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IDTox™ Lactate Dehydrogenase (LDH) Enzyme Cytotoxicity Kit For Cell Culture Supernatant Samples

SUP6009 192 Wells

Enzyme Immunoassay for the determination of the Lactate Dehydrogenase (LDH) enzyme in cell culture supernatant samples.

PRODUCT DESCRIPTION

Lactate Dehydrogenase (LDH) is a ubiquitously-expressed intracellular enzyme which catalyzes the reversible oxidation of lactate to pyruvate. Since LDH is predominantly in the cytosol, the enzyme is released into the supernatant upon cell damage or lysis. Therefore, this enzyme can be used to detect cytotoxicity and cell number in *in vitro* cell culture systems. Therefore, monitoring levels of LDH enzyme has become a routine and fundamental means of investigating the cytotoxicity effects of experimental drug formulations and cell transfection.

The IDTox™ LDH Enzyme Cytotoxicity Kit is a colorimetric plate-based assay to directly determine the amount of Lactate Dehydrogenase enzyme in cultured cell supernatant samples. This kit enables biomedical researchers to determine accurate LDH levels in cultured cell supernatants from a broad range of cell lines. The assay utilizes a simple, proven colorimetric (UV) enzymatic assay to specifically detect LDH.

The kit is designed to be used with a microplate reader. It contains an assay standard to construct a linear calibration curve and verify assay performance, and it contains sufficient materials to test 84 samples in duplicate.

The kit uses a spectrophotometric, kinetic assay to detect changes in aspartate transaminase levels directly from serum samples. The features of the kit are:

- High sensitivity
- A flexible, versatile assay format that can be modified to meet user needs
- High compatibility across a broad range of cell lines
- A robust enzyme-based assay which does not require expensive instrumentation
- High reproducibility
- Specially formulated IDEnhancer™ reagent which significantly improves assay signal

PROCEDURE OVERVIEW

The IDTox™ Lactate Dehydrogenase (LDH) Enzyme Toxicity Kit measures the concentration of LDH using a direct, plate-based, colorimetric reaction. When cell culture supernatant is added to the reaction mix, the LDH in the sample converts the lactate and NAD+ in the mix to pyruvate and NADH. The concentration of LDH in each sample is then directly determined from the increase in absorbance at 340 nm over a 30 minute assay length. Dilutions of the LDH Control, included in the kit, can be used to construct a standard curve to calibrate the assay and confirm assay linearity. This is described in more detail in Section, “Data Analysis.”

Although LDH is present in a host of human tissues, its release varies among cell types. Therefore, the most useful analysis of cytotoxicity in a cultured cell line involves measuring cytotoxicity as a percentage of damaged or lysed cells. In this analysis, the TREATED sample is compared to both an UNTREATED culture, and a LYSED control culture.

Example calculation for percent cytotoxicity:

Cytotoxicity (%) = [(TREATED sample – UNTREATED sample) / (LYSED sample – UNTREATED sample)] X 100.

Where TREATED sample, UNTREATED sample, and LYSED sample represent the LDH activities of the treated, untreated and lysed samples.

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KIT REAGENTS SUPPLIED

The IDTox™ LDH Enzyme Cytotoxicity Kit has the capacity for 192 determinations or testing of 84 samples in duplicate (using 12 wells for standards). The kit also contains enough material to construct four standard curves. Upon receipt of the kit, store the standards and buffer at -20°C and the remainder of the kit at 4°C. Once the LDH Reagent Mix is reconstituted the shelf life of the kit is 3 months when properly stored. For more details, see "Preparation of Reagent Mix". The shelf life of the kit is noted on the label, when the kit is properly stored.

Kit Contents	Amount	Storage
Clear Microtiter Plate	2 x 96-well Plate (8 wells x 12 strips)	Room temp or 4°C
Opaque Plate	2 x 96-well Plate (500 µl capacity)	Room temp or 4°C
LDH Reagent Mix	2 bottles	4°C
IDEnhancer™ Reagent	1 tube	4ºC
Standard	Vial	- 20°C
Standard Dilution Buffer	7 ml	- 20°C

MATERIALS REQUIRED BUT NOT PROVIDED

Microtiter plate reader (340 nm).
Deionized or distilled water.
1.5 ml microfuge tubes.
Multichannel pipette or repeating pipettor (*recommended but not required*).

WARNINGS AND PRECAUTIONS

It is strongly recommended that you read the following warnings and precautions to ensure your full awareness of the techniques and other details you should pay close attention to when running the assays. Periodically, optimizations and revisions are made to the kit and manual. Therefore, it is important to follow the protocol coming with the kit.

Add standards to plate only in the order from low concentration to high concentration, as this will minimize the risk of compromising the standard curve.

