User Manual

# LDH Cytotoxicity Detection Kit User Manual

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# I. Introduction

Cell death is typically assayed by quantifying plasma membrane damage. Many standard methods are based on the uptake or exclusion of vital dyes (Cook & Mitchell, 1989; Yuhas *et al* 1974; Parks, *et al*. 1979; Jones & Senft, 1985), or on the release of radioactive isotopes, fluorescent dyes, or calcein-AM from prelabeled target cells (Oldham *et al*, 1977; Leibold & Bridge, 1979; Kolber *et al*, 1988; Danks *et al*, 1992). Other assays measure cytoplasmic enzymes released by damaged cells, where the amount of enzyme activity detected in the culture supernatant correlates with the proportion of lysed cells (Decker & Lohmann-Mathes, 1988; Szekeres *et al*, 1981; Masanet *et al*, 1988; Martin & Clynes, 1991). Enzyme release assays have been described for alkaline and acid phosphatase, glutamate-oxalacetate transaminase, glutamate pyruvate transaminase and arginosuccinate lyase. However, their use has been hampered by the low amount of these enzymes present in many cells, and by the elaborate kinetic assays required to quantitate most enzyme activities.

In contrast, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the culture supernatant. The **LDH Cytotoxicity Detection Kit** provides a simple and precise colorimetric method to measure LDH activity. Cell-free culture supernatant is collected and incubated with the reaction mixture from the kit. LDH activity is determined by a colorimetric assay: In the first step, $NAD^+$ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, a catalyst included in the reaction mixture (diaphorase) transfers $H/H^+$ from $NADH/H^+$ to the tetrazolium salt INT, which is reduced to a formazan dye (Figure 1).

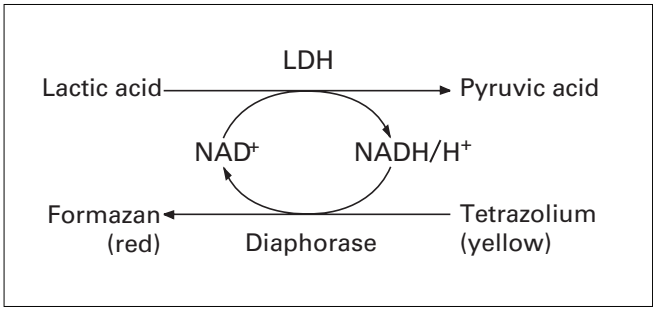


**Figure 1. A two-step enzymatic reaction quantifies cell lysis and cell death.**

An increase in the number of dead or plasma membrane-damaged cells leads to increased LDH activity in the culture supernatant, which directly correlates with the amount of formazan produced in a defined time period. Therefore, the amount of dye produced is proportional to the number of lysed (dead or plasma membrane-damaged) cells. The red formazan dye product is water soluble and shows a broad absorbance maximum around 500 nm, while the tetrazolium salt INT shows little absorbance at this wavelength (Figure 2).

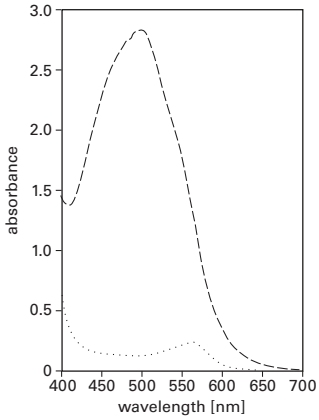


**Figure 2. Absorbance spectra of the LDH Cytotoxicity Detection Kit Reaction Mixture.** The reaction mixture from the LDH Cytotoxicity Detection Kit was added to RPMI 1640 media with 1% BSA and the absorbance spectra was measured in the absence (...) and presence (---) of LDH.

