INSTRUCTIONS

Pierce LDH Cytotoxicity Assay Kit

88953 88954 2503.2

Number	Description
88953	**Pierce LDH Cytotoxicity Assay Kit**, sufficient for 200 reactions in 96-well plates or 800 reactions in 384-well plates

Kit Contents:

Substrate Mix, lyophilizate, store at -20°C protected from light

Assay Buffer, 0.6mL, store at -20°C protected from light

10X Lysis Buffer, 2.5mL, store at 4°C

Stop Solution, 12mL, store at 4°C

LDH Positive Control, 6µL, store at 4°C

88954	**Pierce LDH Cytotoxicity Assay Kit**, sufficient for 1000 reactions in 96-well plates or 4000 reactions in 384-well plates

Kit Contents:

Substrate Mix, 5 × lyophilizate, store at -20°C protected from light

Assay Buffer, 5 × 0.6mL, store at -20°C protected from light

10X Lysis Buffer, 12mL, store at 4°C

Stop Solution, 60mL, store at 4°C

LDH Positive Control, 30µL, store at 4°C

Storage: Upon receipt store individual kit components at the temperatures indicated above. Kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific™ Pierce™ LDH Cytotoxicity Assay Kit provides a simple, reliable colorimetric method for quantifying cellular cytotoxicity assays. The kit can be used with different cell types for measuring cytoxicity mediated by chemical compounds as well as assaying cell-mediated cytotoxicity.

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types. Plasma membrane damage releases LDH into the cell culture media. Extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD^+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

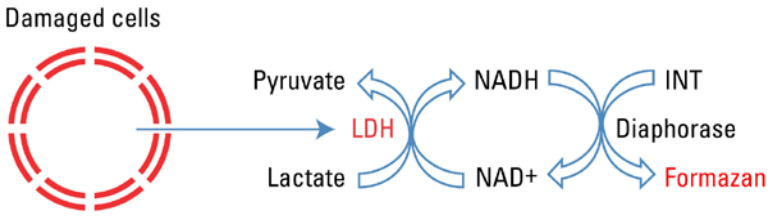

Figure 1. Schematic of LDH cytotoxicity assay mechanism.

Procedure Summary

Cultured cells are incubated with chemical compounds (e.g., Actinomycin D) or effector cells (e.g., natural killer cells) to induce cytotoxicity and subsequently release LDH. The LDH released into the medium is transferred to a new plate and mixed with Reaction Mixture. After a 30 minute room temperature incubation, reactions are stopped by adding Stop Solution. Absorbance at 490nm and 680nm is measured using a plate-reading spectrophotometer to determine LDH activity.

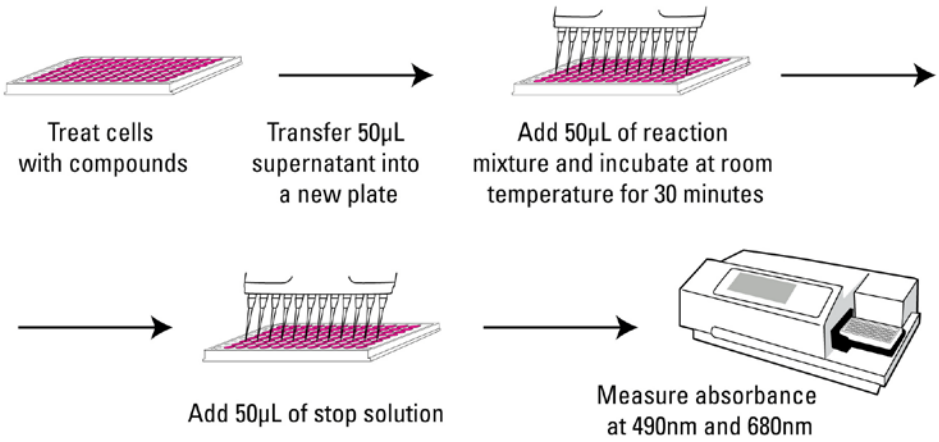

Figure 2. Procedure schematic for Thermo Scientific Pierce LDH Cytotoxicity Assay.

Important Product Information

- LDH activity varies in different sera (e.g., horse serum, fetal bovine serum and calf serum) commonly used to maintain different types of mammalian cell lines. Therefore, it is important to measure LDH activity in culture media with serum. The inherent LDH activity present in serum causes background signal in the assay. To reduce background signal, use the minimum serum percentage appropriate for each cell line; however, ensure the minimum serum percentage does not affect cell viability for the assay period.
- All procedures are written for 96-well plates; for 384-well plates, reduce all volumes by four-fold.

