, see section, **Measurement of cell-mediated cytotoxicity**).

Substance control II

Determines whether the test substance itself interferes with LDH activity.
To perform this control:

1. To each control sample (assayed in triplicate) in an optically clear, 96-well, flat-bottom microplate, add 50 µl assay medium containing the test substance.

2. Add 50 µl/well LDH standard solution (0.05 U/ml).

3. Add 100 µl/well Reaction mixture and measure absorbance with an ELISA reader as described in the Protocols below. This is related to the procedure used in section, **Determination of the optimal cell concentration for the assay**, and the other assays in the protocol section.

4. Compare the absorbance values in these controls with absorbance values obtained in separate (triplicate) LDH control samples that contain only 50 µl/well assay medium, 50 µl/well LDH standard solution (0.05 U/ml), and 100 µl/well Reaction mixture.

Overview of the controls

⚠ ***The background, low, and high controls must be determined in each experimental setup.***

Contents of the Well	Background Control [µl]	Low Control [µl]	High Control [µl]	Substance Control I [µl]	Substance Control II [µl]	Experimental Sample [µl]
Cell-free culture medium	100	50	50	–	–	–
Cells	–	50	50	–	–	50
Lysis buffer[(1)]	–	–	5	–	–	–
Test substance or effector cells diluted in culture medium	–	–	–	100	50	50
LDH standard solution	–	–	–	–	50	–

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

Calculations with the controls

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

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

Fig. 1: Percent cytotoxicity formula.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:

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

Fig. 2: Percent cell-mediated cytotoxicity formula.

General Considerations

Potential sources of test interference

- Inherent LDH activity may be found in serum or test substances, see section, **Controls**.
- In cell-mediated cytotoxicity assays, the amount of LDH released from damaged effector cells may influence the assay results, see sections, **Controls** and **Measurement of cell-mediated cytotoxicity**.
- Substances which inhibit the LDH or diaphorase enzyme activity influence the assay. Include appropriate controls in the assay, see section, **Controls**.
- Pyruvate is an inhibitor of the LDH reaction and is contained in some culture media, such as some formulations of DMEM, Ham's F12, or Iscove's.

Number of tests

- Kit 04 744 926 001 is for 400 tests in 96-well plates (1,600 tests in 384-well plates).
- Kit 04 744 934 001 is for 2,000 tests in 96-well plates (8,000 tests in 384-well plates).

Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis/ Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

Content	Reconstitution/Preparation of Working Solution	Storage and Stability
Catalyst (Bottle 1)	Reconstitute the lyophilizate in 1 ml double-distilled water for 10 minutes; mix thoroughly.	▪ The lyophilizate is stable at +2 to +8°C. ▪ After reconstitution, store 4 weeks at +2 to +8°C. ▪ Store 2 days at +15 to +25°C. ▪ For long-term storage up to 3 months, store at –15 to –25°C. ⚠ ***Freezing and thawing the solution up to 3 times will not significantly reduce performance.***
Dye solution (Bottle 2)	Ready-to-use solution.	▪ Once thawed, store 4 weeks at +2 to +8°C. ▪ Store 2 days at +15 to +25°C. ▪ For long-term storage up to 3 months, store at –15 to –25°C. ⚠ ***Freezing and thawing the solution up to 3 times will not significantly reduce performance. However, if you observe crystals in the Dye solution (Bottle 2), shake the bottle at least 1 hour at +37°C. Remaining precipitates will not influence the performance. Since the precipitates are formed during the freezing of the product, avoid repeated freezing and thawing.***
Lysis solution (Bottle 3)	Ready-to-use solution.	▪ Once thawed, store 4 weeks at +2 to +8°C. ▪ Store 2 days at +15 to +25°C. ▪ For long-term storage up to 3 months, store at –15 to –25°C. ⚠ ***Freezing and thawing the solution up to 3 times will not significantly reduce performance.***
Stop solution (Bottle 4)	Ready-to-use solution.	▪ Once thawed, store 4 weeks at +2 to +8°C. ▪ Store 2 days at +15 to +25°C. ▪ For long-term storage up to 3 months, store at –15 to –25°C. ⚠ ***Freezing and thawing the solution up to 3 times will not significantly reduce performance.***
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); mix thoroughly.	Always prepare fresh before use; do not store. ⚠ ***If you observe crystals in the Dye solution (Bottle 2), shake the bottle at least 1 hour at +37°C. Remaining precipitates will not influence the performance. Since the precipitates are formed during the freezing of the product, avoid repeated freezing and thawing.***

2.2. Protocols

Protocol overview

For an overview of the procedure, see Figure 3.

