Kinetic Lactate Dehydrogenase Assay for Detection of Cell Damage in Primary Neuronal Cell Cultures

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[Abstract] The aim of many *in vitro* models of acute or chronic degenerative disorders in the neurobiology field is the assessment of survival or damage of neuronal cells. Damage of cells is associated with loss of outer cell membrane integrity and leakage of cytoplasmic cellular proteins. Therefore, activity assays of cytoplasmic enzymes in supernatants of cell cultures serve as a practicable tool for quantification of cellular injury (Koh and Choi, 1987; Bruer *et al.*, 1997). Lactate dehydrogenase (LDH) is such a ubiquitously expressed cytosolic enzyme, which is very stable due to a very long protein half-life (Hsieh and Blumenthal, 1956; Koh and Cotman, 1992; Koh *et al.*, 1995).
Keywords: LDH assay, Primary neurons, Cell damage, LDH release, Cell culture, Cell death, Survival, Oxygen-glucose deprivation, Glutamate toxicity

[Background] LDH catalyzes the formation of lactate and Nicotinamidadenindinucleotid (NAD$^+$) from pyruvate and reduced Nicotinamidadenindinucleotid (NADH) in a reversible biochemical reaction. NADH has an absorption on the wavelength of 340 nm. The basis of this kinetic LDH activity assay is the decrease of optical density at the specific wave length caused by a decrease of NADH. The amount of LDH in the supernatant is calculated using a standard enzyme solution with known LDH activity. Different cell densities or metabolic activation rates might be confounders; therefore normalization of LDH activity is recommended. This is achieved by assessment of LDH activity after outer cell membrane lysis that does not block LDH activity itself ('full kill' with 0.5% Triton-X). Finally, percentage of absolute LDH activity from LDH activity by full kill indicates the rate of damaged or dead cells in the cell culture well.

Materials and Reagents

1. 96 well plate, flat bottom (SARSTEDT, catalog number: 82.1581)
2. Pipette tips
3. Neuronal cell cultures which was treated by noxes (*e.g.*, oxygen and glucose deprivation, glutamate or other toxins) (Koh and Choi, 1987; Bruer *et al.*, 1997; Harms *et al.*, 2001; Ruscher *et al.*, 2002; Harms *et al.*, 2004; Meisel *et al.*, 2006; Harms *et al.*, 2007; Datwyler *et al.*, 2011; Schweizer *et al.*, 2015; Donath *et al.*, 2016)
   Note: This protocol works for primary neuronal cultures that were derived from various regions of the brain including septum, hippocampus, striatum, spinal cord, cortex, cerebellum or raphe

nuclei and that were seeded in various densities or with various media formulations or coatings of the plates. However, we achieve best results with cultures that are at least cultivated for more than 7 days in vitro (DIV) and that were seeded in a reasonable density as illustrated below (Figures 1 and 2). These cultures contain approximately 10% glial cells and this does not impact on the possibility to analyze LDH release in the supernatant medium.
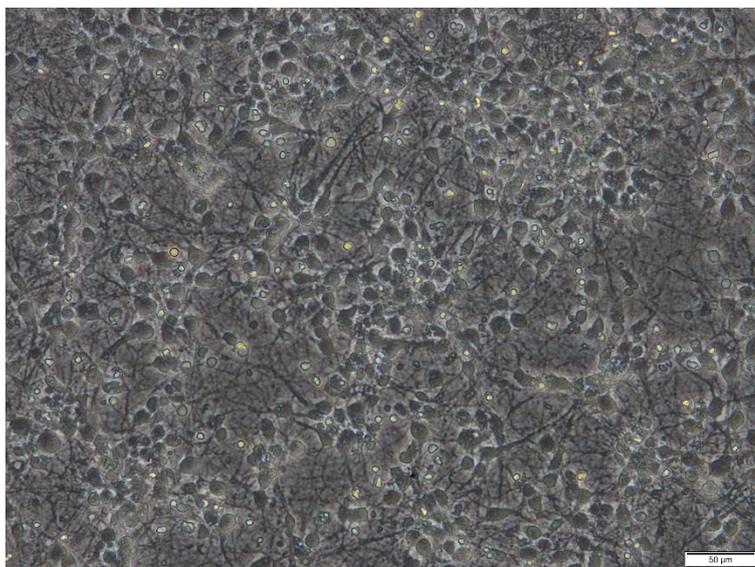
Figure 1. Primary cortical neuronal cultures derived from mouse E15 embryos after 7 days *in vitro* (DIV). Scale bar = 50 μm.
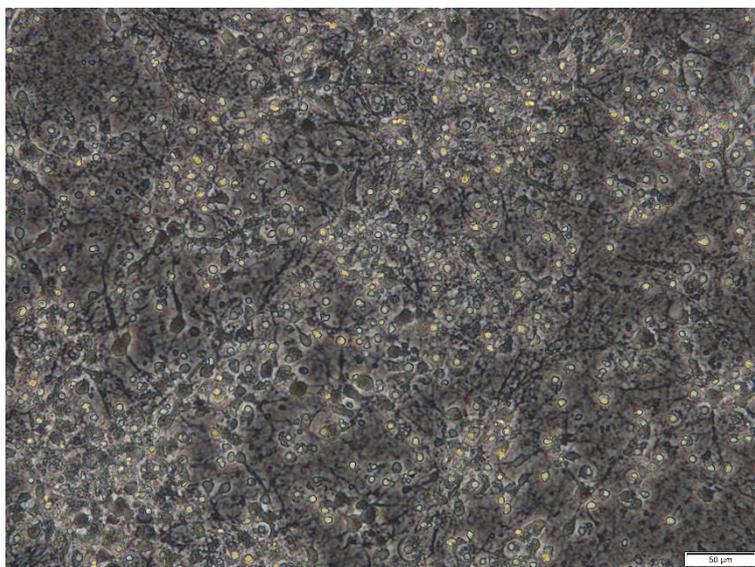
Figure 2. Primary cortical neuronal cultures that were treated with 50 μM glutamate for 24 h on DIV 8. Scale bar = 50 μm.