Additional Materials Required

- Cultured cell line
- Tissue culture 96-well plate
- Flat-bottom, clear 96-well plate compatible with spectrophotometry
- 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)
- Multichannel pipette
- Spectrophotometer (ELISA) plate reader capable of reading 490nm and 680nm absorbance

Reagent Preparation

Reaction Mixture

1. Dissolve one vial of the Substrate Mix (lyophilizate) with 11.4mL of ultrapure water in a 15mL conical tube. Mix gently to fully dissolve lyophilizate.
2. Thaw one vial of the Assay Buffer (0.6mL) to room temperature.

   Note: Protect Assay Buffer from light and do not leave at room temperature longer than necessary.
3. Prepare Reaction Mix by combining 0.6mL of Assay Buffer with 11.4mL of Substrate Mix in a 15mL conical tube. Mix well by inverting gently and protect from light until use.

   Note: One vial of the Reaction Mixture is sufficient for two 96-well plates. The remaining Reaction Mixture can be stored at -20°C protected from light for 3-4 weeks with tolerance for three freeze/thaw cycles without affecting the activity within the storage period.

1X LDH Positive Control

Dilute 1µL of LDH Positive Control with 10mL of 1% BSA in PBS.

Determination of Optimum Cell Number for LDH Cytotoxicity Assay

Different cell types have different levels of LDH activity. For optimal results, perform a preliminary experiment to determine the optimum number of cells to ensure LDH signal is within the linear range. Depending on the individual cell type used, use 2000-20,000 cells/well of a 96-well plate for most experiments.

1. Prepare a serial dilution of cells (0-20,000 cells/100µL media) in two sets of triplicate wells in a 96-well tissue culture plate.

   Note: Include a complete medium control without cells to determine LDH background activity present in sera used for media supplementation. Include a serum-free media control without cells to determine the amount of LDH activity in sera.
2. Incubate the plate in an incubator at 37°C, 5% CO_2 overnight.
3. Add 10µL of sterile, ultrapure water to one set of triplicate wells containing cells.
4. Add 10µL of Lysis Buffer (10X) to the other set of triplicate cell-containing wells and mix by gentle tapping.

   Note: Water-treated cells are referred to as Spontaneous LDH Activity Controls and 10X Lysis Buffer-treated cells are referred to as Maximum LDH Activity Controls.

   Note: Do not create bubbles by pipetting; bubbles inhibit absorbance readings.
5. Incubate the plate in an incubator at 37°C, 5% CO_2 for 45 minutes.
6. Transfer 50µL of each sample medium (e.g., serum-free medium, complete medium, Spontaneous LDH Activity Controls, Maximum LDH Activity Controls and 1X LDH Positive Control) to a 96-well flat bottom plate in triplicate wells.

   Note: To perform an LDH Positive Control assay (optional), use 50µL of 1X LDH Positive Control in triplicate wells.
7. Transfer 50µL of Reaction Mixture to each sample well and mix by gentle tapping.

8. Incubate the plate at room temperature for 30 minutes protected from light.
9. Add 50µL of Stop Solution to each sample well and mix by gentle tapping.

   Note: Break any bubbles present in wells with a syringe needle and/or centrifugation before reading.
10. Measure the absorbance at 490nm and 680nm. To determine LDH activity, subtract the 680nm absorbance value (background signal from instrument) from the 490nm absorbance.
11. Plot the Maximum LDH Release Control absorbance value minus the Spontaneous LDH Release Control absorbance value versus cell number to determine the linear range of the LDH cytotoxicity assay and the optimal number of cells.

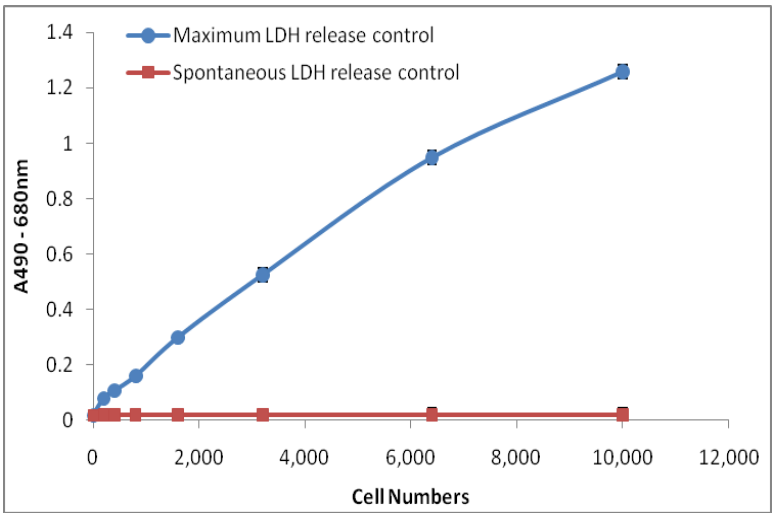

Figure 2. Graph of CHO-K1 cell LDH activity vs. cell number. CHO-K1 cells were diluted in a cell culture plate with RPMI 1640 medium containing 10% fetal bovine serum.