The flexibility of this kit allows users a great degree of freedom in choosing their cell culture parameters. In general, a cell culture outline may prove useful for most cell lines, however it is understood that many users cannot modify their experiments to fit a specific cytotoxicity assay. Users can modify the parameters of the IDTox™ LDH Cytotoxicity assay to fit their experiments. For users who do not have rigid cell culture parameters, a general cell culture preparation guide is provided below. For those who cannot alter their cell culture parameters, some LDH Optimization Tips are provided in the Assay Procedure section (below).

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ASSAY PROCEDURE

PREPARATION OF REAGENTS

Cell Culture Preparation

The suggested seeding concentration for most adherent cell lines is between 5.0 x 10^4 and 2.5 x 10^5 cells per ml. We suggest that users perform each experimental condition in either duplicate or triplicate.

1. Seed cells in either a 96-well plate in 100 µl of culture medium, or in a 24-well plate in 400 µl of culture medium.
2. Culture the cells in a CO_2 incubator for at least 16 hours, and then replace the cell culture media. Allow cells to grow to at or near confluence.
3. Add test substances to wells designated "TREATED", and incubate for 2 – 24 hours.

4. Upon completion of desired experimental incubation, for 96-well plate samples add 5 µl of IDEnhancer™ reagent to the wells designated "LYSED" and pipet up and down. Add 5 µl of dH_2O to all other wells. For 24-well plate samples use 20 µl of IDEnhancer™ reagent and dH_2O.
5. After a 20 minute incubation, pipet the "LYSED" wells up and down, and transfer cell culture supernatants from all wells to the provided opaque 96-well plate. Discard the cell culture plate.
6. For 96-well plate samples add 5 µl of IDEnhancer™ reagent to each well. For 24-well plate samples add 20 µl. Gently triturate (pipet up and down) each sample 4 – 5 times to mix.

Typical Cell Culture Plate Layout (96-well plate)						
	1, 2	**3, 4**	**5, 6**	**7, 8**	**9, 10**	**11, 12**
ROW A	UNTREATED culture	LYSED culture	TREATED culture (dose 1)	TREATED culture (dose 2)	TREATED culture (dose 3)	TREATED culture (dose 4)

Note: Rows B – H can be used for other test substances and/or extra dosing concentrations

ASSAY PROCEDURE

Set up

Make sure you read "Warnings and Precautions" section above. All reagents and the microtiter plate should be brought up to room temperature before use (30 minutes - 1 hour at 20–25°C/68–77°F).

Reagent Preparation

1. Turn on the plate reader, allow light source to warm up, and set the absorbance wavelength to 340 nm.
2. Warm up kit reagents to room temperature for 30 minutes.
3. Reconstitute the LDH Reagent Mix: Add exactly 30 ml of deionized or distilled water to the LDH Reagent Mix powder. Mix by swirling or inverting the bottle 10 times. Allow contents to dissolve for 10 minutes at room temperature.

IMPORTANT: The reconstituted Reagent Mix can be left at room temperature for short periods (30 min) prior to use. Between uses, the reconstituted Reagent Mix should be stored at 4 °C (for up to 3 months). Discard the Reagent Mix 3 months after reconstitution.

Preparation of Standards

1. Label six clean microcentrifuge tubes 1, 2, 3, 4, 5 and 6 (Neg).
2. Dissolve contents of Standard vial in 3.68ml of Standard Dilution Buffer. Mix well and transfer 150 µl of dissolved Standard to Tube 1. Unused remaining portion in vial can be stored at -80°C for 6 months.
3. Serially dilute the standard by adding the appropriate volumes of Standard and Standard Dilution Buffer:

Standard Tube #	Preparation	Relative Dilution*
1	Add 150 µl of dissolved Standard.	1
2	Add 75 µl from Standard Tube #1 + 75 µl of Standard Dilution Buffer. Mix thoroughly.	2
3	Add 75 µl from Standard Tube #2 + 75 µl of Standard Dilution Buffer. Mix thoroughly.	4
4	Add 75 µl from Standard Tube #3 + 75 µl of Standard Dilution Buffer. Mix thoroughly.	8
5	Add 75 µl from Standard Tube #4 + 75 µl of Standard Dilution Buffer. Mix thoroughly.	16
6 (Neg)	Add 100 µl of Standard Dilution Buffer only.	NA

*Only needed for the generation of the Standard Curve.

Assay Optimization Tips

This guide provides users with a general outline to maximize the versatility of the Cytotoxicity Kit. Since LDH expression differs across cell types, it is critical to calibrate the assay parameters so that the change of absorbance of the LYSED control sample remains linear across the length of the assay. This allows the LYSED control well to provide an accurate measure of total LDH concentration of a cell culture well.

