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A Geno Technology, Inc. (USA) brand name

Cytoscan™-LDH Cytotoxicity Assay

A Colorimetric Assay for Cellular Cytotoxicity

(Cat. # 786-210, 786-324)



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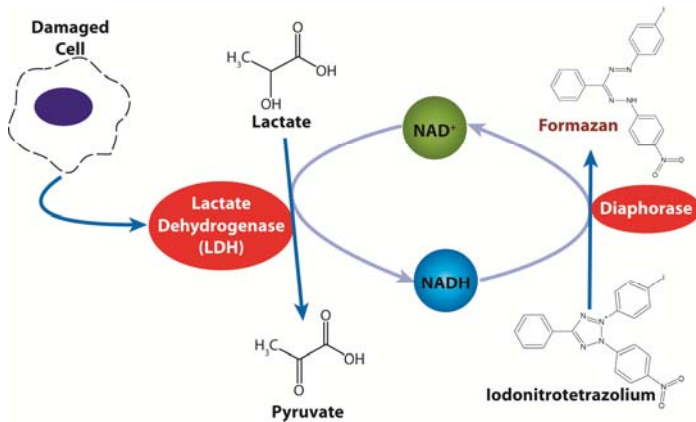
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INTRODUCTION

The Cytoscan™ LDH Assay is a colorimetric method of assaying cellular cytotoxicity, which is an alternative to ⁵¹Cr release cytotoxicity assays. The kit combines the advantages of reliability and simple evaluation, characteristic of radioisotope release assays, with the convenience of accuracy and avoidance of radioactivity. The Cytoscan™ LDH Assay can be used with different cell types for assaying cell mediated cytotoxicity as well as cytotoxicity mediated by chemicals and other test compounds.

The assay quantitatively measures a stable cytosolic enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (INT) into a red color formazan. The LDH activity is determined as NADH oxidation or INT reduction over a defined time period (see below). The enzymatic reactions associated with the assay are as follows:



The resulting formazan absorbs maximally at 492nm and can be measured quantitatively at 490nm using a micro-plate reader or spectrophotometer.

ITEM(S) SUPPLIED

Description	Cat. # 786-210 1000 assays	Cat. # 786-324 200 assays
Cytoscan™ Substrate Mix	5	1
Cytoscan™ -LDH Assay Buffer	5	1
Cytoscan™ -LDH Lysis Buffer [10X]	12ml	2.5ml
Cytoscan™ -LDH Stop Solution	60ml	12ml
LDH Positive Control	30µl	6µl

STORAGE CONDITION

The kit is shipped at ambient temperature. Store Cytoscan™ Substrate Mix and Assay Buffer at -20°C. LDH Positive Control, 10X Lysis buffer and Stop Solution can be stored at 4°C. The kit is stable for 12 months, when stored as recommended.

ADDITIONAL ITEMS REQUIRED

- Microplate reader
- 96 well tissue culture plate
- Multichannel pipette
- PBS containing 1% BSA (bovine serum albumin) solution

PROTOCOL

The Cytoscan™ LDH Assay protocol is divided into three sections - Target Cell Number Optimization, Cell-Mediated Cytotoxicity Assay and Chemical Induced Cytotoxicity Assay. The various steps of the protocol are as follows:

(A) TARGET CELL NUMBER OPTIMIZATION

It is highly recommended to do a preliminary experiment using your target cell population(s) to determine the optimum number of target cells to be used with the Cytoscan™ LDH Assay as various target cell types contain different amounts of LDH. The supplied LDH may be used as a positive control to verify that LDH assay is functioning properly.

I. Assay Plate Setup

1. First prepare a serial dilution (0, 5000, 10,000 and 20,000/100µl) of target cell type in triplicate sets of wells. Use the same medium and final volume that will be used for cytotoxicity assays. For example, if the co-culture volume is 50µl/well of target cells with 50µl/well of effector cells, prepare serial dilutions in 100µl/well.
2. Prepare a triplicate set of wells for the culture medium background without cells.
3. **Optional LDH Assay:** To perform a LDH positive control assay, mix the supplied LDH Positive Control properly and then dilute it 1:10,000 (1µl of LDH into 10ml of PBS containing 1% BSA). For LDH positive control assay, use the same assay volume (100µl) as in the wells containing cells in triplicate.

