Cat. No. 304

CellToxi®

LDH Cytotoxicity Assay kit Product Information

Quick Facts

1. Storage conditions before opening:
   Reagent **A**：**-20 ℃, protected from light**；The rest reagents：**4 ℃.**
2. Measure the absorbance at **450** nm.
3. Prepare working solution by mixing Reagent **A** and **B**.
4. Suggested volumes for working, sample, lysis (Reagent C) and stop solutions (Reagent D)：**100, 10, 10** and **10 µL.**

Introduction

This product, LDH cytotoxicity assay kit, is designed for the determination of cytotoxicity by measuring the lactate dehydrogenase (LDH) activity. LDH is a stable enzyme presented in all types of cells, and rapidly released into the cell medium upon the damaged plasma membrane. Therefore, we can measure the activity of LDH with this product according to **Fig. 1.**

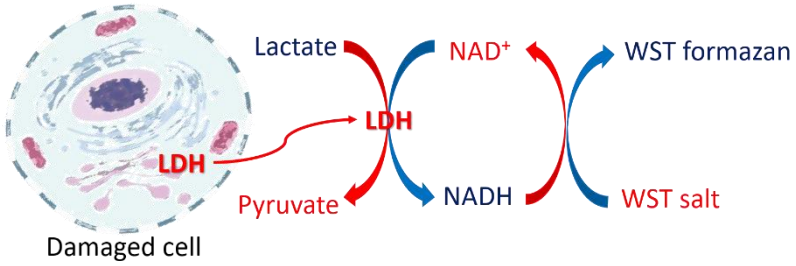


Fig. 1 The principle of LDH cytotoxicity assay.

LDH catalyzes dehydrogenation of lactate to pyruvate thereby reducing NAD^+ to NADH. NADH reduces a water-soluble tetrazolium salt (WST) into an orange-color formazan. The intensity of the generated orange-color correlates directly with the number of lysed cells. Since the advanced WST is more sensitive than traditional tetrazolium, such as INT, less amount of culture medium (10 µL) is required for assay, and the background from serum and culture medium is significantly reduced. In addition, since the generated color is stable, the reaction can be read multiple times by spectrometer or plate reader at 450 nm (OD_{450}).

Kit Contents, Shipping and Storage Information

	Package	Shipping	Storage
Dye mix (Reagent A)	1 vial	4 °C with protection from light.	12 months under -20 °C with protection from light.
Assay buffer (Reagent B)	55 mL	4 °C with protection from light.	
Cell Lysis solution (Reagent C)	5.2 mL		
Stop solution (Reagent D)	5.2 mL		

Protocol

1. Collect adherent or suspension cells：

1.1 Collect cells (adherent or suspension) and wash once with fresh culture medium, then seed 100 µL solution (with 2-10 x 10^3 cells per well) in a 96-well plate.

Note：Trypsin may be used to remove adherent cells from a culture surface before seeding to a 96-well plate.

2. Prepare working solution：

2.1 Warn the assay kit to room temperature (take ~30 mins).

2.2 Add 5 mL of assay buffer (Reagent B) to Dye mix vial (Reagent A).

2.3 Dissolve the contents completely by inverting.

2.4 Transfer all of the solution in the Reagent A vial to assay buffer bottle (Reagent B, 55 mL).

2.5 Close the cap and mix the bottle well.

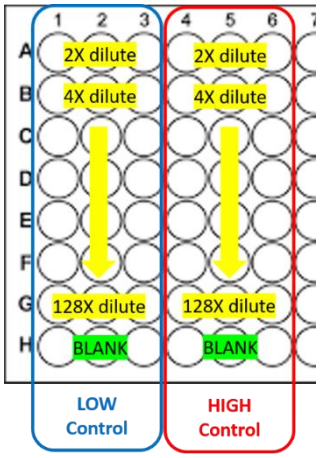
Note：After preparing the working solution, please store it at 4 °C and protect from light. Under this condition, it is stable for 1 month.

3. Assay procedure：

3.1 Determine the optimum cell concentration for LDH cytotoxicity assay：

Different cell types may contain different amounts of LDH. Therefore, the optimum cell concentration for a specific cell type should be determined in a preliminary experiment if you use this product for the first time or use an unfamiliar cell line. In this optimum cell concentration, the difference between the low and high control is at a maximum, then use this concentration for the subsequent assay. With most cell lines, the optimal cell concentration is between 2-10 x10^3 cells per well (100 µL).

