



## LDH Cytotoxicity Assay Kit

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Item No. 601170

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## GENERAL INFORMATION

### Materials Supplied

This kit will arrive packaged as a -20°C kit. After opening the kit, store the individual components as stated below.

| Item Number | Item                           | 96 wells Quantity/Size | 480 wells Quantity/Size | Storage |
|-------------|--------------------------------|------------------------|-------------------------|---------|
| 10009318    | LDH Diaphorase                 | 1 vial/1 ea            | 1 vial/5 ea             | -20°C   |
| 10009319    | LDH NAD <sup>+</sup> (100X)    | 1 vial/120 µl          | 1 vial/550 µl           | 4°C     |
| 10009320    | LDH Lactic Acid (100X)         | 1 vial/120 µl          | 1 vial/550 µl           | 4°C     |
| 10009328    | LDH INT (100X)                 | 1 vial/120 µl          | 1 vial/550 µl           | -20°C   |
| 10009321    | LDH Positive Control           | 1 vial                 | 1 vial                  | -20°C   |
| 10009322    | Cell-Based Assay Buffer Tablet | 1 tablet               | 1 tablet                | RT      |
| 601171      | Triton X-100 (10%)             | 1 vial/10 ml           | 1 vial/10 ml            | RT      |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



**WARNING:** THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

## Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

Please read these instructions carefully before beginning this assay.

## If You Have Problems

### Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888  
Fax: 734-971-3641  
Email: techserv@caymanchem.com  
Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored at the temperatures outlined in **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

## Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 490-520 nm
2. A plate centrifuge

## INTRODUCTION

### Background

Cell death can occur either by apoptosis, a highly regulated pathway involving signal transduction cascades, or by necrosis. Necrosis is accompanied by mitochondrial swelling and increased plasma membrane permeability, while apoptosis involves an articulated breakdown of the cell into membrane-bound apoptotic bodies.<sup>1</sup> There are a number of screening techniques available that detect cytotoxicity and cell death, independent of mechanism. Most of these assays assess cell viability by measuring plasma membrane permeability.<sup>2</sup>

Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium upon cell damage or lysis. LDH activity in the culture medium can, therefore, be used as an indicator of cell membrane integrity, and thus a measurement of cytotoxicity.

## About This Assay

Cayman's LDH Cytotoxicity Assay Kit measures cell death in response to chemical compounds or environmental factors using a coupled two-step reaction. In the first step, LDH catalyzes the reduction of  $\text{NAD}^+$  to  $\text{NADH}$  and  $\text{H}^+$  by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses the newly-formed  $\text{NADH}$  and  $\text{H}^+$  to catalyze the reduction of a tetrazolium salt (INT) to highly-colored formazan which absorbs strongly at 490-520 nm. The amount of formazan produced is proportional to the amount of LDH released into the culture medium as a result of cytotoxicity.

In a typical cytotoxicity assay, target cells are cultured with a cytotoxic chemical agent or a cytotoxic cell (NK cells, cytotoxic T cells) to induce target cell death and LDH release. The LDH-containing supernatants are transferred to wells of a new 96-well assay plate and mixed with the LDH Reaction Solution. After an incubation of 30 minutes at room temperature, the absorbance at 490 nm (A490) is read using a plate reader.

The results of each experiment are calculated as "% cytotoxicity", or a percentage of the total amount of LDH contained within the target cells. Thus, for each experiment it is necessary to have a set of control wells in which all of the target cells are killed using 10% Triton X-100 solution provided in the kit. These are the "maximum release" wells. Also, in each experiment it is necessary to have a set of control wells in which no cytotoxic agents or cytotoxic cells are added, resulting in only the lowest possible (spontaneous or background) LDH release. These are the "spontaneous release" wells. Cells treated with cytotoxic agents or cytotoxic cells will release an amount of LDH that falls between the maximum release level and the spontaneous release level. That level will be calculated as a % cytotoxicity using the following formula:

% Cytotoxicity of test sample =

$$\left[ \frac{(\text{Experimental Value A490}) - (\text{Spontaneous Release A490})}{(\text{Maximum Release A490}) - (\text{Spontaneous Release A490})} \right] \times 100$$

### Assay-Specific Considerations

#### 1. Assay Medium

Serum used to supplement growth medium (fetal calf serum, etc.) contains LDH that will react with the LDH Reaction Solution and induce a "background" color change (A490), even in the absence of cell death. The higher the percentage of serum in the medium, the higher the background signal will be. There are two solutions to this background problem; grow the cells in the absence of serum, or subtract the background signal from all wells prior to calculation of % cytotoxicity. Removal of serum from a growth medium can have a negative impact on overall cell viability, so this may not be an option for all cell types. Subtraction of the background signal is easier, requiring simply the addition of wells to the assay that contain medium only, without added cells. The LDH A490 signal resulting from the medium-only controls can be subtracted from all test wells after reading the plate.