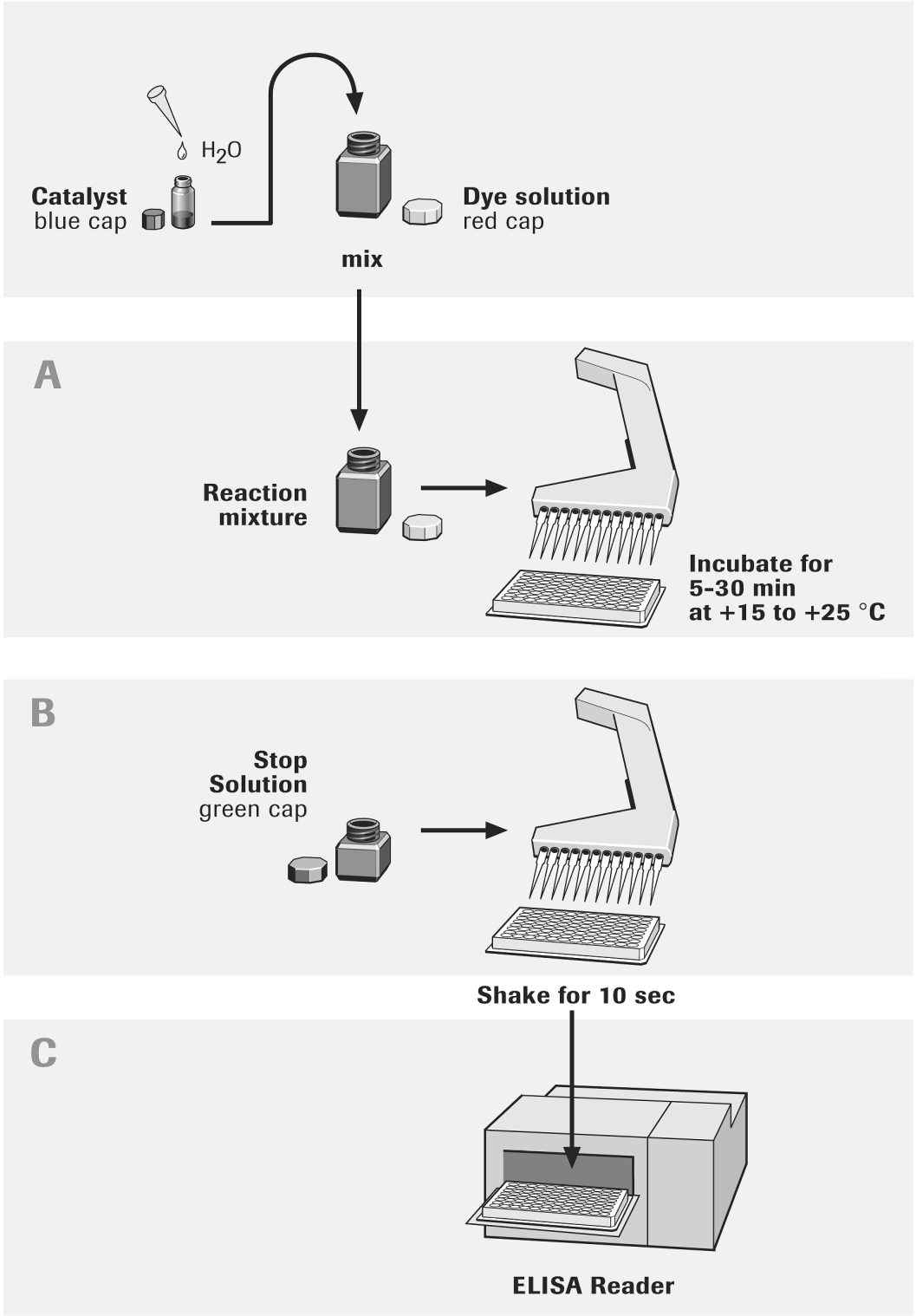

Fig. 3: Overview of protocol.

