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Laboratory Protocol Lactate Dehydrogenase Cytosolic Marker Enzyme

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Section Page

1 Background

Lactate dehydrogenase (EC 1.1.1.27) is an enzyme, which catalyzes the last step in glycolysis. LDH is a soluble enzyme and localized in the cytosol (cytoplasm). LDH, therefore, is used as a quantitative marker enzyme for the intact cell, its activity providing information on cellular glycolytic capacity (Renner et al., 2003). Measurement of LDH release (leakage) is an important and frequently applied test for cellular membrane permeabilization (rupture) and severe irreversible cell damage. LDH leakage normally correlates well with CK release and the trypan blue viability test.

1.1 Enzymatic reaction catalyzed by LDH

$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{L-Lactate} + NAD^+ \quad (1)$$

1.2 Principle of spectrophotometric enzyme assay

(Bergmeier 1970)

Absorbance and enzyme activity: The optical density, *OD,* of a liquid sample is related to the absorbance, *A,* by the optical path length, *l* [cm],

$$OD = A / l = \varepsilon_B \cdot c_B \quad (2)$$

The unit of *A* is a dimensionless number. The path length is fixed by the dimension of the spectrophotometric cuvette. The molar extinction coefficient of the absorbing substance B, ε_B [$mM^{-1} \cdot cm^{-1}$], is specific for the compound studied at a particular wavelength, and absorbance increases with molar concentration, c_B [mM], in the final solution contained in the cuvette. The rate of increase or decrease of the absorbance is the slope, r_A = dA/d*t*, which is proportional to enzyme activity.

LDH assay: In the spectrophotometer, the reaction catalyzed by LDH (Eq. 1) is associated with oxidation of the absorbing at 340 nm compound NADH:

$$\text{NADH} \rightarrow NAD^+ \quad (3)$$

The reaction product NAD (oxidized NADH) is not an absorbing at this wavelength substance. Therefore, the working wavelength is 340 nm. The absorbance decreases linearly with time, (over 100 s of measurement).

1.3 Temperature of enzyme assay

When LDH activity is used as a marker cytosolic enzyme or/and for detection of the damage of cellular membrane (LDH leakage), it is not critical to choose a physiological temperature. A constant reference temperature has to be applied for comparability of measurements. Commonly measurements can be performed at room temperature, but possibly more frequently at 30 °C.

2 Reagents and buffers

2.1 Prepare every month new and store at 4 °C

Tris-HCl buffer (100 mM, pH 7.1): 1.2114 g/100 ml a.d., adjust to pH 7.1 with 37 % HCl (ca. 600 µl/100 ml).

Triton X-100 (10 % solution): reagent solution is 100 %, add 90 ml a.d. to 10 g (ca 10 ml) Triton X-100.

2.2 Prepare samples of NADH and pyruvate, store at 4 °C

5 x 11.5 mg NADH, 5 x 110.0 mg Pyruvate, store at 4° C.

2.3 Prepare fresh every day

NADH stock solution (15 mM): 11.5 mg NADH + 1 ml a.d.

Pyruvate stock solution (1.0 M): 110 mg Pyruvate + 1 ml a.d. Store on ice during measurement.

2.4 Chemicals

Name	FW	Source No.	Stock Solution	Comments
Tris [Tris-(hydroxymethyl)-aminomethan], C4H11NO3	121.14	Merck 8382	0.1 M; 1.2114 g/100 ml a.d.	Adjust to pH 7.1 by HCl, to obtain Tris-HCl buffer.
NADH (□□Nicotin-amide adenine dinucleotide reduced) Disodium salt, Trihydrate C21H27N7Na2O14P2. 3 H2O	763.46	Fluka 43423	15 mM; 11.50 mg/1 ml a.d.	Electron donor. Hydroscopic, sensitive to air. Keep at 4 °C.

Pyruvate (Pyruvic acid, a-Ketopropionic acid) Sodium salt C3H3O3Na	110.00	Sigma P-2256	1.0 M; 110 mg/1 ml a.d	Keep at 4 °C. Desiccate.
Triton X-100, C34H62O11	646.87	Serva 37238	10%; 10 g/100 ml a.d.	Viscous liquid; detergent. Keep at 4 °C.
L –Lactic Dehydrogenase, LDH Typ II EC 1.1.1.27 From Rabbit Muscle	134,000 tetramer	Sigma L-2500	11 mg prot./ml (Biuret)	From porcine heart, stored at 4 °C. Do not freeze. Avoid contact and inhalation. Crystalline suspension in 3.2 M (NH4)2SO4, pH 6. Specific activity: 950 IU/mg protein at 37 °C, varies with Lot Number.

3 Sample preparation

Freeze sample in liquid nitrogen and store frozen at -80 °C. During measurement store on ice. LDH activity of cells is stable during storage (a few hours) on ice. Incubate suspension at 31 °C for 5-10 min before measurement.

15 to 20 samples, including standards, can be processed as a batch.