Chemical Compound-Mediated Cytotoxicity Assay

1. Plate the optimal number of cells/well in 100µL of medium (as determined in preliminary experiments) in triplicate wells in a 96-well tissue culture plate.

   Note: Include a complete medium control without cells to determine LDH background activity present in sera used for media supplementation. Include a serum-free media control to determine the amount of LDH activity in sera. Plate additional cells in triplicate wells for Spontaneous LDH Activity Controls (water) and Maximum LDH Activity Controls (10X Lysis Buffer).
2. Incubate the plate in an incubator at 37°C, 5% CO_2 overnight.
3. Prepare samples in the following manner:
   - Spontaneous LDH Activity Controls: Add 10µL of sterile, ultrapure water to one set of triplicate wells of cells.
   - Maximum LDH Activity Controls: Add nothing to one set of triplicate wells of cells.
   - Chemical Compound: Add 10µL of vehicle containing chemical compound to one set of triplicate wells of cells.
4. Incubate the plate in an incubator at 37°C, 5% CO_2, as needed.
5. To the set of triplicate wells serving as the Maximum LDH Activity Controls, add 10µL of Lysis Buffer (10X), and mix by gentle tapping.

   Note: Do not create bubbles by pipetting; bubbles may inhibit absorbance readings.
6. Incubate the plate in an incubator at 37°C, 5% CO_2 for 45 minutes.
7. Transfer 50µL of each sample medium (e.g., complete medium, serum-free medium, Spontaneous LDH Activity Controls, compound-treated and Maximum LDH Activity Controls) to a 96-well flat-bottom plate in triplicate wells.
8. Transfer 50µL of Reaction Mixture to each sample well and mix using a multichannel pipette.
9. Incubate the plate at room temperature for 30 minutes protected from light.

10. Add 50µL of Stop Solution to each sample well and mix by gentle tapping.

    Note: Break any bubbles present in wells with a syringe needle and/or centrifugation before reading.

11. Measure the absorbance at 490nm and 680nm. To determine LDH activity, subtract the 680nm absorbance value (background) from the 490nm absorbance before calculation of % Cytotoxicity [(LDH at 490nm) - (LDH at 680nm)].

12. To calculate % Cytotoxicity, subtract the LDH activity of the Spontaneous LDH Release Control (water-treated) from the chemical-treated sample LDH activity, divide by the total LDH activity [(Maximum LDH Release Control activity) – (Spontaneous LDH Release Control activity)], and multiply by 100:

$$\% \text{ Cytotoxicity} = \frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100$$

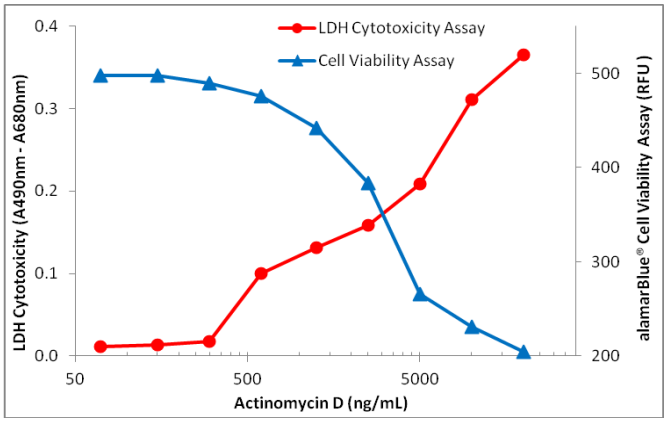

Figure 3. Determination of LDH cytotoxicity of Actinomycin D in CHO-K1 cells. CHO-K1 cells (10,000 cells per well) were plated in a 96-well plate in RPMI 1640 medium supplemented with 10% serum and incubated overnight in an incubator at 37°C, 5% CO_2. After 24 hours, different concentrations of Actinomycin D were added to the culture media and incubated for 18 hours at 37°C, 5% CO_2. LDH cytotoxicity was measured using the Pierce LDH Cytotoxicity Assay Kit. In a separate set of wells in the same 96-well plate, cell viability was measured using Thermo Scientific alamarBlue Cell Viability Assay Reagent (Product No. 88951-2).

Cell-Mediated Cytotoxicity Assay

Perform triplicate sets of experimental and control assays in a 96-well assay plate as follows:

1. Set up experimental wells containing a constant number of target cells (e.g., Jurkat, K562, etc.) as previously optimized and add various numbers of effector cells (e.g., natural killer cells, lymphokine-activated killer cells, cytotoxic T lymphocytes or other cell lines) to test several target:effector cell ratios. The final volume is 100µL/well.

