LDH-Cytox™ Assay Kit

A Colorimetric Cytotoxicity Measuring Kit

Cat. No. 426401

LDH-Cytox™ Assay Kit can be used to measure cytotoxicity *in vitro*

BioLegend, Inc

Biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Introduction

LDH-Cytox™ Assay Kit is used to determine cytotoxicity by quantitatively measuring Lactate Dehydrogenase (LDH) activity in damaged cells. LDH is a stable cytoplasmic enzyme present in all cells. Once cells are impaired by stress, injuries, chemicals, or intercellular signals, LDH is rapidly released into the cell culture medium through the damaged plasma membrane. The cytotoxicity detection mechanism of this kit is based on the reduction of NAD^+ to NADH when LDH catalyzes dehydrogenation of lactate to pyruvate. NADH further reduces a cell impermeant, water-soluble tetrazolium salt in the presence of an electron mediator to produce an orange formazan dye. The intensity of the formazan dye thus formed is proportional to that of released LDH in the medium, which is an indication of cytotoxicity.

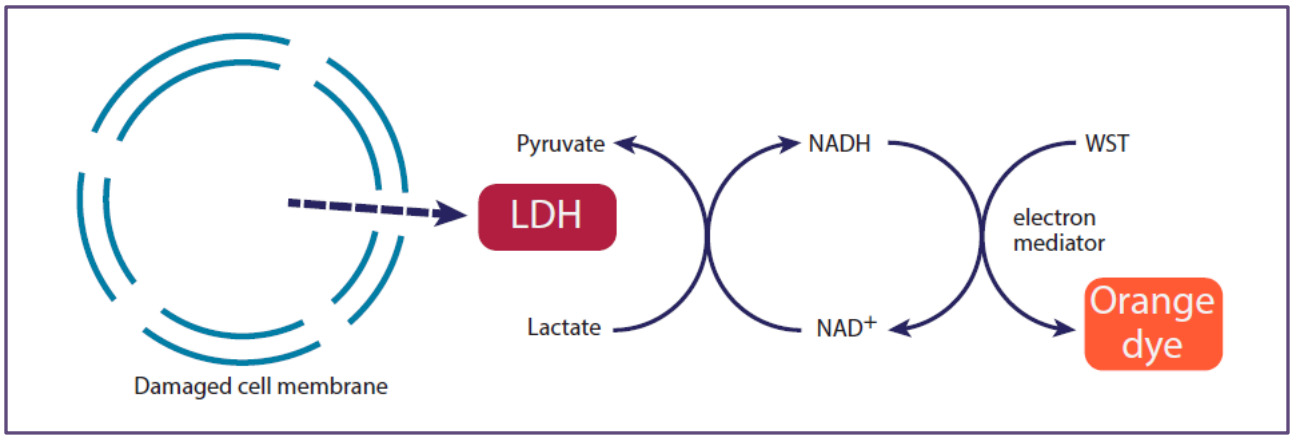

Figure 1. Principle of the assay

Materials Provided (500 tests)

- Dye Mixture – lyophilized, 1 vial
- Assay Buffer – 55 ml
- Lysis Buffer – 5.5 ml
- Stop Solution – 27.5 ml

Storage

The kit is stable for 6 months at 2-8°C.

Materials to be Provided by the End-User

- 96-well tissue culture plate (flat-bottomed, optically clear). For suspension cells in non-homogeneous assay: round or V-bottomed plate
- Appropriate cell culture media
- 20 and 100-200 µl multichannel pipettes
- Plate centrifuge (if using cell supernatant (non-homogenous assay protocol)

- Microplate reader (490 nm filter)
- CO_2 incubator

Precaution:

This kit contains a glass bottle with an aluminum cap. Use protective gloves as needed and use caution in handling.

After reconstitution of the dye mixture with the assay buffer, make sure to transfer and keep the reconstituted dye mixture in the amber assay buffer vial protected from light.