4. Triton X-100 solution (Sigma-Aldrich, catalog number: 93443)

5. Potassium phosphate monobasic (KH_2PO_4) (MW 136.1) (Sigma-Aldrich, catalog number: P5655)

6. Dibasic potassium phosphate (K_2HPO_4; MW 174.2) or potassium phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$; MW 228.2) (Sigma-Aldrich catalog number: P5504)

7. Distilled water

8. Na-pyruvate (MW 110) (Sigma-Aldrich, catalog number: P2256)

9. LDH standard (TruCal U) (DiaDys Diagnostic Systems, catalog number: 5 9100 99 10 063)

10. -NADH (MW 709.4, reduced form) (Sigma-Aldrich, catalog number: N8129)

11. 10x LDH-buffer (see Recipes)

12. 1x LDH-buffer (see Recipes)

13. LDH-standard (see Recipes)

14. Na-pyruvate-solution (see Recipes)

15. -NADH-solution (see Recipes)

Equipment

1. Pipette

2. CO_2-incubator for cell cultures

3. Multi-pipette (Eppendorf, model: Multipette® plus)

4. Spectrophotometer for 96 well plate that can measure 340 nm and kinetic measurement (*e.g.*, Dynex Technologies, model: MRX Microplate Reader)

Procedure

1. Preparation of solutions (see Recipes)

2. Measurement

   a. Warm up NADH and an appropriate aliquot of Na-pyruvate-solution (for one 96 well plate you need about 2.5 ml Na-pyruvate-solution) to room temperature or max. 37 °C, thaw appropriate amount of LDH-standard aliquots.

   b. Pipette 50 µl cell culture medium (probe) in each well. A duplicate measurement of each cell culture well is recommended.

   c. Pipette 25 µl LDH standard in 2 wells per plate. It is very important that you perform a standard measurement on each 96 well plate.

   d. Add 200 µl NADH solution to each well and remove bubbles if necessary. The final concentration of NADH in the assay is 153.8 µM.

   e. Add 25 µl Na-pyruvate solution to each well. The enzymatic reaction starts promptly. The final concentration of Na-pyruvate in the assay is 2.063 mM. Do not mix samples with pipets. Mixing should be initiated by using automated shaking by the plate reader before and in

