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Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) Activity

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Summary

Apoptosis and necrosis are two major forms of cell death observed in normal and disease pathologies. Although there are many assays for detection of apoptosis, relatively few assays are available for measuring necrosis. A key signature for necrotic cells is the permeabilization of plasma membrane. This event can be quantified in tissue culture settings by measuring the release of the enzyme lactate dehydrogenase (LDH). When combined with other methods, measuring LDH release is a useful method for detection of necrosis. In this chapter, we describe the step-by-step procedure for detection of LDH release from necrotic cells using a microtiter plate based colorimetric absorbance assay.

Keywords

programmed necrosis; necroptosis; membrane leakage; lactate dehydrogenase

1. Introduction

Necrosis is a type of cell death that is morphologically characterized by swelling and rupture of intracellular organelles, eventually leading to the breakdown of the plasma membrane (1–3). Plasma membrane leakage from necrotic cells causes the release of intracellular contents into extracellular milieu. Therefore, necrotic cell death evokes inflammatory responses and is closely associated with inflammatory diseases (4). In contrast to apoptosis, necrosis was once considered as a passive and accidental form of cell death (5). However, several recent studies have clearly established that necrosis can be a programmed event (6–8). Death cytokines in the tumor necrosis factor (TNF) family are potent triggers of necrosis. In this chapter, we describe a convenient, non-radioactive and high-throughput method to detect necrosis, including secondary necrosis, based on the leakage of a cytoplasmic enzyme, lactate dehydrogenase (LDH), from the damaged cells.

Historically, necrotic cell death is evaluated by determining damage of the plasma membrane. The cytotoxicity assays for measuring necrosis are principally divided into two categories: those that are based on differential uptake of DNA binding dyes that do not traverse the plasma membrane of living cells, such as propidium iodide; and those that are based on the leakage of intracellular molecules through impaired plasma membrane. LDH is

[3]Pyruvate is a common culture media supplement. However, because it is an intermediate product of the LDH reaction, it should not be included in culture media to avoid interference of the LDH reaction.

[17]After harvest, cell-free culture supernatants can be stored at 2 – 8°C for a few days without appreciable loss of LDH activity.

[19]To minimize the variation in incubation time between different wells when large number of sample wells is involved, use a multichannel pipette to dispense the 2X LDH assay buffer to the assay wells.

a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged (9). To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor (10). The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (*see* Note 1).

2. Materials

2.1. Equipment

1. Microtiter plate reader (FluoStar Optima, BMG LABTECH Inc, Ortenberg, Germany).
2. Centrifuge with plate adaptors (Sorvall Legend RT, DJB Labcare Ltd, Buckinghamshire, England).
3. Multichannel pipettes (Brand, Wertheim, Germany) and repeat pipettor (Eppendorf, Hamburg, Germany).

2.2. Reagents and Supplies

1. Flat bottom or U-bottom 96-well microtiter plate.
2. RPMI 1640, Dulbecco's Modified Eagle Medium, or any other media depending on the cell type used (Invitrogen, CA, USA) (*see* Notes 2–[4]).
3. Fetal bovine serum (Any vendors) (*see* Note 5).
4. Tris Buffer, 200 mM, pH 8.0. Dissolve 24.2 g Tris base in 1 L milli-Q water. Adjust pH to 8.0 with hydrochloric acid. Sterilize by autoclave.
5. Prepare the 2X LDH assay buffer as follows: Dissolve 223 mg INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride), 57 mg PMS (N-methylphenazonium methyl sulfate) (*see* Note 6), 575 mg NAD (nicotinamide adenine dinucleotide), and 3.2 g lactic acid in 480 mL 200 mM Tris buffer solution, pH 8.0 (*see* Notes 7–[8]). Store the 2X LDH assay buffer in small aliquots at −20°C (*see* Notes 9–[10]).

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[1]It is noteworthy that the LDH release assay described here does not distinguish between primary necrosis and secondary necrosis as a consequence of apoptotic cell death. Other assays, such as those that measure caspase activity or DNA strand breaks in apoptotic cells, can be performed to distinguish whether any detected LDH release is due to secondary necrosis from apoptotic cells.

[2]Phenol red is a common pH indicator included in tissue culture media. Phenol red-free media should be used to minimize interference with absorbance measurement.

[4]INT can be reduced non-enzymically by strong reducing agents. Hence, reducing agents such as ascorbate, 2-mercaptoethanol and dithiothreitol should be excluded in culture media.

[5]Animal serum contains different quantities of LDH. The amount of LDH present in the serum varies from batch to batch. In general, human AB serum is relative low in LDH activity, whereas fetal bovine serum contains relatively high levels of LDH. We therefore recommend that no more than 5% fetal bovine serum should be used to reduce background LDH activity.

[6]PMS is an electron carrier for the second step of the LDH reaction. Instead of PMS, other electron carriers such as Mendola blue or diaphorase can also be used.

[7]The chemicals may not completely dissolve immediately. Care should be taken to completely dissolve all chemicals prior to assay development.

[8]Commercial vendors sometimes provide the components in a lyophilized or concentrated form. Follow the manufacturer's instructions for reconstitution of the components to ensure the optimal LDH assay performance.