# I. Introduction continued

Proven uses of LDH assays include:

- Determination of the cytotoxic potential of compounds (Dubar *et al.*, 1993; Kondo, *et al.*, 1993; Murphy *et al.*, 1993; Courjault *et al.*, 1993; Shrivastava *et al.*, 1992; Gelderblom *et al.*, 1993; Thomas *et al.*, 1993; Sasaki *et al.* 1992).
- Detection and quantification of cell-mediated cytotoxicity induced by cytotoxic T-lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer (LAK) cells or monocytes (Decker & Lohmann-Matthes, 1988; Korzeniewski & Callewaert, 1983).
- Determination of mediator-induced cytolysis (Decker & Lohmann-Matthes, 1988).
- Measurement of antibody-dependent cellular cytotoxicity and complement-mediated cytolysis.
- Determination of cell death in bioreactors (Goergen *et al.*, 1993; Legrand *et al.*, 1992; Racher *et al.*, 1990).

The LDH Cytotoxicity Detection Kit provides several advantages over other cell proliferation assay reagents:

- **Safety:** No radioactive isotopes are used.
- **Accuracy:** Assay results obtained with this kit strongly correlate with the number of damaged cells. Furthermore, there is a good correlation between results from the LDH Cytotoxicity Detection Kit assay and the [$^{51}$Cr] release assay (Decker & Lohmann-Matthes, 1988; Korzeniewski & Callewaert, 1983).
- **High sensitivity:** Low numbers of dead cells can be detected (e.g., 2 x $10^3$–2 x $10^4$ cells/well, in a 96-well plate).
- **Speed:** The assay takes only 0.5–1 hr, including harvesting supernatants and performing and measuring the enzymatic reaction. Large numbers of samples can be processed simultaneously with a multiwell plate reader.
- **Simple procedure:** No need for prelabeling or washing steps. Cleanup and waste disposal are simplified since this kit does not employ radioactive isotopes.

# II. List of Components

Store unopened LDH Cytotoxicity Detection Kit reagents at –20°C. Dissolved catalyst and thawed dye solution can be stored for several weeks at 4°C.

The LDH Cytotoxicity Detection Kit contains 5 bottles of Catalyst (lyophilized) and 5 bottles of Dye Solution, enough for 2,000 reactions.

**LDH Cytotoxicity Detection Kit (Cat. No. 630117)**

- 5 bottles Catalyst (lyophilized)
- 5 bottles Dye Solution (45 ml each)

**Supporting Documents**

- LDH Cytotoxicity Detection Kit User Manual (PT3947-1)

Visit our web site at **www.clontech.com** for a current list of Cell Signaling/Apoptosis products.

# III. Additional Materials Required

The following materials are required but not supplied:

- Incubator (37°C)
- Centrifuge with rotor for 96-well plates
- Multiwell plate reader with a 490 or 492 nm filter. If a reference wavelength is to be subtracted, an additional filter above 600 nm is required.
- Multichannel pipettor (100 µl)
- Sterile pipette tips
- 96-well plates (sterilized, cell culture quality) for measurements of cell-mediated lysis and for the analysis of cytotoxic compounds:
  - For suspension cells: round or V-bottom 96-well plates
  - For adherent cells: flat-bottom 96-well plates
  - For color development *in all assays*: optically clear, flat-bottom 96-well plates
- Assay Medium (e.g., medium containing 1% serum or 1% bovine serum albumin). Both human and animal sera contain LDH, which may increase the background absorbance of the assay. Therefore, we recommend performing the assay in the presence of low serum concentrations (e. g., 1%) or replacing the serum with 1% bovine serum albumin (w/v).
- Triton X-100 lysing solution (2% Triton X-100 in assay medium). The maximum LDH activity is determined by lysing the cells with Triton X-100 (final concentration 1%). At this concentration, Triton X-100 does not interfere with LDH activity.
- HCl stop solution (1 N). The reaction product can be measured without adding a stop solution. Alternatively, the enzyme reaction can be stopped by adding 50 µl of 1 N HCl to each well (final concentration: 0.2 N HCl).
- LDH standard solution. If the released LDH activity must be calculated as Units/ml instead of percent cytotoxicity, we recommend that you prepare a standard curve using an LDH standard solution.
- Microscope
- Hemacytometer

# IV. LDH Cytotoxicity Detection Kit Protocol Overview

***PLEASE READ ALL PROTOCOLS IN THEIR ENTIRETY BEFORE BEGINNING.***
***Successfully implementing the LDH Cytotoxicity Detection Kit consists of performing the steps listed below, all of which are included in this user manual.***

## A. Protocol Overview

The LDH Cytotoxicity Detection Kit is a simple colorimetric assay to quantitate cytotoxicity/cytolysis, and is based on the measurement of LDH activity released from damaged cells into the supernatant (Table I). The cell-free culture supernatant is collected and incubated with the Reaction Mixture to determine LDH activity and quantify cell death. Specific protocols are included for four different types of experiments:

- Measuring cytotoxicity caused by soluble compounds for suspended cells.
- Measuring cytotoxicity caused by soluble compounds for adherent cells.
- Measuring cell-mediated cytotoxicity caused by effector cells.
- Measuring cell death in fermentation cultures.