Determination of the optimal cell concentration for the assay

Different cell types may contain different amounts of LDH, therefore, determine the optimal cell concentration for a specific cell type in a preliminary experiment (Fig. 4). In general, the 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 to 1 × 10^4 cells/100 µl assay (= 0.25 to 1 × 10^5 cells/ml).
⚠ ***Perform all test samples in triplicate.***

Protocol for a 96-well microplate

To adapt the protocol to a 384-well plate, use 25 µl/well instead of 100 µl/well in Steps 2 to 4. For example, in Step 2, add 25 µl assay medium to each well of a 384-well plate.

1. Wash cells with assay medium.
   – Adjust cell suspension to a concentration of 2 × 10^6 cells/ml in assay medium.

2. Add 100 µl/well assay medium to each well of an entire 96-well, tissue-culture plate.

3. 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. Leave at least 3 wells cell-free to use as a Background control.***
   – For each cell dilution, designate 3 wells as a Low control (= spontaneous LDH release) and 3 wells as a High control (= maximum LDH release).
   – For an overview of the controls, see section, **Controls**.

4. Incubate the plate in an incubator at +37°C, 5% CO_2, and 90% humidity.
   ⚠ ***Use the same incubation time that will be used in the final assay.***

5. 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.***

6. To determine the LDH activity, add 100 µl freshly prepared Reaction mixture to each well on the 96-well plate, and incubate 5 to 10 minutes at +15 to +25°C for high cell numbers or up to 30 minutes for low cell numbers (<100 cells/well).
   – For a 384-well plate, use 25 µl/well freshly prepared Reaction mixture.
   ⚠ ***Protect the microplate from light during this incubation period, see Figure 3, A.***

7. 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 **Figure 3, B**.

8. Measure the absorbance of the samples at 490 or 492 nm according to the available filters using an ELISA reader.
   ⚠ ***Use a reference wavelength of >600 nm, see Figure 3, C.***

9. Determine the optimal cell concentration for the assay, that is the concentration with the greatest difference between the High control and Low control values.

Results

Figure 4 shows the linear relationship between cell number and absorbance at 492 nm obtained with the Cytotoxicity Detection Kit^{PLUS} (LDH) in 384- and 96-well plates. U937 cells were diluted in microplates as described above to obtain the cell concentrations indicated in the figure. Culture medium was added to determine the spontaneous release of LDH activity (control) and Lysis solution was added to determine the maximal release of LDH activity (lysed). The LDH reaction was allowed to continue for 15 minutes.
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 <100 cells/well on 384-well plates and from <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.

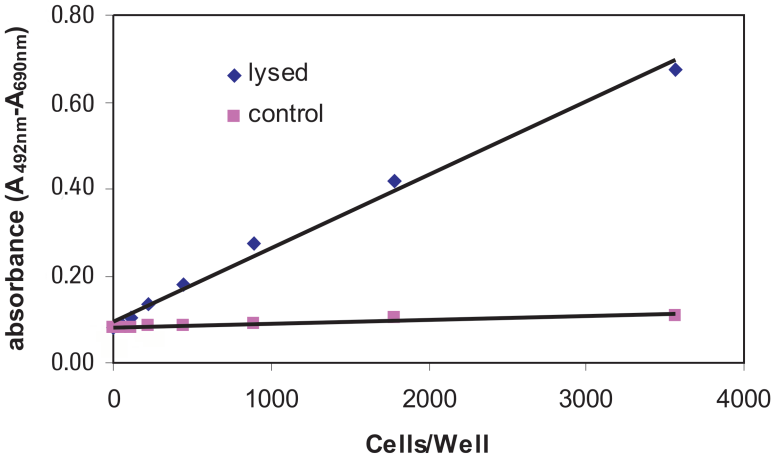

Fig. 4A: 384-well microplate
Measurement of the cytotoxic potential of soluble substance

Measurement of the cytotoxic potential of soluble substances

Protocol for a 96-well microplate

To adapt this protocol to a 384-well plate, use only ¼ the amount of cells and assay medium in Steps 1 to 3. For example, in Step 1, start with 25 µl cells/well and resuspend in 12.5 µl medium.

⚠ ***Perform all test samples in triplicate.***

1. Grow cells to the concentration determined in section, **Determination of the optimal cell concentration for the assay**.
   – 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 prepared in Step 3 below in triplicate, and prepare the two cell-containing controls listed in Step 4.***

2. Wash cells with assay medium.

3. Immediately before the experiment, use a separate microplate to prepare serial dilutions of the test substance, such as 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).