II. Cell Lysis and Harvesting Supernatant

To all wells (100µl medium), add 10µl of lysis buffer (10X) to lyse the cells. Incubate in a humidified chamber at 37°C, 5% CO₂ for 45 minutes. Then centrifuge the plate for 5 minutes at 250x g.

III. LDH Measurement

1. First dissolve one vial of the provided Substrate Mix in total volume of 11.4ml DI water in batches by inverting and mixing gently and transferring it to a 15ml tube.
2. Allow the 0.6ml assay buffer to warm to room temperature (**protect from light**) and add it to the reconstituted substrate mix, then mix well. One bottle of substrate mix is sufficient for two 96 well plates.

NOTE: *Keep the reconstituted substrate mix protected from strong direct light. Reconstituted Substrate Mix can be stored for 3-4 weeks at -20°C*

3. After centrifugation (step II above), transfer 50µl aliquots (supernatant) from all wells to a fresh 96 well flat bottom plate, using a multichannel pipette.
4. Add 50µl of the reconstituted Substrate Mix to each well and mix it thoroughly. Cover the plate with foil to protect it from light and incubate at room temperature (RT) for 30 minutes or at 37°C for 20 minutes.
5. Add 50µl of Stop Solution to each well. Mix it thoroughly and record the absorbance at 490nm within an hour after the addition of Stop Solution.
6. Determine the concentration of target cells yielding absorbance values at least two times the background absorbance of the medium control.

NOTE: *If you co-culture 100µl/well of target cells with 100µl/well of effector cells, target cell sensitivity can be increased by increasing the cell density (co-culturing the equal number of cells in 50µl/well volumes). Thus the concentration of released LDH is increased.*

(B) CELL-MEDIATED CYTOTOXICITY ASSAY

I. Background Absorbance Corrections

In tissue culture medium, two factors can contribute to background absorbance in Cytoscan™ LDH Assay, phenol red from medium and LDH from animal sera. This can be corrected by including a culture medium background control. The absorbance value from this control is used to normalize the absorbance values obtained from the other samples. Background absorbance from phenol red may also be eliminated by using a phenol red-free medium.

II. Assay Plate Setup

Perform each control and experimental assay in triplicate, after setting up the 96 well assay plate as follows:

1. **Experimental Wells:** Add a constant number of target cells (as optimized previously) to all experimental wells. Add various numbers of effector cells to triplicate set of wells to test several effector:target cell ratios. The final combined volume should be 100µl/well.
2. **Effector Cell Spontaneous LDH Release (Control):** This control corrects for spontaneous release of LDH from effector cells. Add effector cells at each concentration used in the experimental setup to a triplicate set of wells containing medium. Adjust the final volume to 100µl/well with culture medium.
3. **Target Cell Spontaneous LDH Release (Control):** This control corrects for spontaneous release from target cells. Add target cells (as optimized previously) to a triplicate set of wells containing culture medium. Adjust the final volume to 100µl/well with culture medium.
4. **Target Cell Maximum LDH Release (Control):** This control is required in calculations to determine 100% release of LDH. Add target cells (as optimized previously) to a triplicate set of wells containing culture medium. The final volume must be the same i.e. 100µl/well (adjust the volume with culture medium). Add 10µl of 10X Lysis Buffer per 100µl culture medium. Incubate target cells with lysis buffer for 45 minutes before harvesting the supernatant.
5. **Volume Correction (Control):** This control is recommended for correcting the volume increase caused by addition of 10X Lysis Buffer. This volume change affects the concentration of phenol red and serum, which contribute to the absorbance values. For this add 10µl of 10X Lysis Buffer to a triplicate set of wells, containing 100µl of culture medium (without cells).
6. **Culture Medium Background (Control):** This control is required to correct for the contributions caused by phenol red and LDH activity that may be present in serum containing culture medium. Add 100µl of culture medium to a triplicate set of wells.