1. Add 20 µl of the LYSED positive control sample to a microplate well
2. Add 280 µl of Reagent Mix to the well
3. Immediately measure the absorbance of each sample at 340 nm (= initial reading). After exactly 30 min, measure the absorbance again (= final reading)

If the absorbance change between the "final reading" and the "initial reading" is greater than 2.0 (with an "initial reading" of 1.0 or less), decrease the sample size used in the assay to 10 µl. If the absorbance change remains above 2.0, continually shorten the assay length to 15 min, 10 min, and 5 min, until the "initial reading" is below 1.0 and the absorbance change is below 2.0.

the absorbance change between the "final reading" and the "initial reading" is less than 0.1, increase the sample size used in the assay to 50 µl. If the absorbance change remains below 0.1, increase the assay length to 60 minutes. If by using the 60 min assay length, the change of absorbance remains below 0.1, consider altering your cell culture conditions to increase cell density.

Note: In these cases, the parameter changes determined using this optimization procedure should then replace all parameters in the Assay Protocol below.

Assay Protocol

1. Add 20 µl of each sample or standard (in duplicate) to the microplate wells.
2. Add 280 µl of reconstituted LDH Reagent Mix to the well. (Using a multichannel pipet or repeating pipettor is recommended).
3. Immediately measure the absorbance of each sample at 340 nm (= initial reading). After exactly 30 min, measure the absorbance again (= final reading).

CALCULATION OF RESULTS

Cytotoxicity Analysis (%)

Use the formula below to calculate percent cytotoxicity of a treated cell culture supernatant.

1. Calculate the absorbance change for each sample by subtracting the "final reading" from the "initial reading".
2. Calculate the average absorbance change for each condition run in duplicate, including UNTREATED, LYSED, and TREATED conditions.
3. Substitute the values into the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{TREATED sample} - \text{UNTREATED control}}{\text{LYSED control} - \text{UNTREATED control}} \times 100$$

Where TREATED sample, UNTREATED control, and LYSED control represent the average absorbance change of their respective samples.

Standard Curve Construction (Optional)

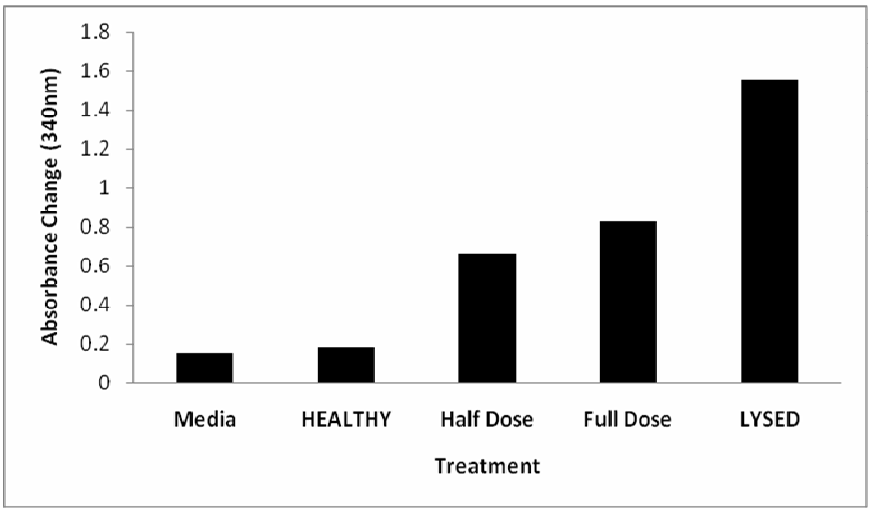
A calibration curve to confirm assay linearity can be constructed using the calibration standards supplied with the kit as follows:

1. For each calibration point, calculate the *average corrected absorbance* by subtracting the average "FINAL" absorbance of the "Neg" point from the average "FINAL" absorbance of each point in the calibration. This calculation should include subtracting the average "Final" absorbance of the "Neg" value from itself, which is zero.
2. For each standard, plot the average change in absorbance along the y-axis (from lowest in value to highest in value) and the inverse value of the relative dilution number* (i.e. 0.0625, 0.125, 0.25, 0.5 and 1) on the x-axis. For Tube #6 (Neg) use "0".

APPENDIX: Results of LDH Assay Kit

A. Cells were seeded in a 24-well plate at a concentration of 2.5 X 10^5 / ml, and allowed to grow to confluence over 48 hours. Cell culture media was replaced and Cytotoxic Transfection Agent X was added in two increasing doses to the wells. Cells were further incubated 16 hours before harvesting cell culture supernatant.

1. HeLa cells



2. A549 cells