4. For an overview of the different controls, see section, **Controls**.
   – On the same plate, prepare the following controls in triplicate:

Controls	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.

5. Incubate the cells in an incubator at +37°C, 5% CO_2, and 90% humidity.
   ⚠ ***Depending on the experimental setup, use incubation times of 2 to 24 hours.***

6. 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 minutes.
   ⚠ ***Shaking the plates during lysis speeds up the process.***

7. To determine the LDH activity, add 100 µl freshly prepared Reaction mixture to each well on the 96-well plate, and incubate for up to 30 minutes at +15 to +25°C, see **Figure 3, A**.
   – For a 384-well plate, use 25 µl/well Reaction mixture.
   ⚠ ***Protect the microplate from light during this incubation period.***

8. Add 50 µl Stop solution to each well on the 96-well plate, see **Figure 3, B**.
   – For a 384-well plate, use 12.5 µl/well Stop solution.
   – Shake the plate for 10 seconds.

9. Measure the absorbance of the samples at 490 or 492 nm according to the available filters using an ELISA reader, see **Figure 3, C**.
   ⚠ ***Use a reference wavelength of >600 nm.***

10. Calculate the percent cytotoxicity for each sample as described in section, **Calculations with the controls**.

Results

The results from Figures 5 and 6 show experiments with a suspension cell line U937 and an adherent cell line WEHI 164.

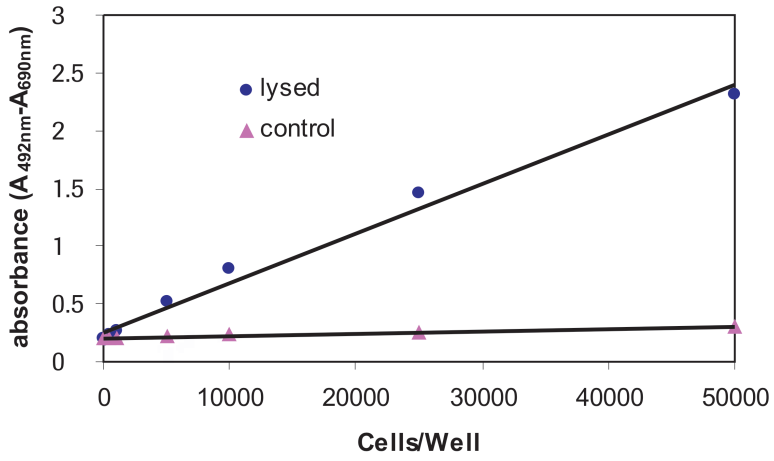

Fig. 4B: 96-well microplate

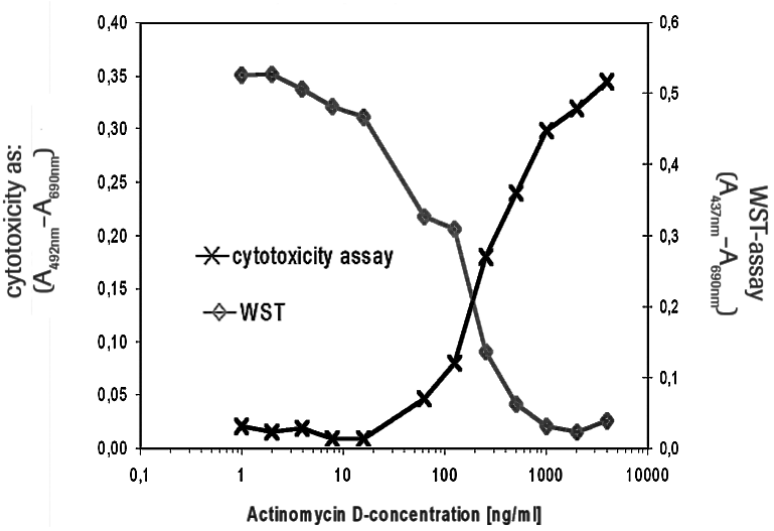

Fig. 5: Determination of cytotoxic activity of actinomycin D on U937 suspension cells. U937 cells were seeded into fresh medium at a density of 10,000 cells/well in a 96-well plate. Different concentrations of actinomycin D were added to the cultures and incubated for 16 hours, and cytotoxicity was measured with the Cytotoxicity Detection Kit^{PLUS} (LDH). In a separate set of control wells, treated in the same way, cell proliferation was measured with the Cell Proliferation Reagent, WST-1*. The results show that both types of assays give similar IC_{50} values.

