# NMDA-induced Excitotoxicity and Lactate Dehydrogenase Assay in Primary Cultured Neurons

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**[Abstract]** N-Methyl-D-aspartic acid receptor (NMDAR)-mediated excitotoxicity is thought to contribute to the pathogenesis of a large number of chronic neurodegenerative disorders (such as Alzheimer's and Huntington's diseases to mental illnesses) in addition to acute brain insults such as stroke and brain trauma. Understanding the mechanisms underlying NMDAR-mediated excitotoxicity may lead to development of novel therapeutics for treating neurological diseases. Stimulation of primary cultured neurons with excessive NMDA is widely used as an *in vitro* model for studying NMDAR-mediated excitotoxicity, which allows careful dissection of the detailed cellular mechanisms underlying excitotoxic neuronal death.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme which can convert NAD into NADH. LDH is released from cells into culture medium when the plasma membrane integrity is compromised. Therefore, the amount of released LDH represents the degree of cell death. In our current study, the extracellular LDH level was measured using an *in vitro* Toxicology Assay Kit obtained from Sigma-Aldrich. The basis of this LDH assay is: 1) LDH reduces NAD into NADH, 2) the resulting NADH is then utilized in the stoichiometric conversion of a tetrazolium dye, and 3) the resulting colored compound is measured by a spectrophotometric microplate reader at a wavelength of 490 nm. The cell death rate was expressed as a percentage (%) between the absorbance of treated group and that of control group.

## Materials and Reagents

1. Primary cultured neurons
2. NMDA (Sigma-Aldrich, catalog number: M3262-25MG)
3. Neurobasal medium (Life Technologies, Invitrogen™, catalog number: 21103)
4. LDH Assay Substrate Solution
5. LDH Assay Dye Solution
6. LDH Assay Cofactor Preparation
7. *In vitro* Toxicology Assay Kit (Sigma-Aldrich, catalog number: TOX-7)

## Equipment

1. 37 °C, 5% $CO_2$ Cell culture incubator
2. 96-well plate (Sigma-Aldrich, catalog number: SIAL0596-50EA)
3. Spectrometer for 96 well plate that can measure 490 nm and 690 nm (Molecular Devices, model: SpectraMax M2e Multi-Mode Microplate Reader)

## Procedure

A. NMDA-induced Excitotoxicity

1. Prepare fresh NMDA 1,000x stock solution (25 mM) with fresh neurobasal medium. Old NMDA stock solution is less effective in inducing excitotoxicity, and higher dose might be required.
2. Immediately prior to NMDA treatment, half of the conditioned medium (old medium) is taken out and placed in the incubator to keep warm and pH balanced. The conditional medium taken out is saved for replacement of NMDA-containing medium after the excitotoxicity treatment. The reason to use conditional medium rather than fresh medium for replacement is that primary culture neurons are very sensitive to environment change, and using the conditional medium can minimize additional stimulation to neurons other than the NMDA treatment during the whole procedure.
3. NMDA is added directly to the culture medium to initiate excitotoxicity stimulation of neurons. The working concentration of NMDA is 25 μM. If the culture medium in the plate is 10 ml, the NMDA stock (25 mM) added will be 1/1,000 of 10 ml, which is 10 μl. Neurons are kept in the incubator during the treatment.
4. After 60 min incubation with NMDA, neurons are washed with fresh neural basal medium (warm and pH balanced) for once and then returned to the previously saved conditional medium.
5. Neurons are allowed to recover for different periods of time, ranging from 0 h to 24 h until further experiments.
6. Significant neuronal death could be observed after 6 h recovery and reaches its maximum level after 24 h.

   *Note: The conditioned medium is essential to minimize the stress for neurons.*

B. Lactate dehydrogenase assay to detect cell death

1. Prepare the Lactate Dehydrogenase Assay Mixture immediately before performing the assay by mixing equal volume of LDH Assay Substrate Solution, LDH Assay Dye

 

Solution and LDH Assay Cofactor Preparation according to Sigma manufacture protocol. Store the mixture on ice.

2. Remove cultures from incubator.
3. Transfer proximately 100 µl culture medium from each condition into a micro-centrifuge tube respectively and spin down the culture medium at 13,000 rpm for 1 min to deposit the cell debris.
4. Transfer 50 µl cultured medium from each condition into 96 well plate. Add 100 µl Lactate Dehydrogenase Assay Mixture to each sample. The volume of culture medium and Lactate Dehydrogenase Assay Mixture could be adjusted proportionally.
5. Cover the plate with Aluminum Foil to avoid light exposure.
6. Incubate at room temperature for several minutes to several hours depending on the concentration of LDH in the culture medium. For the first time user, reading the results every 20 min to determine the optimal end point is highly recommended. To obtain comparable results between different batches of cultures, a similar end point should be used. As the culture conditions (cell viability, cell density and medium volume) vary across different batches of neurons, it is sometimes hard to determine the end point using the length of reaction time. For example, in one batch of neurons, it may take 1 hour to achieve a reading of 1.0 at 490 nm in the control group (non-treated group); while in another batch, it takes 1.5 h. Therefore, it's highly recommended that the reaction should be read every 20 min during the whole LDH reaction, and only the data collected at the point when the reading of control group (*e.g.* 1.0 ± 0.1) is comparable to other batches should be used. As the reading is just arbitrary number, the data is only meaningful when comparing the treatment group with its control group.
7. Spectrophotometrically measure absorbance at a wavelength of 490 nm. Measure the background absorbance of 96 well plate at 690 nm. Subtract reading of 690 nm from that of 490 nm to obtain the final reading.
8. If plate reader is not available, samples could be transferred to appropriate sized cuvettes for spectrophotometric measurement.

**Notes**

1. The concentration of extracellular LDH depends on cell viability, cell density and medium volume. Ideally, cells density and medium volume in different culture wells should be exactly the same to avoid fluctuation of the results.
2. Some culture medium contains a significant level of LDH activity. In this case, blank medium should be measured and subtracted from the final results. Alternatively,

 

serum-containing medium could be replaced with serum-free medium before any treatment.

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