3.1.1. Prepare a serial dilution of cells (0-10,000 cells per well in 100 µL media) in two sets of triplicate wells in a 96-well culture plate. One set of the serial dilutions is lysed and used to determine the Maximum LDH release (**High Control**). The second set of serial dilutions is used to determine the spontaneous LDH release (**Low Control**).



3.1.2. For adherent cell, incubate the cells overnight (37 ℃, 90% humidity and 5% CO_2) to attach the cell to plate. Remove the supernatant and add 100 µL fresh media into each well to wash the cell；For suspension cell, skip this incubation procedure.

3.1.3. Add 10 µL of lysis buffer (Reagent C) into the High Control dilution series, then mix by gentle tapping; Add 10 µL of sterile water into the **Low Control** dilution series, then mix by gentle tapping.

Note：Do not create bubbles when pipetting because bubbles prevent accurate absorbance reading.

3.1.4. Incubate the plate in a 37 ℃, 90% humidity, 5% CO_2 incubator for the appropriate time. Gently shake the plate at the end of the incubation to ensure LDH is evenly distributed in the culture medium.

3.1.5. For suspension cells, centrifuge the plate at 250 x g for 2 mins to deposit the cell. For adherent cell, skip this step.
3.1.6. Transfer 10 µL of the supernatant from each well into an optically clear flat-bottom 96-well plate.
3.1.7. Add 100 µL working solution (step **2.5**) into each well, then mix by gentle tapping. Protect the plate from light and incubate it at room temperature for 30 mins.
3.1.8. Measure the absorbance of all controls at OD_{450}.
Note：The reaction time can be shorter or longer than 30 mins. It depends on the color development. The plate can be read at multiple time point until the desired reading is observed. The optimized reaction time and cell concentration can be determined as the OD_{450} of **high control** should be around 2.0；while, the **low control** should be <0.8. The difference between the low and high control is at a maximum value (more than 0.2).
3.1.9. The reaction can be stopped by adding 10 µL of stop solution (Reagent D) and mix by gentle tapping. With protection the plate from light and evaporation, the reaction may be read within 24 hours without significant changes. Even so, it is still recommended that you measure the OD_{450} value as soon as possible to avoid unnecessary errors.
3.2 Cytotoxicity Assay：
3.2.1. See the following **Table 1.** to design the experiment.
Note：Please use the optimum cell concentration as step **3.1.8**. For adherent cells, please incubate the plate overnight to attach the cell on the plate. After the incubation, remove the supernatant and add 100 µL fresh media to each well to wash the cell；For suspension cell, skip this incubation procedure.
Note：If the test substances are not dissolved in the medium, a solvent control may be performed by addition of the same amount of solvent in triplicate without testing substances.

Table 1. Overview of all the controls

Volume (µL)	Background	Low control	High control	Test substance
Medium	110	10	0	0
Cell suspension	0	100	100	100
Test substance	0	0	0	10
Lysis buffer	0	0	10	0

3.2.2. Incubate the plate in a 37 ℃, 90% humidity, 5% CO_2 incubator for the appropriate time determined for the test substance. Gently shake the plate at the end of the incubation to ensure LDH is evenly distributed in the culture medium.
3.2.3. For suspension cells, centrifuge the plate at 250 x g for 2 mins to deposit the cell. For adherent cell, skip this step.
3.2.4. Transfer 10 µL of the supernatant from each well into an optically clear flat-bottom 96-well plate.
3.2.5. Add 100 µL working solution (step **2.5**) into each well, then mix by gentle tapping. Protect the plate from light and incubate it at room temperature for 30 mins. Please see the step **3.1.8.**

for the optimized reaction time.

3.2.6. Measure the absorbance of all controls at OD_{450}.

3.2.7. The reaction can be stopped by adding 10 µL of stop solution (Reagent D) and mix by gentle tapping. With protection the plate from light and evaporation, the reaction may be read within 24 hours without significant changes. Even so, it is still recommended that you measure the OD_{450} value as soon as possible to avoid unnecessary errors.