Preparation of Reagents:

1) To prepare a Working Solution, add 5 ml volume of Assay Buffer to the Dye Mixture vial. Close the cap and dissolve the contents completely. The working solution is stable for up to 6 months after mixing

2) Add back the whole volume of the mixture prepared in 1) to the Assay Buffer bottle.

(Note: The entire content of the lyophilized powder in the Dye Mixture is reconstituted with 55 ml of the Assay Buffer)

Store the Working Solution at 4℃ and protected from light. The ready-to-use solution after preparation is stable for 6 months.

General Protocol:

A. Optimization of cell concentration

Note: As different cell types have different levels of LDH activity, the cell density for the assay will depend on the cell type used and will need to be optimized by the end-user to obtain accurate experimental results

A.1. Preparation of cells

For optimal results, perform a preliminary experiment to determine the optimum number of cells to ensure LDH signal is within the linear range.

1) Collect cells and wash them with cell culture media. Using lower concentration of serum (5%) in the media might help to reduce background. Prepare cell suspension to 5×10^5 cells/ml in the cell culture media. Note: Suspended cells might need higher starting cell concentration.

2) Add 100 µl of the cell culture medium to each well of a flat-bottom 96-well tissue culture plate.

3) Add the cell suspension (5×10^5 cells/ml) to the top wells (row A) and mix by pipetting. Prepare 2-fold serial dilution in triplicates for the "high-control" (LDH released from lysed cells), "low control" (LDH from viable cells - no lyse) and "background control" (medium only) (Refer to Fig.2 for the plate arrangement).

[Serial Dilution Procedure]

After adding 100 µl of 5×10^5 cells/ml to the top row A wells, these wells will contain the maximum number of cells (2.5×10^4 cells/well). Transfer 100 µl from the top wells to the next set of wells in row B, and mix by pipetting. Repeat this procedure by going down the rows (A to H).

4) Incubate the plate in the 37°C incubator (5% CO2, 90% humidity) for the same amount of time the cells are exposed to the test substance treatment in the cytotoxicity assay (refer to B.1. step 3)

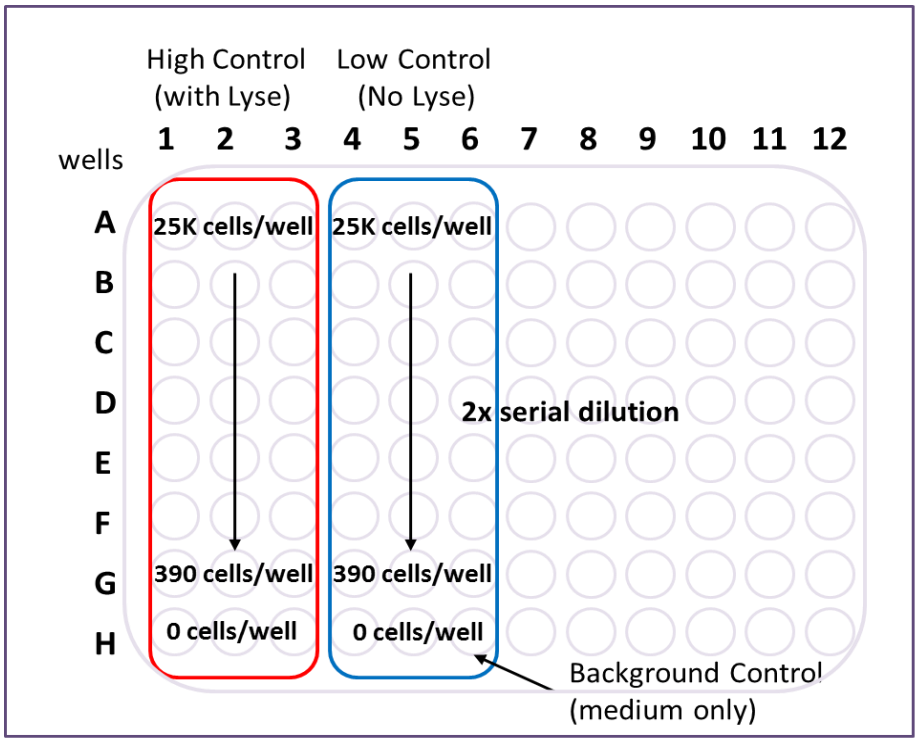

Figure 2. ***Assay Plate Layout***

A.2. LDH measurement

5) Add 20 µl of the Lysis Buffer to each well of the "high control" panel only.
6) Incubate the plate in the 37°C incubator for 30 minutes.