#### 2. Target Cells

Different cell types contain different amounts of LDH. For cells with high LDH levels, fewer cells per well will be required to produce a strong A490 value than for cells with relatively low LDH levels. Therefore, we recommend performing an initial titration experiment to determine the optimal number of cells per well of the target cell you plan to use. Figure 3, on page 13, shows the results of a titration of HL-60 cells seeded at various concentrations per well and treated with the Triton X-100 provided in the kit to generate the maximum release LDH value for each cell titration. For HL-60, lysis of 200,000 cells per well produces a strong A490 value of ~0.4, whereas the LDH release from 10,000 cells per well or fewer is undetectable. Thus, if 200,000 HL-60 target cells are used per well in a cytotoxicity assay, it will be possible to detect the lysis of >10,000 cells (5% cytotoxicity or more), but it will not be possible to detect less than 5% cytotoxicity.

## Reagent Preparation

### 1. Assay Buffer Preparation

Dissolve the Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of distilled water. This buffer should be stable for approximately one year at room temperature.

### 2. LDH Diaphorase

**1 ea vial LDH Diaphorase (96-well kit; Item No. 10009318):** Reconstitute with 150  $\mu$ l UltraPure water.

OR

**5 ea vial LDH Diaphorase (480-well kit; Item No. 10009318):** Reconstitute with 600  $\mu$ l UltraPure water.

Keep on ice during use. Store at  $-20^{\circ}\text{C}$  when not in use. Freezing and thawing of this solution should be limited to a single time. If you plan to use this solution in multiple experiments, we recommend that you aliquot it for storage at  $-20^{\circ}\text{C}$ .

### 3. LDH Reaction Solution

To make 10 ml of LDH Reaction Solution, sufficient for use on one 96-well plate, add 100  $\mu$ l of the following to 9.6 ml of Assay Buffer:

NAD<sup>+</sup> (100X) (Item No. 10009319)

Lactic Acid (100X) (Item No. 10009320)

INT (100X) (Item No. 10009328)

Reconstituted Diaphorase (prepared in Step 2)

Any leftover LDH Reaction Solution should be discarded after use, as it is not stable. If less than a full 96-well plate is to be used in an experiment, adjust the volumes of each of the reactants accordingly. Store remaining LDH INT at  $-20^{\circ}\text{C}$ . Store remaining LDH NAD<sup>+</sup> and LDH lactic acid at  $4^{\circ}\text{C}$ .

### 4. LDH Positive Control Preparation (*optional*)

The LDH Positive Control in solution is very temperature-sensitive.

Immediately prior to use, reconstitute the LDH Positive Control (Item No. 10009321) in 1.8 ml ice-cold UltraPure water. Do not vortex, mix gently. Maintain reconstituted LDH Positive Control on ice while performing the assay. Aliquot and immediately freeze unused reconstituted LDH Positive Control at  $-80^{\circ}\text{C}$ . Frozen aliquots must be thawed on ice and thawed only once.

## ASSAY PROTOCOL

### Determination of optimal target cell number and growth medium

1. Seed target cells in a 96-well plate at a density of  $10^3$ - $10^6$  cells/well in 200  $\mu$ l of culture medium. Prepare six wells at each cell concentration.
2. Add 200  $\mu$ l of medium only (without cells) to three wells (background control).
3. Add 20  $\mu$ l of Triton X-100 (10%) (Item No. 601171) to three wells, and 20  $\mu$ l of Assay Buffer to three wells, and incubate at room temperature for one hour.
4. Centrifuge the 96-well tissue culture plate at 400 x g for five minutes (optional but recommended).
5. Transfer 100  $\mu$ l of cell supernatant to a new 96-well assay plate.
6. Add 100  $\mu$ l of LDH Reaction Solution (prepared in #3, see page 8) to each well.
7. Incubate the plate with gentle shaking on an orbital shaker for 30 minutes at 37°C.
8. Read the absorbance at 490 nm with a plate reader.
9. Repeat the assay above with culture medium containing low serum (0-2%) and high serum (10-20%) to assess the effects of serum on cell viability (spontaneous release) and background, or include extra wells in the initial assay to accommodate this variable.