   Effector Cell Spontaneous LDH Release Control corrects for spontaneous release of LDH from effector cells. Add effector cells at various numbers used in the experimental wells. Adjust the final volume to 100µL/well with culture medium.

   Target Cell Spontaneous LDH Release Control corrects for spontaneous release from target cells. Add the same number of target cells used in the experimental wells. Adjust the final volume to 100µL/well with culture medium.

   Target Cell Maximum LDH Release Control is required in calculations to determine 100% release of LDH. Add the same number of target cells used in experimental wells. The final volume must be 100µL/well (Step 4 adds 10µL of 10X Lysis Buffer).

   Volume Correction Control corrects the volume increase caused by addition of 10X Lysis Buffer. This volume change affects the concentration of serum, which contributes to the absorbance values. In Step 4, add 10µL of 10X Lysis Buffer to a triplicate set of wells containing 100µL of culture medium (without cells).

   Culture Medium Background Control is required to correct for the contributions caused by LDH activity that may be present in serum containing culture medium. Add 100µL of culture medium to a triplicate set of wells (without cells).

2. Add 10µL of sterile, ultrapure water to one set of triplicate wells containing Effector and Target Cell Spontaneous LDH Release Controls.

3. Incubate the plate in an incubator at 37°C, 5% CO_2 for an appropriate time.
4. Forty five minutes before harvesting the supernatant, add 10µL of Lysis Buffer (10X) to another set of triplicate wells containing Target Cell Maximal LDH Release Control and Volume Correction Control.
5. At the end of incubation, centrifuge the plate at 250 × *g* for 3 minutes.
6. Transfer 50µL of each sample medium to a 96-well flat bottom plate in triplicate wells.
7. Transfer 50µL of Reaction Mixture to each sample well and mix by gentle tapping.
8. Incubate the plate at room temperature for 30 minutes protected from light.
9. Add 50µL of Stop Solution to each sample well and mix by gentle tapping.
10. Measure the absorbance at 490nm and 680nm. To determine LDH activity, subtract the 680nm absorbance value (background signal from instrument) from the 490nm absorbance value.

    Note: Break any bubbles present in wells with a syringe needle and/or centrifugation before reading.
11. To calculate the corrected values, subtract the average value of the culture medium background control from average values of the Experimental, Effector Cell Spontaneous LDH Release Control and Target Cell Spontaneous LDH Release Control. The average value of the Volume Correction Control is then subtracted from the average value of the Target Cell Maximum LDH Release Control.
12. To calculate % Cytotoxicity for each Effector:Target cell ratio, use the equation below with the corrected values:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental value} - \text{Effector Cells Spontaneous Control} - \text{Target Cells Spontaneous Control}}{\text{Target Cell Maximum Control} - \text{Target Cells Spontaneous Control}} \times 100$$

Troubleshooting

Problem	Possible Cause	Solution
High medium control absorbance	High inherent LDH activity in animal sera in cell culture media	Reduce serum concentration to 1-5%
High spontaneous control absorbance	High cell density	Repeat determination of optimum cell number for assay
	Vigorous pipetting during cell plating	Gently handle cell suspension during plate set-up
Low absorbance value in experiment	Cell density was too low	Repeat determination of optimum cell number as described in Preliminary Experiment 2
High variability of absorbance well-to-well	Bubbles were in wells	Centrifuge the plate for a longer time or at a higher speed
		Break bubbles with a syringe needle

Related Thermo Scientific Products

88951 **alamarBlue™ Cell Viability Assay Reagent**

88952 **alamarBlue Cell Viability Assay Reagent**

8410000* **Live Cell Green**

8410200* **Live Cell Violet**

8410100* **Live Cell Blue**

K0200011* **Cell Viability Kit**

51119000* **Multiskan™ FC Microplate Photometer**

51119100* **Multiskan FC Microplate Photometer with incubator**

5250030* **Varioskan Flash Multimode Reader with top reading**

5250040* **Varioskan Flash Multimode Reader with top and bottom reading**

5250500*	**Varioskan Flash Multimode Reader with LumiSens Option, factory-fitted (also enabling luminometric spectral scanning)**
5250510*	**Varioskan Dispenser Option**

* Products can be found at *www.thermoscientific.com*

General References

Decker, T. and Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Meth* **115:**61-9.

Korzeniewski, C. and Callewaert, D.M. (1983). An enzyme-release assay for natural cytotoxicity. *J Immunol Meth* **64:**313-20.

Nachlas, M.M., *et al*. (1960). The determination of lactic dehydrogenase with a tetrazolium salt. *Anal Biochem* **1:**317-26.