7A) ***For Homogenous Assay using viable cells***:

- Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at room temperature for 30 minutes*. Or,

7B) ***For Non-Homogenous Assay using cell supernatant***:

- Centrifuge the plate at 250 × g for 2 minutes to precipitate the cells (for suspension cells).
- Transfer 100 µl of the supernatant from each well to an optically clear 96-well plate.
- Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at the room temperature for 30 minutes*.

8) Add 50 µl of the Stop Solution to each well. The absorbance will be stable for 48 hours with protection from light.

9) Measure the absorbance at 490 nm.

* The reaction time can be decreased or increased depending on the color development. The plate can be measured at multiple time points until the desired value is observed.

A.3. Determination of optimum cell concentration for the assay

Plot the determined absorbance for the “low control” and “high control” against the corresponding number of cells/well. The absorbance at 490 nm of “low control” should be lower than 0.8. The cell concentration corresponding to the greatest difference between the absorbance of the “high control” and “low control”, that is in the linear range of the assay, is the optimum cell concentration for the LDH measurement assay. The optimal concentration for most cell lines is between $2.5x10^4$ – $2.5x10^5$ cells/ml.

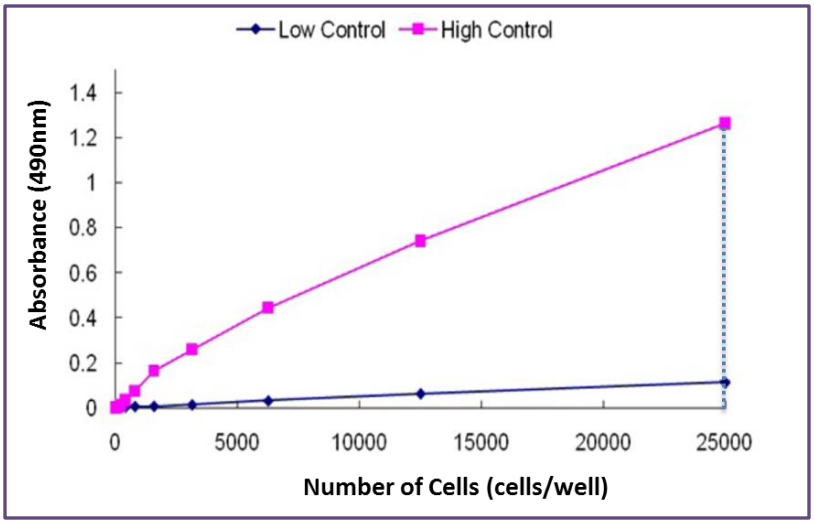

Figure 3. ***Optimum cell concentration plot.*** Optimum cell concentration is at 25,000 cells/well in this experiment.

B. Cytotoxicity Assay

B.1. *For Homogenous Assay using Viable Cells*: *(Refer to Table 1)*

1) Add 50 µl of cell suspension (optimum cell concentration determined as described above) to each well of a flat-bottom 96-well tissue culture plate except the "background control" wells.

Note:

- Perform each test in at least triplicates
- Using lower concentration of serum (5%) in the cell culture medium will reduce background.
- For adherent cells, incubate the plate at 37℃ overnight in a CO_2 incubator to allow the cells to adhere and then replace the medium with 50 µl of fresh cell culture medium.

2) Add 50 µl of cell culture medium to 'high control" and "low control" wells, and 100 µl of medium in "background control" wells.