**Table I: Protocol Overview**

| Step | Procedure | Time |
|---|---|---|
| Optimizing Experimental Conditions | | |
| 1. | Prepare working solutions | 15 min |
| 2. | Optimize cell concentration | Incubation time + approx. 45 min |
| Performing the Experiment | | |
| 1. | Incubate cells with test substance or cytotoxic effector cells | 2–24 hr |
| 2. | Prepare working solutions | 15 min |
| 3. | Centrifuge the 96-well plate containing the cells | 10 min |
| 4. | Transfer cell-free culture supernatant to optically clear, flat-bottom 96-well plate | |
| 5. | Incubate the supernatant with freshly prepared Reaction Mixture containing the tetrazolium salt, INT | approx. 10–30 min |
| 6. | (Optional) Stop the reaction by adding 1 N HCl to each well | |
| 7. | Measure absorbance at 490 or 492 nm (reference wavelength 690 nm) and calculate percent cytotoxicity | |

## B. Experiment Controls

To calculate the percent cytotoxicity, you must perform the following three controls in *every* experiment:

- **Background Control:** Measures the LDH activity present in the assay medium. The background absorbance must be subtracted from all other absorbance measurements.
- **Low Control:** Measures the level of spontaneous LDH release from untreated cells.
- **High Control:** Measures the maximum LDH activity that can be released from the 100% dead cells (in response to Triton X-100).

In addition, the following two controls may be required to perform your experiment or for troubleshooting:

- **Substance Control I:** Measures the LDH activity contained in the test substance. Alternatively, if cell-mediated toxicity is measured, this control provides information on the LDH activity released from the effector cells (e.g., NK cells, LAK cells, CTLs).
- **Substance Control II:** Determines whether the test substance itself interferes with LDH activity.

**Note:** Perform all assays, including controls, in triplicate.

# V. Sample Preparation and Experiment Setup

## A. Protocol: Preparing Working Solutions

Prepare working solutions as directed in Table II.


Recipes

**Table II: Working Solutions**

| Solution | Preparation | Storage and Stability |
|---|---|---|
| Catalyst (bottle 1; blue cap) | Reconstitute the lyophilate in 1 ml distilled water and mix thoroughly for 10 min. | Several weeks at 4° C. |
| Dye Solution (bottle 2; red cap) | Thawed INT dye solution is ready to use. | Several weeks at 4° C. |
| Reaction Mixture | **For 100 tests:** Shortly before use, mix 250 µl of Catalyst (bottle 1) with 11.25 ml of Dye Solution (bottle 2).<br>**For 400 tests:** Shortly before use, mix the total volume (1 ml) of *one bottle* of Catalyst (bottle 1) with the total volume (45 ml) of *one bottle* of Dye Solution (bottle 2). |  Prepare immediately before use. The reaction mixture cannot be stored. |

## B. Protocol: Determining the Optimal Cell Concentration


**Protocol Incubation time, plus 1 hr**

LDH release varies among cell types. Therefore, you must determine the optimal cell concentration (where the difference between the High and Low Controls is at a maximum) for each cell type. The optimal cell concentration for most cell lines is between 5 x $10^3$ and 2 x $10^4$ cells/well in 200 µl of media (2.5 x $10^4$–1 x $10^5$ cells/ml).