between of each measurement cycle. The reaction is within the linear range, if excess NADH is available for the enzymatic reaction. This lasts about 7-10 min. Ideally, pipetting of pyruvate and measurement of all cycles should be accomplished within this period. Do not start the reaction in more than one plate at once. It might be necessary to dilute the sample as specified in Figure 3.
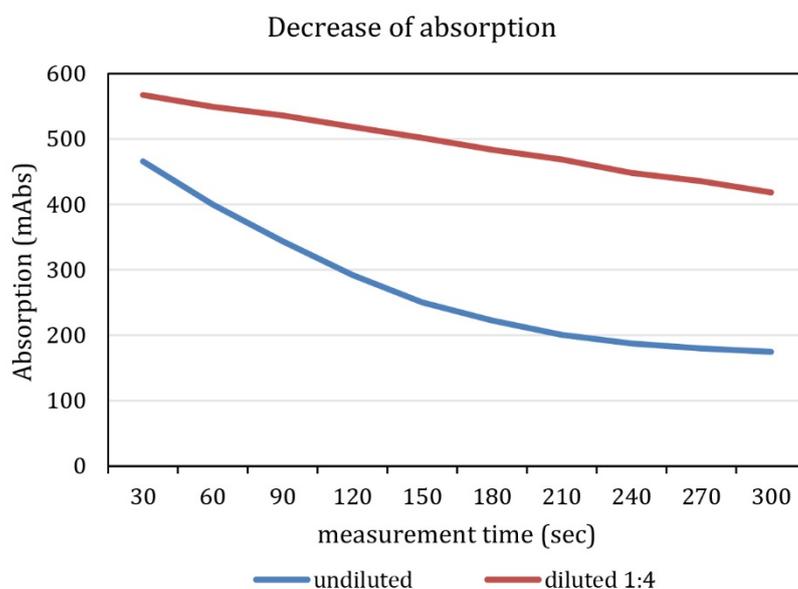
Figure 3. Representative graph of a measurement. The blue line indicates an undiluted and the red line a diluted (F1:4, red) kinematic curve. Note that the undiluted sample shows a non-linear decrease in absorption at 340 nm.

    f. Immediately start measurement of absorption with following parameters:
- i. Wavelength: 340 nm.
- ii. Measurement cycles: 10.
- iii. Cycle time: 30 sec.
- iv. Shaking time: 5 sec.
- v. Temperature: 37 °C (see also Notes).

3. Total lysis
   a. Add Triton X-100 to the rest of cell culture medium. The final concentration should be 0.5%.
   b. Incubate on 37 °C for 20 min. It is possible to incubate a longer time until to 4-6 h or overnight. This is not critical as long as you treat all groups equally.
   c. Repeat steps 2b-2f from paragraph 2 (Measurement).

Data analysis

1. Calculate the decrease of absorption for each well in mAbs/min. In our hands the value is about 10-50 mAbs/min.

Note: Check, if the decrease of absorption is linear over the whole time. Otherwise, reduce the volume of probe or dilute them.

2. The decrease of absorption in LDH standard equates an LDH activity about 500 U/l. The LDH activity in standard is a little bit different in different batches. Calculate according to manufactures specifications.

3. Calculate the LDH activity for your probes using a rule of proportion.

   Example:

   a. Decrease of absorption in probe: 15 mAbs/min.

   b. Decrease of absorption in LDH standard: 43 mAbs/min.

   c. LDH activity in standard 466 U/l.

   Equation: (15 mAbs/min x 466 U/l/43 U/l) = 162.5 U/l

4. Adjust the LDH activity for probe on the relation of probe volume and LDH standard volume.

5. Finally, calculate LDH activity as a percentage of LDH activity in the same cell culture sample after assessing LDH activity after total LDH release ('full kill').

Notes

1. The reaction rate is dependent on the temperature. In case of high LDH activity in your probe and an additional high temperature, an exhaustion of -NADH is possible and leads to a non-linear decrease of absorption.

2. Assessment of total LDH release after cell lysis ('full kill') is recommended for each cell culture well. There are some treatments, which lead to an increase of protein synthesis in cells, so that the amount of total LDH in the cells is increased as well. Normalization helps for better correlation as an indirect measure of cell death in such cases.

3. It is possible to store samples of supernatants at 4 °C if samples are sealed to avoid evaporation for two to three days with a minimal loss of LDH activity. Do not freeze samples.

Recipes

1. 10x LDH-buffer

   45.3 g KH_2PO_4

   116.1 g K_2HPO_4 or 152 g $K_2HPO_4 \cdot 3H_2O$

   Dissolve in about 800 ml distilled water, adjust pH to 7.4 and fill up to 1,000 ml with distilled water

   Store at 4 °C, The solution is stable for 12 month

2. 1x LDH-buffer

   Dilute 10x LDH buffer with distilled water 1:10

   Store at 4 °C, long-term storage up to 12 months is possible

3. LDH-standard

Dissolve lysate of enzyme according to manufacturer's protocol, prepare aliquots of 105 µl (that's for measurement of two plates) and store at -20 °C. Avoid freeze and thaw cycles

4. Na-pyruvate-solution

Dissolve 1.25 g Na-pyruvate in 500 ml 1x LDH-buffer, results in 22.7 mM Na-pyruvate-solution

Store at 4 °C. The solution is stable for 18 month

5. -NADH-solution

Dissolve 3 mg -NADH in 20 ml 1x LDH-buffer, results in 211.4 µM solution

Note: 20 ml is the amount for one 96 well plate. This solution is stable only for 2 days at 4 °C.

Acknowledgments

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