3) Add 50 µl of cell culture medium containing test substance (cytotoxic agent) that is adjusted to the desired concentration to the "test substance" wells.

3) Incubate the plate in a 37℃ CO_2 incubator for 4 hours or for an appropriate time period

4) Add 10 µl of the Lysis Buffer to each well of the "high control'. Incubate the plate at 37℃ for 30 minutes in a CO_2 incubator.

5) Add 100 µl of the Working Solution to all wells. Protect the plate from light and incubate it at room temperature for 30 minutes.

6) Add 50 µl of the Stop Solution to all wells.

7) Measure the absorbance at 490 nm

8) Cytotoxicity calculation – refer to B.3

Table 1: Volume of solution chart for Homogenous Assay using Viable Cells

	Test Substance	High Control	Low Control	Background Control
Cell culture medium	-	50 µl	50 µl	100 µl
Cell suspension	50 µl	50 µl	50 µl	-
Test substance in culture medium	50 µl	-	-	-
Lysis Buffer	-	10 µl	-	-

B.2. *For Non-Homogenous Assay using Cell Supernatant*: (Refer to Table 2)

1) Add 100 µl of cell suspension (optimum cell concentration determined as described above) to each well of a flat-bottom 96-well tissue culture plate except the "background control" wells.

Note:

- Perform each test in at least triplicates
- Using lower concentration of serum (5%) in the cell culture medium will reduce background.
- For adherent cells, incubate the plate at 37℃ overnight in a CO_2 incubator to allow the cells to adhere and then replace the medium with 50 µl of fresh cell culture medium.

2) Add 100 µl of cell culture medium to 'high control", 120 µl to "low control" wells, 20 µl to "test substance" wells, and 220 µl of medium in "background control" wells.

3) Add 100 µl of cell culture medium containing test substance (cytotoxic agent) that is adjusted to the desired concentration to the "test substance" wells.

3) Incubate the plate in a 37℃ CO_2 incubator for 4 hours or for an appropriate time period

4) Add 20 µl of the Lysis Buffer to each well of the "high control'. Incubate the plate at 37℃ for 30 minutes in a CO_2 incubator.

5) Centrifuge the plate at 250 × g for 2 minutes to precipitate the cells (for suspension cells).

6) Transfer 100 µl of the supernatant from each well to a new optically clear 96-well plate

7) Add 100 µl of the Working Solution to all wells. Protect the plate from light and incubate it at room temperature for 30 minutes.

8) Add 50 µl of the Stop Solution to all wells.

9) Measure the absorbance at 490 nm

10) Cytotoxicity calculation – refer to B.3

Table 2: Volume of solution chart for Non-Homogenous Assay using cell supernatant

	Test Substance	High Control	Low Control	Background Control
Cell culture medium	20 µl	100 µl	120 µl	220 µl
Cell suspension	100 µl	100 µl	100 µl	-
Test substance in culture medium	100 µl	-	-	-
Lysis Buffer	-	20 µl	-	-

B.3. *Cytotoxicity Calculation:*

Calculate the average absorbance from each triplicate set of wells and subtract the background control value. Calculate the percent cytotoxicity by the following equation:

$$\text{Cytotoxicity}(\%) = \frac{(A-C)}{(B-C)} \times 100$$

A: Test Substance
B: High Control
C: Low Control

Example data:

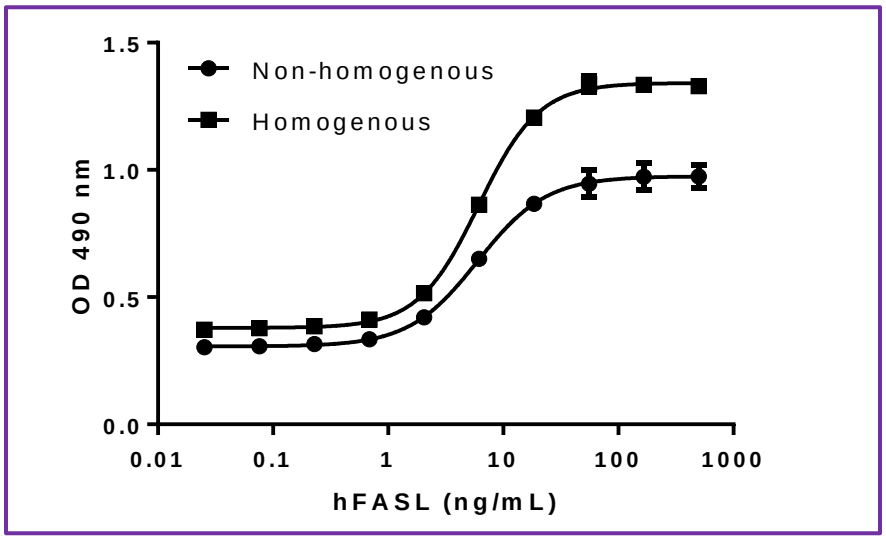

Figure Legend: Human FASL (Cat # 589402) induced cytotoxicity in Jurkat cells (60,000 cells/well) are measured using both Homogenous and Non-Homogenous Assay methods with LDH-Cytox™ Assay Kit.

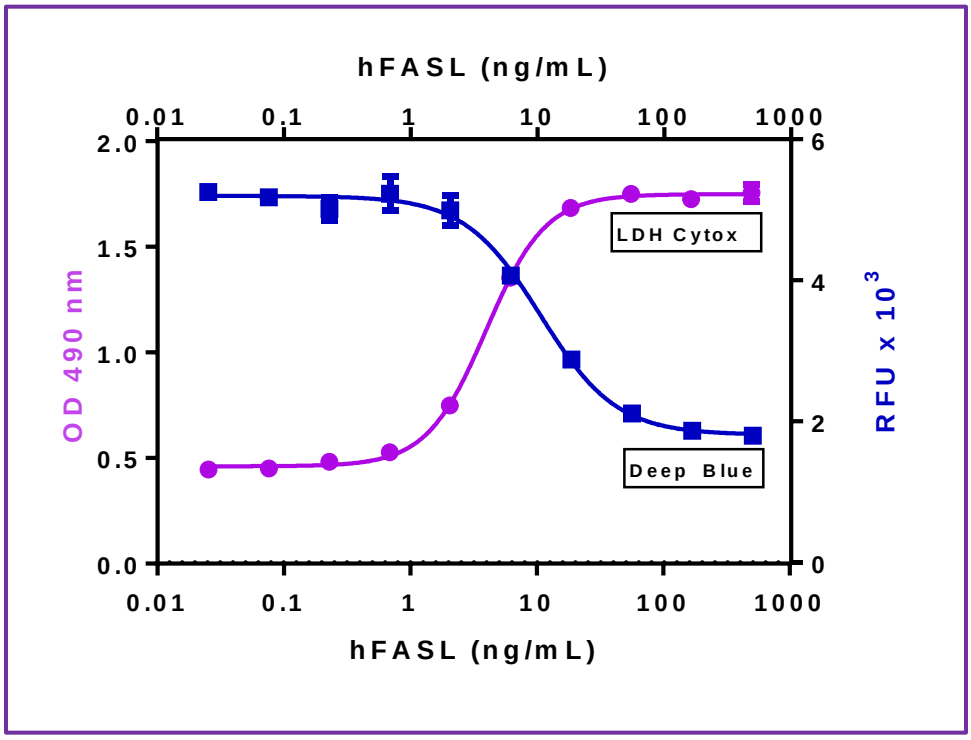

Figure Legend: Recombinant Human FASL (Cat # 589402) induced cytotoxicity in Jurkat cells (60, 000 cells/well) is measured using both LDH-Cytox™ Assay Kit and Deep Blue Cell Viability™ Kit (Cat # 424701).

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