7. **LDH Positive Control (Optional):** This is to verify the performance of other system components. Gently mix the supplied LDH enzyme and then dilute 1:10,000 in PBS containing 1% BSA, which will provide approximately the same level of enzyme found in 13,500 lysed L929 fibroblast cells. Prepare the dilution fresh for each use by adding 1 μ l of supplied LDH in 10ml PBS+1%BSA and use in triplicate set of wells.

III. Cell Culture and Supernatant Harvest

1. Incubate the assay plate for an appropriate time (a minimum of 4 hours incubation is needed for sufficient contact between target and effector cells) in a humidified chamber at 37°C, 5% CO₂.
2. 45 minutes before harvesting the supernatant, add 10 μ l of 10X Lysis Buffer to the wells containing the target cell maximum LDH release control. (NOTE: If the target cells are not completely lysed as determined by microscopy, add 5 μ l more of 10X Lysis Buffer).
3. At the end of incubation, centrifuge the plate at 250x g for 4 minutes.

IV. LDH Measurement

1. First dissolve one vial of the provided Substrate Mix in 11.4ml water by inverting and mixing gently.
2. Thaw the Assay Buffer provided with the kit and remove 0.6ml. Allow the 0.6ml assay buffer to warm to room temperature (*protect from light*) and add to the reconstituted substrate mix, then mix well. One bottle of substrate mix is sufficient for two 96 well plates.

NOTE: *Unused assay buffer must be immediately stored at -20°C. Keep the reconstituted substrate mix protected from strong direct light.*

3. Transfer 50 μ l aliquots (cell lysate) from all wells to a new 96 well flat-bottom plate by using a multichannel pipette.
4. Add 50 μ l of the reconstituted Substrate Mix to each well of the plate and mix it thoroughly. Cover the plate with foil to protect it from light and incubate at room temperature (RT) for 30 minutes or at 37°C for 20 minutes.
5. Add 50 μ l of Stop Solution to each well, mix it thoroughly and record the absorbance at 490nm within an hour after addition of Stop Solution.

NOTE: *Break the bubbles, if any, with a pipette tip before reading.*

V. Calculation of Results

1. Average absorbance values of the culture medium background is subtracted from all absorbance values of Experimental, Target Cell Spontaneous LDH Release and Effector Cell spontaneous LDH release.
2. Then subtract the average absorbance values of the Volume Correction Control from the absorbance values obtained for the Target Cell Maximum LDH Release Control.
3. To compute Percent (%) Cytotoxicity for each effector: target cell ratio, use the corrected values obtained from steps 1 and 2 in the following formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous})}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

(C) CHEMICAL INDUCED CYTOTOXICITY ASSAY:

The Cytoscan™ LDH Assay can also be used to test for high-throughput screening of cell death of a single cell type (without an effector cell) due to cytotoxicity of the test compounds.

1. Make triplicate wells for each sample to be tested (i.e. Experimental), as well as for Maximum (positive) and Spontaneous (negative) controls. Optimize the cell number (if required) as in the Section A of the above protocol.
2. Add the cytotoxic agents to be tested into cell culture (experimental). Incubate the plate for 20-24 hours in a cell incubator. After incubation, spin it and collect the medium samples (i.e. supernatant, 50µl each) into a new plate, to assess the LDH released, due to cell death.
3. For Spontaneous (which corrects for spontaneous release of LDH from cells and the LDH that might be present in the serum), use only culture medium.
4. For Maximum LDH release, lyse the cells along with the culture medium by freezing/thawing (or use Lysis buffer), then assay for the LDH activity. Use 50µl supernatant for both the controls to assay LDH activity.
5. Add 50µl reconstituted substrate mix into each well and incubate the assay plate at RT for 30 minutes or at 37°C for 20 minutes, protected from light. Add 50µl Stop Solution, record the absorbance at 490nm and calculate % Cytotoxicity as follows:

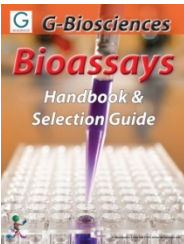
$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental } (OD_{490}) - \text{Spontaneous } (OD_{490}))}{\text{Maximum LDH release } (OD_{490})} \times 100$$

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