Measurement of cell proliferation

To use the Cytotoxicity Detection Kit^{PLUS} (LDH) to perform a proliferation assay, let the cells proliferate, and at the end of an experiment, lyse the cells using the Lysis solution supplied with the kit. Measure the total released LDH with the Reaction mixture in the kit. You do not need to use the Low or High controls.

Protocol for a 96-well microplate

⚠ ***Perform all test samples in triplicate.***

To adapt this protocol to a 384-well plate, see sections, **Determination of the optimal cell concentration for the assay** and **Measurement of the cytotoxic potential of soluble substances**.

1. 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.

2. Wash cells with assay medium.

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

4. For an overview of the different controls, see section, **Controls**.
   – On the same plate, prepare the following controls in triplicate:

Controls	Add to each well
Background control	100 µl assay medium only.
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.

5. Incubate the cells in an incubator at +37°C, 5% CO_2, and 90% humidity for an appropriate time.

6. 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.***

7. To determine the LDH activity, add 100 µl freshly prepared Reaction mixture to each well and incubate for up to 30 minutes at +15 to +25°C, see **Figure 3, A**.
   ⚠ ***Protect the microplate from light during this incubation period.***

8. Add 50 µl Stop solution to each well.
   – Shake the plate for 10 seconds, see **Figure 3, B**.

9. Measure the absorbance of the samples at 490 or 492 nm according to the available filters using an ELISA reader, see **Figure 3, C**.
   ⚠ ***Use a reference wavelength of >600 nm.***

10. Calculate the percent cytotoxicity for each sample as described in section, **Calculations with the controls**.

Results

Figures 4A and 4B in section, **Determination of the optimal cell concentration for the assay** show that the number of cells in the lysed cell control is proportional to the LDH signal intensity.

Measurement of cell-mediated cytotoxicity

Sample arrangement on a 96-well microplate

⚠ ***Perform all test samples 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

Protocol for a 96-well microplate

⚠ ***Perform all test samples in triplicate.***
To adapt this protocol to a 384-well plate, see sections, **Determination of the optimal cell concentration for the assay** and M**easurement of the cytotoxic potential of soluble substances**.

1. Titrate effector cells, such as NK cells, LAK cells, and CTLs into the appropriate assay medium in sterile, 96-well, tissue-culture plates by serial dilutions (final volume of each dilution = 50 µl/well).

2. 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 in section**, Determination of the optimal cell concentration for the assay.**

3. Add 50 µl/well target cell suspension to the dilutions of effector cells (= effector-target cell mix), see **Sample arrangement on a 96-well microplate**.

4. For the the different controls, see section, **Controls**.
   – On the same plate, prepare the following controls in triplicate:

Controls	Add to each well
Background control	100 µl assay medium only.
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	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.***

5. Incubate the cells in an incubator at +37°C, 5% CO_2, and 90% humidity for the appropriate time period.

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

7 To determine the LDH activity, add 100 µl freshly prepared Reaction mixture to each well, and incubate for up to 30 minutes at +15 to +25°C, see **Figure 3, A**.
⚠ ***Protect the microplate from light during this incubation period.***

8 Add 50 µl Stop solution to each well.
– Shake the plate for 10 seconds, see **Figure 3, B**.

9 Measure the absorbance of the samples at 490 or 492 nm according to the available filters using an ELISA reader, see **Figure 3, C**.
⚠ ***Use a reference wavelength of >600 nm.***

10 Calculate the percent cytotoxicity for each sample as described in section, **Calculations with the controls**.

2.3. Parameters

Sensitivity

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

3. Troubleshooting

Observation	Possible cause	Recommendation
Weak color reaction.	Cell concentration is too low.	Titrate cell concentration.
	Substance or assay medium inhibits LDH activity.	Use Substance control II, see section, **Controls**, to test substance and/or assay medium for compounds inhibiting LDH activity.
		Avoid culture media containing pyruvate.
Strong color reaction present in Low controls.	Cell concentration is too high.	Titrate cell concentration.
	Substance or assay medium have LDH activity.	Use Substance control I, see section, **Controls**, to test substance and/or assay medium for compounds with LDH activity.
	High spontaneous release 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 at short incubation times. Increase serum concentration to approximately 1 to 5%.
Strong color reaction with 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 have LDH activity.	Use Substance control I, see section, **Controls**, to test substance and/or assay medium for compounds with LDH activity.
Strong color reaction in effector cells controls.	Poor conditions 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