[9]Exposure to ambient light can degrade the LDH assay buffer substrates and give rise to low absorbance values. Protect the aliquots from light.

6. 10X Lysis solution: 9% Triton X-100 (Thermo Fisher Scientific, NH, USA). Dissolve 9 mL Triton X-100 with 91 mL milli-Q water.
7. Stop solution: 1 M acetic acid (Thermo Fisher Scientific, NH, USA) (*see* Note 11).

3. Methods

All the reactions should be performed at room temperature (22 – 25°C). Warm all the components to 22 – 25°C before starting the experiment.

3.1 Cell culture preparation (see Note 12)

1. Seed cells in a 96-well flat bottom microtiter plate at a density of $1 \times 10^4 – 5 \times 10^4$ cells/well in 100 μL of culture medium. Samples in each experimental group should be prepared in triplicate wells (*see* Notes 13–[14]).
2. On the same plate, prepare the following controls in triplicates.

   Maximum LDH release – Seed the same number of cells in these wells as in step 3.1.1.

   Medium alone – these wells contain only medium but no cells.

   Volume Correction Control – these wells contain only medium but no cells.
3. For adherent cells, allow the cells to incubate for 12 – 16 hours prior to treatment. For suspension cultures, treatment can be initiated when seeding of cells is completed.
4. Culture the cells for the required amount of time in a humidified 37°C incubator equilibrated with 5% CO_2.

3.2 Harvest Supernatants

1. To the maximum LDH release wells and the volume correction control wells, add 10 μL of 10X lysis solution.
2. Return the plate to a humidified 37°C, 5% CO_2 incubator for 45 minutes.
3. Centrifuge the microtiter plate in a centrifuge at 1,500 – 2,000 rpm for 5 minutes (*see* Note 15).

[10]Repeated freezing and thawing of the LDH assay buffer should be avoided. We recommended storing aliquots of the buffer in quantities sufficient for a single assay at −20°C (e.g. 5 ml for one 96-well microtiter plate).
[11]Instead of 1 M acetic acid, 1–5% SDS, other reagents such as strong base (1 N NaOH) or 1 N hydrochloric acid (HCl) can be used to stop the reaction.
[12]The LDH protocol described here can be adapted to measurement of cell-mediated cytotoxicity (11).
[13]Different cell types contain different amounts of LDH. Therefore, the optimal cell density used should be determined empirically by performing a “total lysis” using different number of cells. The resulting LDH absorbance reading can be plotted against the cell number. The optimal cell number should be the one that falls within the linear range of the absorbance curve.
[14]Because LDH can “build up” in the culture media after overnight incubation and contribute to high background LDH activity, we recommend replacing the old media with fresh media prior to induction of cell death for adherent cell cultures.
[15]We recommend centrifugation of the cells to minimize inadvertent inclusion of cell materials during the harvest of the culture supernatants. However, care should be taken to minimize physical damage of the cells during centrifugation, which can lead to increased background “leakage” of LDH into the culture media.

4. With a multi-channel pipette, collect 50 μL of culture supernatant from each well and transfer it to a new microtiter plate (*see* Notes 16–[18]). Be careful not to transfer any cell materials.

3.3 LDH measurement

1. Add 50 μl the reconstituted 2X LDH assay buffer to the supernatants. Mix the reagent by gentle shaking for 30 seconds (*see* Notes 18–[20]).
2. Protect the assay plate from light. Incubate the plate at room temperature (22 – 25°C) for 10 –30 minutes (*see* Note 21).
3. Add 50 μL of Stop Solution to the wells. Mix the reagent by shaking for 30 seconds (*see* Note 22).
4. Measure absorbance between 490 – 520 nm (*see* Note 23).

3.4 Data Analysis

1. The reading from the untreated and test wells is subtracted with the reading from the medium alone control wells. This is the corrected reading for the untreated or test well.
2. Subtract the reading of Volume correction control wells from the reading of the Maximum LDH release wells. This is the corrected maximum release reading.
3. Calculate the percentage of cytotoxicity using the following formula:

$$\%\ \text{Cytotoxicity} = 100 \times (\text{Corrected reading from Test well} - \text{corrected reading from untreated well}) / (\text{Corrected maximum LDH release control} - \text{corrected reading from untreated well})$$

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[16]A smaller volume of culture media can be harvested depending on the sensitivity of the assay. Factors such as the number of cells used, the amount of LDH expressed in a given cell type, and the reagents used can affect assay sensitivity. For 100 μL culture on 96-well flat bottom microtiter plates, we recommended that no more than 50 μL to be harvested to minimize contamination of the culture supernatant with cell materials.
[18]After harvest of culture supernatants, the cells in the original plate can be used for other complementary cell viability assays such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (12).
[20]An optional positive control of LDH standard can be included.
[21]The reaction time can be decreased or increased depending on the color development. Monitor the color conversion periodically. Stop the reaction before the color conversion of the sample wells reach the level of that of the maximum LDH release wells.
[22]Perform measurement of the absorbance within one hour after addition of the stop solution. If the rate of the reaction rate is slow (i.e. slow color conversion), absorbance can be measured without adding the stop solution.
[23]Air bubbles present in wells affect the absorbance readings. Air bubbles can be purged using a needle.

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