1. Fill the entire 96-well plate with 100 µl/well assay medium.
2. Wash the cells with assay medium, and then adjust the cell suspension to a concentration of 2 x $10^6$ cells/ml with assay medium.
3. Table III outlines the plate setup: the 96-well plate is divided in half, with identical dilutions of the cell suspension in wells B1–H6 and B7–H12. Titrate the cells by twofold serial dilutions in assay medium, using a multichannel pipette:
   a. Transfer 100 µl/well cell suspension into wells B1–B3 and B7–B9 (Dilution 1).
   b. Transfer 100 µl of the diluted cell suspension from these wells into C1–C3 and C7–C9 (Dilution 2). Repeat this step 12 times to prepare all the cell suspension dilutions.

**Table III: Plate Layout to Optimize Cell Concentration**

| | 1 2 3 | 4 5 6 | 7 8 9 | 10 11 12 |
|---|---|---|---|---|
| A | Background Control | | | |
| B | 1:1 cells:medium | $2^{-7}$:1 cells:medium | 1:1 cells:Triton X-100 | $2^{-7}$:1 cells:Triton X-100 |
| C | 0.5:1 cells:medium | $2^{-8}$:1 cells:medium | 0.5:1 cells:Triton X-100 | $2^{-8}$:1 cells:Triton X-100 |
| D | 0.25:1 cells:medium | $2^{-9}$:1 cells:medium | 0.25:1 cells:Triton X-100 | $2^{-9}$:1 cells:Triton X-100 |
| E | $2^{-3}$:1 cells:medium | $2^{-10}$:1 cells:medium | $2^{-3}$:1 cells:Triton X-100 | $2^{-10}$:1 cells:Triton X-100 |
| F | $2^{-4}$:1 cells:medium | $2^{-11}$:1 cells:medium | $2^{-4}$:1 cells:Triton X-100 | $2^{-11}$:1 cells:Triton X-100 |
| G | $2^{-5}$:1 cells:medium | $2^{-12}$:1 cells:medium | $2^{-5}$:1 cells:Triton X-100 | $2^{-12}$:1 cells:Triton X-100 |
| H | $2^{-6}$:1 cells:medium | $2^{-13}$:1 cells:medium | $2^{-6}$:1 cells:Triton X-100 | $2^{-13}$:1 cells:Triton X-100 |
| | Low Controls | | High Controls | |

# V. Sample Preparation and Experiment Setup continued

4. Prepare the following controls:
   a. Background control: Add 100 µl assay medium to wells A1–A3.
   b. Low Controls for spontaneous LDH release: Add 100 µl/well assay medium to Rows B–H, Columns 1–6.
   c. High Controls for maximal LDH release: Add 100 µl/well Triton X-100 to Rows B–H, Columns 7–12.
5. Incubate the plate in a humidified atmosphere (37˚C, 5% $CO_2$, 90% humidity) for the time required to assay the test substances in your experiment (typically in the range of 2–24 hr)

   **Note:** Each well contains a total volume of 200 µl: 100 µl of assay medium, plus either 100 µl of cell suspension (steps 2–3) or 100 µl of Control substance (step 4).

6. Centrifuge the plate at 250 x g for 10 min.
7. Remove 100 µl/well supernatant carefully, without disturbing the cell pellet. Transfer the supernatant into the corresponding wells of an optically clear 96-well flat-bottom plate.
8. Add 100 µl Reaction Mixture (freshly prepared; Part V. A) to each well and incubate at room temperature for 30 min, protected from light.
9. Measure the absorbance of the samples at 490 or 492 nm. The reference wavelength should be greater than 600 nm.
10. Calculate the average absorbance value of each triplicate, and subtract the average value for the Background Control from the triplicate average. Compare the Low and High Control values at each cell concentration. The cell concentration with the greatest difference between the absorbance of the Low and High Controls is the optimal cell concentration for your experiment (see Appendix A, part A for an example).

# VI. Measuring Cytotoxicity

Once you have optimized the cell concentration for your experiment (Part V. B), the assay steps are similar in each case, although the sample preparation varies (Protocols A–D below). In brief, the assay steps are:

1. Incubate the optimal concentration of cells in a 96-well plate with the test substance or effector cells (at varying concentrations).
2. Centrifuge the 96-well plate.
3. Transfer cell-free supernatant to an optically clear, flat-bottom 96-well plate.
4. Add freshly prepared Reaction Mixture and incubate to allow the enzymatic reaction to take place (Figure 1).
5. (Optional) Add 1 N HCl (not included in the kit) to stop the reaction.
6. Measure absorbance at 490 or 492 nm (reference wavelength greater than 600 nm)

All assays must be performed in triplicate.