4.1. Test Principle

The cell-free culture supernatant is collected and incubated with the reaction mixture from the kit. LDH activity is determined in an enzymatic test:

1. NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate.

2. The catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan (Fig. 7).

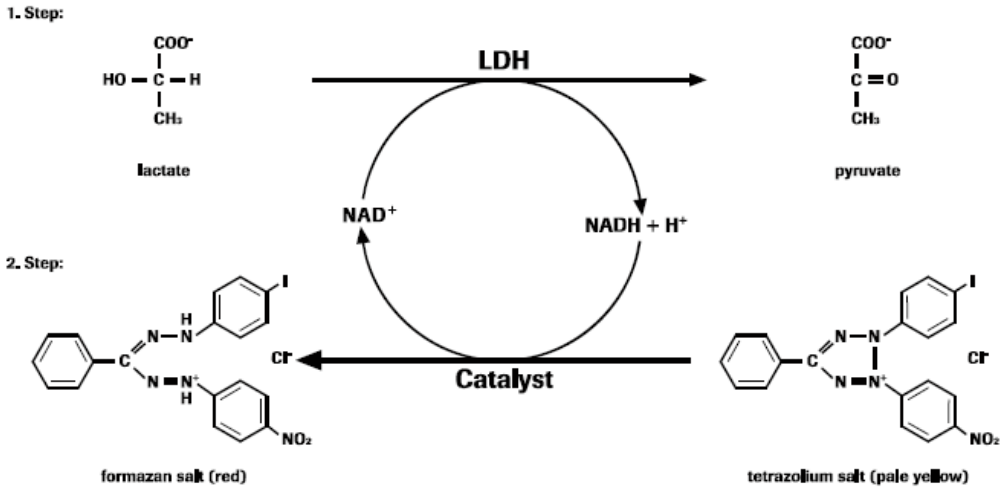

Fig. 7: Assay of released LDH. In the first step, released lactate dehydrogenase (LDH) reduces NAD^+ to $NADH^+/H^+$ by oxidizing lactate to pyruvate. In the second enzymatic reaction 2 hydrogens are transferred from $NADH^+/H^+$ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium 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 approximately 500 nm, whereas the tetrazolium salt INT shows no significant absorption at these wavelengths (Fig. 8).

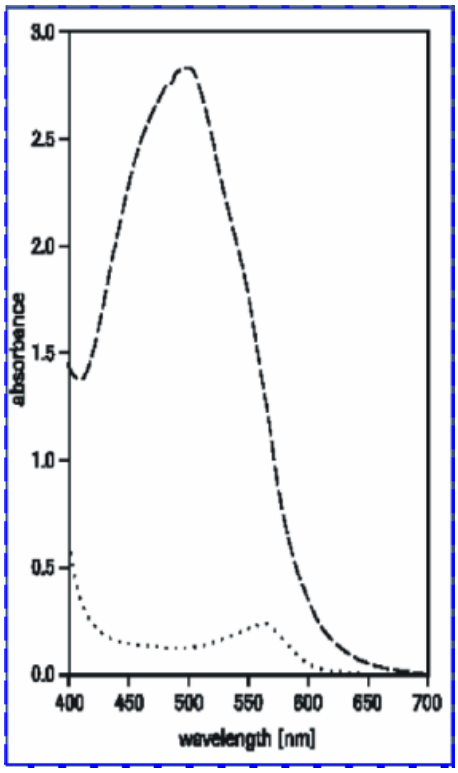

Fig. 8: Absorbance spectra of the working solution of the Cytotoxicity DetectionKit^{PLUS}(LDH). The Reaction mixture of the Cytotoxicity DetectionKit^{PLUS}(LDH) was added to RPMI 1640 with 1% BSA and the absorbance spectra was measured in the absence (.....) and presence (-----) of LDH.

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 for the quantification of 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 proprietary Stop solution increases the transparency 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 kit are suitability for high throughput because fewer handling steps are necessary and no transfer, centrifugation, or prelabeling steps are required.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Layout changes.
Editorial changes.
New information added related to the REACH Annex XIV.
Update to include new safety Information to ensure handling according controlled conditions.

5.3. Trademarks

All product names and trademarks are the property of their respective owners.

5.4. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.5. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.7. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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