| Cells per well | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|----------------|---|---|---|---|---|---|---|---|---|----|----|----|
| 200,000        | A | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 100,000        | B | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 50,000         | C | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 25,000         | D | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 12,500         | E | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 6,250          | F | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 3,125          | G | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |
| 0 (background) | H | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  | ○  | ○  |

Triton X-100    Assay Buffer    Triton X-100    Assay Buffer  
Low serum      Low serum      High serum      High serum

**Figure 1. Recommended plate format for determining optimal target cell number and serum concentration.**

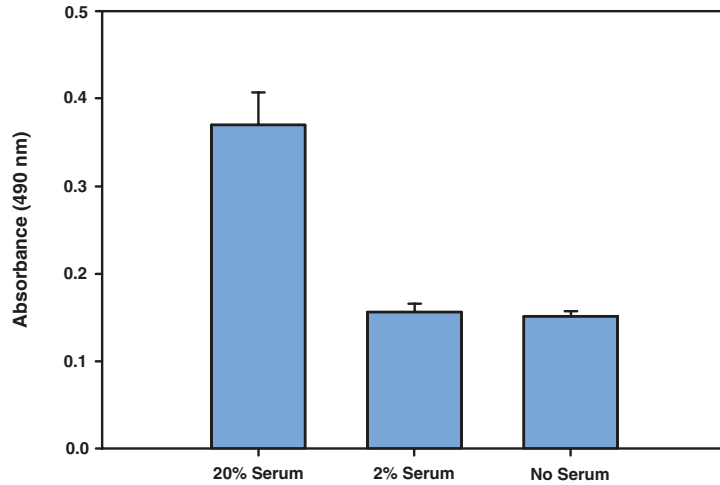


Figure 2. Background LDH in medium with the indicated amounts of FBS.

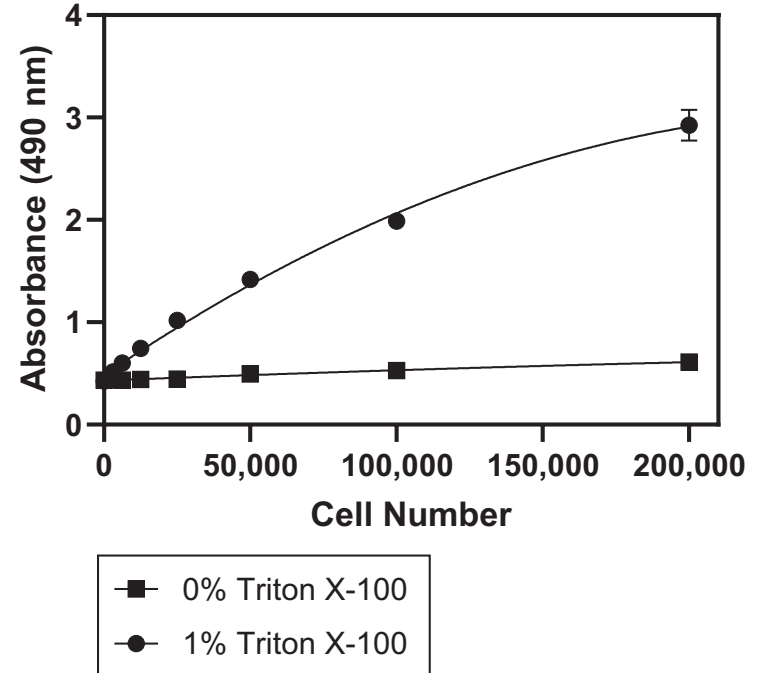


Figure 3. LDH release from Jurkat cells.

## Performing a Cytotoxicity Assay

### Procedure

1. Seed target cells in a 96-well plate at the previously determined optimal density in 200 µl of culture medium.
2. Add 200 µl of medium only (without cells) to three wells for background control, and to three wells for LDH Positive Control (optional).
3. Add 20 µl of 10% Triton X-100 solution to three wells containing cells (maximum release) and 20 µl of Assay Buffer to three wells (spontaneous release). Add 20 µl of the LDH Positive Control to three wells (optional).
4. Add 20 µl of experimental cytotoxic agent to appropriate wells in triplicate.
5. Incubate the plate in a CO<sub>2</sub> incubator at 37°C for the length of time required by your experiment to induce cytotoxicity.
6. Centrifuge the 96-well tissue culture plate at 400 x g for five minutes (optional but recommended).
7. Transfer 100 µl of cell supernatant to a new 96-well assay plate.
8. Add 100 µl of LDH Reaction Solution (prepared in #3, see page 8) to each well.
9. Incubate the plate with gentle shaking on an orbital shaker for 30 minutes at 37°C.
10. Read the absorbance at 490 nm with a plate reader.
11. Subtract background A490 levels from all wells.
12. Calculate % Cytotoxicity as follows:

% Cytotoxicity of test sample =

$$\left[ \frac{(\text{Experimental Value A490}) - (\text{Spontaneous Release A490})}{(\text{Maximum Release A490}) - (\text{Spontaneous Release A490})} \right] \times 100$$

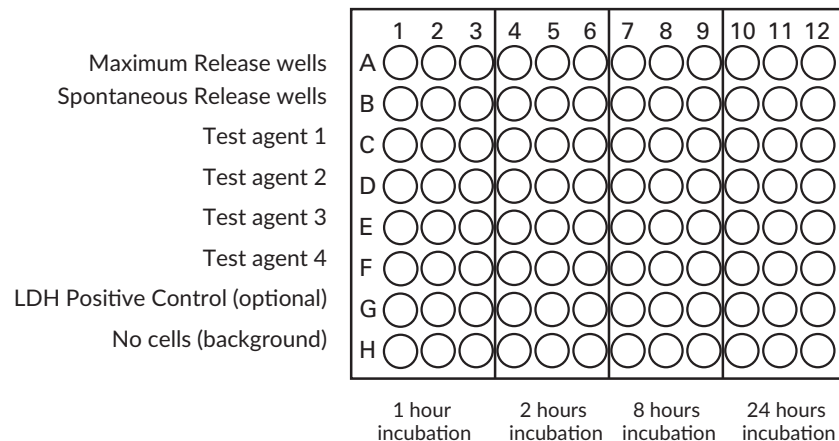


Figure 4. Recommended plate format for testing cytotoxic agents.

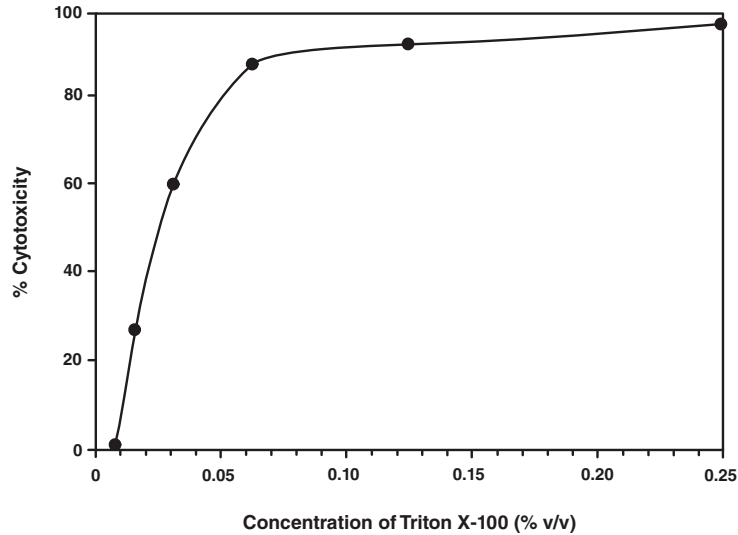


Figure 5. A typical cytotoxicity assay using HL-60 target cells.

## RESOURCES

### References

1. Bonfoco, E., Krainc, D., Ankarcrona, M., *et al.* Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* **92**, 7162-7166 (1995).
2. Haslam, G., Wyatt, D., and Kitos, P.A. Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. *Cytotechnology* **32**, 63-75 (2000).

|    |   |   |   |   |   |   |   |   |
|----|---|---|---|---|---|---|---|---|
| 1  |   |   |   |   |   |   |   |   |
| 2  |   |   |   |   |   |   |   |   |
| 3  |   |   |   |   |   |   |   |   |
| 4  |   |   |   |   |   |   |   |   |
| 5  |   |   |   |   |   |   |   |   |
| 6  |   |   |   |   |   |   |   |   |
| 7  |   |   |   |   |   |   |   |   |
| 8  |   |   |   |   |   |   |   |   |
| 9  |   |   |   |   |   |   |   |   |
| 10 |   |   |   |   |   |   |   |   |
| 11 |   |   |   |   |   |   |   |   |
| 12 |   |   |   |   |   |   |   |   |
|    | A | B | C | D | E | F | G | H |

## NOTES

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