**Protocol**
**30 min**

## A. Protocol: Preparing Cell Suspensions to Measure Cytotoxicity Due to Soluble Compounds

1. Titrate test substance (in triplicate) in assay medium into wells B1–G6 of a sterile 96-well plate by twofold serial dilution (final volume: 100 µl/well).

   **Note:** Do not add test substance to the Control wells (A1–A6 and H1–H6; see Table IV).

2. Wash the cells in assay medium and dilute the cell suspension to the optimal concentration determined in Part V. B. Add 100 µl/well cell suspension to the dilutions of the test substance.

   **Note:** Do not add cells to the Control wells (A1–A6 and H1–H6; see Table IV).

3. Prepare the following controls on the plate. (See Table IV)
   a. Background Control: Add 200 µl assay medium to triplicate wells A1–A3.
   b. Substance Control I: Add 100 µl test substance (at the maximum concentration used in the experiment) and 100 µl assay medium to triplicate wells A4–A6.
   c. Low Control for spontaneous LDH release: Add 100 µl cell suspension and 100 µl assay medium to triplicate wells H1–H3.
   d. High Control for maximum LDH release: Add 100 µl cell suspension and 100 µl Triton X-100 solution to triplicate wells H4–H6.
4. Proceed to Protocol E.

# VI. Measuring Cytotoxicity continued

**Protocol overnight, plus 30 min**

## B. Protocol: Preparing Adherent Cells to Measure Cytotoxicity Due to Soluble Compounds

1. Plate 100 µl cell suspension (in culture medium, at the optimal concentration determined in Part V. B) into wells B1–H6 of a sterile 96-well plate (see Table IV).

   **Note:** Do not add cells to the Background Control or Substance Control I wells (A1–A6; see Table IV).

2. Incubate the cells overnight (e.g. 37°C, 5% $CO_2$, 90% humidity) to allow the cells to adhere tightly.
3. Immediately before use, titrate the test substance in assay medium in wells B1–G6 *of a separate 96-well plate* by twofold serial dilution (final volume of 200 µl/well).

   **Note:** Do not add the test substance to the wells corresponding to the Control wells (A1–A6 and H1-H6; see Table IV).

4. Remove the culture medium from the adherent cells (to remove LDH released from the cells during the overnight incubation). Add 100 µl fresh assay medium to each well.
5. Transfer 100 µl of the test substance dilutions into the corresponding wells containing adherent cells.

   **Note:** Do not add the test substance to the Control wells (A1–A6 and H1-H6; see Table IV).

6. Prepare the following controls on the plate (see Table IV).
   a. Background Control: Add 200 µl assay medium to triplicate wells A1–A3.
   b. Substance Control I: Add 100 µl test substance (at the maximum concentration used in the experiment) and 100 µl/well assay medium to triplicate wells A4–A6.
   c. Low Control for spontaneous LDH release: Add 200 µl assay medium to triplicate wells H1–H3.
   d. High Control for maximum LDH release: Add 100 µl assay medium and 100 µl Triton X-100 solution to triplicate wells H4–H6.
7. Proceed to Protocol E.

**Table IV: Plate Layout to Measure Cytotoxicity Due to Soluble Compounds**

| | 1 2 3 | 4 5 6 | 7 8 9 | 10 11 12 |
|---|---|---|---|---|
| A | Background Control | Substance Control I | | |
| B | 1:1 test substance:cells | $2^{-6}$:1 test substance:cells | | |
| C | 0.5:1 test substance:cells | $2^{-7}$:1 test substance:cells | | |
| D | 0.25:1 test substance:cells | $2^{-8}$:1 test substance:cells | | |
| E | $2^{-3}$:1 test substance:cells | $2^{-9}$:1 test substance:cells | | |
| F | $2^{-4}$:1 test substance:cells | $2^{-10}$:1 test substance:cells | | |
| G | $2^{-5}$:1 test substance:cells | $2^{-11}$:1 test substance:cells | | |
| H | Low Control | High Control | | |

**Table IV. 96-well plate layout to assay cytotoxicity (Columns 1–6).** Note that a second test substance could be assayed in columns 7–12.