4. Calculation of cytotoxicity：

$$\text{Cytotoxicity}(\%)=\frac{OD_{450}\text{ of test substance}-OD_{450}\text{ of low control}}{OD_{450}\text{ of high control}-OD_{450}\text{ of low control}}\ X100\ \%$$

Results

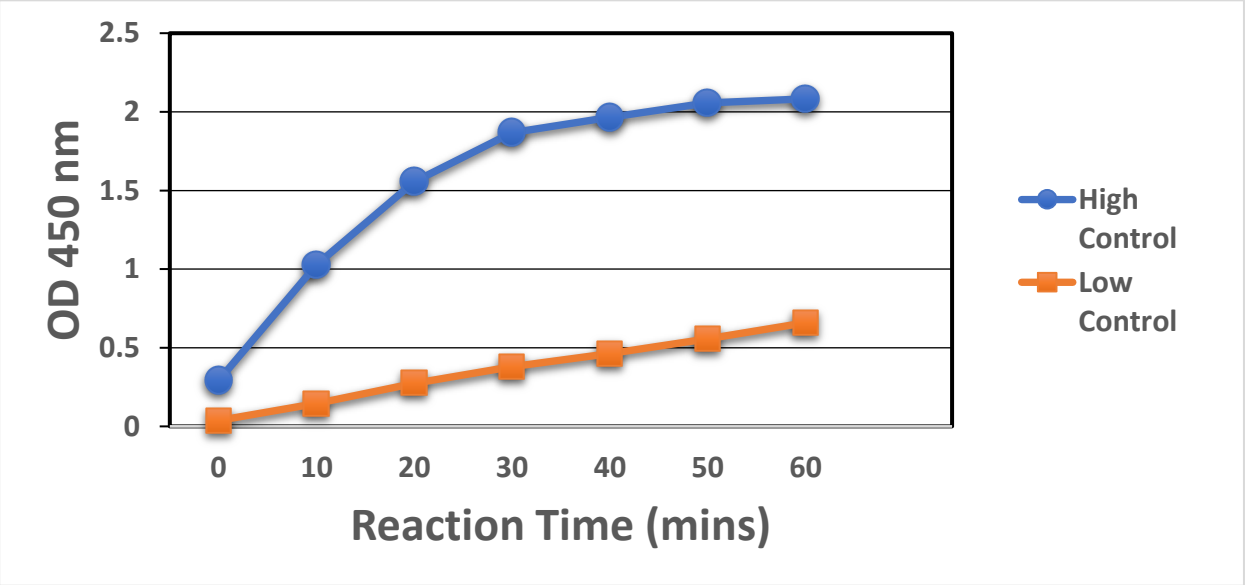


Fig. 1. The LDH activity of A549 cells (5,000 cells per well). A549 cells were diluted in 96-well cell culture plate with DMEM medium containing 10% FBS.

Troubleshooting

Problems	Reason	Solution
High background values	Medium contains high concentration of LDH.	Use the serum-free medium or the medium contains the serum less than 5 %.
	Test substance or medium has reductive activity.	Some reductive compounds such as ascorbic acid react directly with WST. Use medium such as DMEM, RPMI, F-12. These media do not contain reductive ingredients.
Strong color reaction	Cell concentration is too high	Titrate cell concentration.
	Test substance or medium	Some reductive compounds such as ascorbic

also in low controls	has reductive activity.	acid react directly with WST. Use medium such as DMEM, RPMI, F-12. These media do not contain reductive ingredients.
	High spontaneous release may be due to bad condition of the cells used in the assay.	Check culture conditions：some cell lines do not survive in serum free media, even at short incubation time. Increase serum concentration to about 1-5%.
Low color reaction	Cell concentration is too low	Titrate cell concentration.
	Test substance or medium inhibit LDH activity or the colorimetric reaction.	--Use substance control to test the substance and/or medium for compounds inhibiting LDH activity.*1 --Avoid pyruvate containing in the media.
Large variation of absorbance	There are bubbles in the medium.	Please break bubbles by using a needle or centrifuge the plate with 1000 x g for 1 min.
	The concentration of medium has been changed by evaporation of the solution.	Evaporation occurs easily in the outermost wells of the plate. If the incubation time is long, please add medium in the outermost wells and do not use them for analysis.
	Reagent do not mix evenly.	The volume of lysis buffer is added less than the other reagents, and it affects the high control set significantly. Please tapping all the solutions evenly and gently.
	The volume of reagents is not accurate.	Calibrate the pipett.
	Reaction time varies among wells by using a single channel pipette.	Multichannel pipettes are recommended to add working and stop solution.

*1 To test the substance and/or the medium interfere with LDH activity, the following control set is added in Table 1.：add 25 µL/well test substance solution, 25 µL/well medium and 50 µL/well LDH solution (0.05 U/mL, not provided in this kit) in triplicate.

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