# VI. Measuring Cytotoxicity continued

Protocol
30 min

## C. Protocol: Preparing Samples to Measure Cell-Mediated Cytotoxicity

1. Table V outlines the plate setup: the 96-well plate is divided in half, with identical dilutions of the effector cell suspension in wells B1–G6 and B7–G12. Titrate the effector cells (in triplicate) by twofold serial dilutions in assay medium (final volume 100 µl/well), using a multichannel pipette.

   **Note:** Do not add effector cells to the Control wells (Rows A & H; see Table V).

2. Wash the test cells in assay medium, and dilute them to their optimal concentration (Part V. B).
3. Add 100 µl/well test cell suspension to the effector cell dilutions in wells B1–G6 (see Table V).

   **Note:** Do not add test cells to the Control wells (A1–A6 and H1–H6; see Table V) or to the effector-cell only wells (B7–G12).

4. Prepare the following controls on the plate (see Table V).
   a. Background Control: Add 200 µl assay medium to triplicate wells A1–A3.
   b. Low Control for spontaneous LDH release: Add 100 µl test cell suspension and 100 µl assay medium to triplicate wells H1–H3.
   c. High Control for maximum LDH release: Add 100 µl test cell suspension and 100 µl Triton X-100 solution to triplicate wells H4–H6.
   d. Spontaneous LDH release for each effector cell concentration: Add 100 µl assay medium to wells B7–G12.

   **Note:** Spontaneous LDH release must be determined for *each effector cell concentration* used in the assay.

5. Proceed to Protocol E.

**Table V: Plate Layout to Measure Cell-Mediated Cytotoxicity**

| | 1 2 3 | 4 5 6 | 7 8 9 | 10 11 12 |
|---|---|---|---|---|
| A | Background Control | | | |
| B | 1:1 effector:test cells | $2^{-6}$:1 effector:test cells | 1:1 effector cells:medium | $2^{-6}$:1 effector cells:medium |
| C | 0.5:1 effector:test cells | $2^{-7}$:1 effector:test cells | 0.5:1 effector cells:medium | $2^{-7}$:1 effector cells:medium |
| D | 0.25:1 effector:test cells | $2^{-8}$:1 effector:test cells | 0.25:1 effector cells:medium | $2^{-8}$:1 effector cells:medium |
| E | $2^{-3}$:1 effector:test cells | $2^{-9}$:1 effector:test cells | $2^{-3}$:1 effector cells:medium | $2^{-9}$:1 effector cells:medium |
| F | $2^{-4}$:1 effector:test cells | $2^{-10}$:1 effector:test cells | $2^{-4}$:1 effector cells:medium | $2^{-10}$:1 effector cells:medium |
| G | $2^{-5}$:1 effector:test cells | $2^{-11}$:1 effector:test cells | $2^{-5}$:1 effector cells:medium | $2^{-11}$:1 effector cells:medium |
| H | Low Control | High Control | | |

Protocol
Culture time,
then 30 min

## D. Protocol: Assaying Cell Death in Fermentation Cultures

1. Collect samples from the cell culture (0.5–1 ml) at regular intervals of 12–24 hr.
2. Centrifuge each sample and carefully remove the culture supernatant without disturbing the cell pellet.

   **Note:** The cell-free supernatant can be stored at 4°C for several days without loss of LDH enzyme activity.

3. Titrate the culture supernatants with culture medium by serial dilutions into an optically clear 96-well flat-bottom plate (final volume 100 µl/well).
4. Proceed to Protocol E, *step 4*.

# VI. Measuring Cytotoxicity continued

**Protocol**
**Incubation time, plus 45 min**

### E. Protocol: Measuring LDH Release

1. Incubate the plate in an incubator (e.g. 37°C, 5% $CO_2$, 90% humidity) for the time required to assay the test substance or effector cells (2–24 hr).
2. After incubation, centrifuge the plate at 250 x g for 10 min.
3. Carefully remove 100 µl of supernatant from each well (do not disturb the cell pellet) and transfer into the corresponding wells of an optically clear 96-well flat-bottom plate.
4. Add 100 µl freshly prepared Reaction Mixture (Part V.A) to each well and incubate for up to 30 min at room temperature, protected from light.
5. Measure the absorbance of the samples at 490 or 492 nm using a multiwell plate reader. The reference wavelength should be more than 600 nm.
6. Calculate the percent cytotoxicity according to Protocol F or Protocol G, as appropriate.

### F. Protocol: Calculating Cytotoxicity Due to Soluble Substances

Use this formula to calculate cytotoxicity due to the addition of a potentially cytotoxic substance (based on the results of Part VI, Protocols A, B, or D).

1. Calculate the average absorbance value for each triplicate. Subtract the average Background Control value from the average absorbance value for each triplicate to determine absorbance due to cell lysis (or other factors, in the case of Control triplicates).
2. Substitute the resulting values into the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{Triplicate Absorbance - Low Control}}{\text{High Control - Low Control}} \times 100$$

### G. Protocol: Calculating Percent Cell-Mediated Cytotoxicity

Use this formula to calculate cytotoxicity due to effector cells (based on the results of Part VI, Protocol C).

1. Calculate the average absorbance value for each triplicate. Subtract the average Background Control value from the average absorbance value for each triplicate to determine absorbance due to cell lysis (or other factors, in the case of Control triplicates).
2. Substitute the resulting values into the following equation, using the Effector: Test Cell Mix and Effector Cell Control values for a given concentration of effector cells.

$$\text{Cytotoxicity (\%)} = \frac{\text{(Effector:Test Cell Mix - Effector Cell Control) - Low Control}}{\text{High Control - Low Control}} \times 100$$

# VII. Troubleshooting

**Table VI: Troubleshooting the LDH Cytotoxicity Detection Kit**

| Description of Problem | Explanation | Solution |
|---|---|---|
| No color reaction | Cell concentration may be too low | Check cell concentration; titrate if necessary. |
| | Test substance or assay medium inhibit LDH activity | Use Substance Control II to measure test substance and/or assay medium for intrinsic LDH inhibition. |
| | | Avoid using media which includes pyruvate (an LDH inhibitor) |
| Strong color reaction in Low Controls | Cell concentration may be too high | Check cell concentration; titrate if necessary. |
| | Test substance or assay medium have LDH activity | Use Substance Control I to measure test substance and/or assay medium for intrinsic LDH activity. |
| | Spontaneous LDH release caused by cell death due to assay conditions | 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 | Test substance or assay medium have LDH activity | Use Substance Control I to measure test substance and/or assay medium for intrinsic LDH activity. |
| Strong color reaction in Effector Cell Controls | Spontaneous LDH release caused by effector cell damage or death, due to culture conditions or isolation method | Improve cell culture conditions. |
| | | Separate viable from dead effector cells by density gradient centrifugation. |

- **Substance Control I:** Measures the LDH activity contained in the test substance or effector cells. The procedure to perform Substance Control I is described in detail in the Part VI Protocols.
- **Substance Control II:** Determines whether the test substance itself interferes with LDH activity. To perform this control proceed as follows:

  a. Add 50 µl test substance solution (diluted in assay medium), 50 µl LDH solution (0.05 U/ml) and 100 µl freshly prepared Reaction Mixture (Part V.A) to triplicate wells.

  b. Add 50 µl assay medium, 50 µl LDH solution (0.05 U/ml) and 100 µl freshly prepared Reaction Mixture to a second set of triplicate wells (e.g., the control triplicate).

  c. Incubate for up to 30 min at room temperature, protected from light.

  d. Measure the absorbance of the samples at 490 or 492 nm using a multiwell plate reader. The reference wavelength should be more than 600 nm.

  e. Compare the average absorbance value of the triplicate containing the test substance with the average absorbance value of the control triplicate.

# VIII. References


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# Appendix A: Examples of LDH Cytotoxicity Detection Kit Results

## A. Optimizing Cell Concentration

K562 cells were titrated in 96-well plates at the concentrations indicated on the horizontal axis of Figure 3. Culture medium (o) was added to measure spontaneous LDH release (Low Control), and Triton X-100 (●) was added (final concentration 1%) to measure the maximum LDH release (High Control). The optimal cell concentration in this experiment was between 1 x $10^4$ and 1 x $10^5$ cells/well.

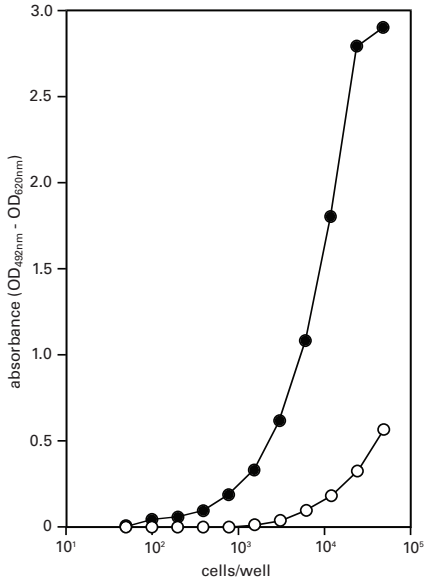


**Figure 3. Optimizing K562 cell concentration.**

## B. Measuring Detergent Cytotoxicity

Three detergents (Synperonic® F68 (■), Triton X-100 (▲) and Nonident P40 (▼) were titrated with culture medium in a 96-well plate to the final concentrations indicated on the horizontal axis of Figure 4. Subsequently, P815 cells were added (final concentration 1 × $10^4$ cells/well). The cells were incubated for 18 hr and LDH release was measured.

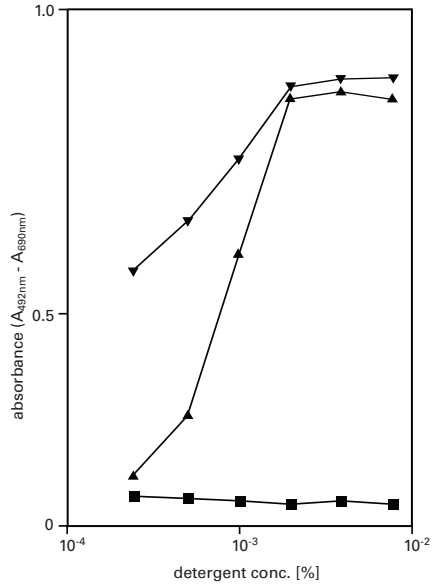


**Figure 4. Measuring detergent cytotoxicity.** Synperonic® F68 (■), Triton X-100 (▲) and Nonident P40 (▼).

# Appendix A: Examples of LDH Cytotoxicity Detection Kit Results continued

## C. Measuring Cell-Mediated Toxicity

Spleen cells of C57/Bl 6 mice (H-2b) were stimulated *in vitro* with P815 cells (H-2d). Viable cytotoxic T lymphocytes (CTLs) were purified by ficoll density gradient, washed and titrated in a 96-well plate. $1 \times 10^4$ P815 test cells/well were added to the effector CTL cells. The cell mixture was centrifuged and incubated for 4 hr. 100 µl of culture supernatant was collected to measure LDH activity.

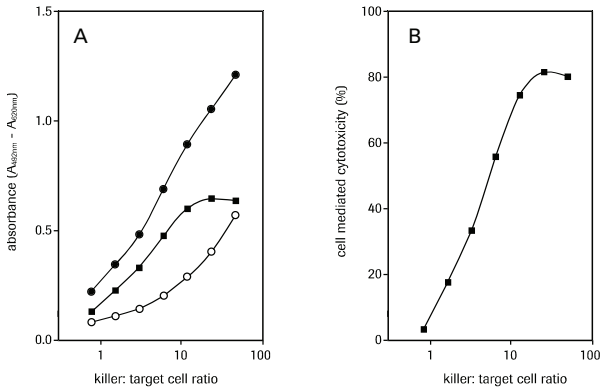


**Figure 5. Measuring the cytolytic activity of allogen-stimulated, cytotoxic T lymphocytes. Panel A.** Absorbance values. Effector cell control (O), effector-test cell mix (●), effector-test cell mix minus effector cell control (■). **Panel B.** Percent cell-mediated cytotoxicity, calculated as described in Part VI.G.