3.1 LDH Standard

Preparation: As a standard, commercial LDH is diluted 1:500 in 0.1 M Tris-HCl buffer, pH 7.1 (RT). Accurate dilution is critical and is achieved by adding 2 µl of LDH standard (using a 10 µl Hamilton syringe) to 1000 µl of buffer. Starting with a protein concentration of 11 mg·cm^{-3} in the undiluted LDH standard, this yields a final protein concentration of 0.022 mg·cm^{-3} in the sample, of which 5 µl are added to a volume of 995 µl of incubation medium. Use freshly diluted enzyme. During measurement store on ice.

Application: The LDH standard serves as a check of chemicals and assay conditions, and may even be used for final correction of results. A standard (at least in replicate) is included at each day of measurement, if a large number of samples is processed collectively.

Different LDH standards: The age of the standard has to be checked critically. If a new LDH standard is applied, the Lot Number is noted, together with the specific activity

and the protein concentration provided by the supplier (Sigma).

3.2 Suspended cells

For typical cells (HUVEC, TEC) at 1-2·10^6 cells/ml, take replicates of 100-200 µl samples into Eppendorf tubes, freeze in liquid nitrogen, and store until measurement.

3.3 Sample preparation for the assessment of LDH in cardiac muscle or liver homogenates

Mince frozen tissue samples (40-100 mg) with scissors, place into ice-cold 0.1 M K-phosphate buffer, pH 7.4 at 25 °C (buffer is added to yield a concentration of 30 mg tissue per ml). Homogenize for 30 s with Ultra-Turrax homogenizer at maximum speed at 0 °C. Freeze samples in liquid nitrogen and store frozen at -80 °C or in liquid nitrogen. LDH activity of cardiac homogenates is stable after one freezing/thawing step and during storage (a few hours) on ice. For the measurement, as a rule use 10 µl of heart and 20 µl liver homogenates.

4 Measurement: Spectrophotometer Beckman DU 640

4.1 Switch on the spectrophotometer

Switch on the spectrophotometer ca. 10 min before measurement.
Power up diagnostic **--> *quit***
Routine measurement **--> *kinetics/time***
--> *method name: A:\LDH.* There is possibility to change options, simply click on desired option.
--> *exit*

4.2 Blank-measurement

--> *vis on* [visible lamp, light will be switched on immediately, the sign becomes red]. ***uv on*** [mercury lamp, light will be switched with delay, the sign becomes also red].
Add 1 ml a.d. into quartz cuvette. Do not use plastic cuvettes, since these yield lower activities compared to quartz.
Insert cuvette into spectrophotometer.
--> *blank* [value of blank will be saved automatically, blue sign „reading blank"].

4.3 Preparation of incubation medium

To obtain a total volume, $V_{cuvette}$, of 1000 µl in the cuvette (including the volume of sample, V_{sample}), add into glass tubes:

1. (975 µl - V_{sample}) Tris-HCl buffer
2. 10 µl 1 M Pyruvate (final concentration 10 mM)
3. 20 µl 15 mM NADH (final concentration ca. 0.3 mM)
4. In case of intact cells, 25 µl 10 % Triton X-100 (final concentration ca. 0.25 %)

Mix carefully.
Pre-incubate medium in thermostat at 30 °C (5-10 min) for measurements at 30 °C.

4.4 Measurement of changes in absorbance

Transfer incubation medium into pre-thermostated spectrophotometric quartz cuvette.
Immediately put the cuvette into the cell holder of spectrophotometer.
Add the sample, V_{sample}, i.e. 100-200 µl cell suspension, or 5 µl LDH standard (see Section 3), into prepared quartz cuvettes.
Mix carefully with plastic stick, wash stick with a.d.
--> read sample, --> start.
The linear decrease of absorbance is measured over ca. 100 s.
Another possible way for mixing is to add sample directly into pre incubated in thermostat medium, mix with Wortex and immediately transfer into quartz cuvette pre-thermostated (5 min, 30 °C) in spectrophotometer cuvette holder.
Optional: After ca. 100 s (middle of time range) it is possible to make a second sample addition (the same volume), in this case the activity must be proportional to the amount of the sample added, taking into account the dilution factor. (normally this is proportional up to 0.15 units of absorbance per minute).
--> rates [squares show data points].
Change *A* scale if necessary [click on upper or lower y-axis value], or use ***zoom.***
--> trace [click on trace and define your starting and final times, cursor is placed on your defined starting or final points], change initial and final time points to measure the rate within linear part of the curve.
Write into the protocol the measured rate of absorbance change, $r_A = dA/dt$ (see Section 5).

--> *exit*
--> *save clear -*
There is a possibility to save the data ***file name*** [choose file name] ***- OK - OK***
Use ***save*** in exceptional cases, due to very limited memory of Beckman's computer.
For non-saving mode make square empty (click with mouse).
Device is ready for measurement of next sample.

5 Data analysis: Calculation of specific LDH activity

5.1 Absorbance, concentration and rate of reaction

The rate of concentration change of the absorbing compound B in the cuvette, dc_B/dt, is calculated from the rate of the absorption change (Eq. 2),

$$dc_B / dt = \frac{dA/dt}{l \cdot \varepsilon_B} = \frac{r_A}{l \cdot \varepsilon_B} \qquad (4)$$

The reaction flux per unit volume, J_V, in the cuvette is,

$$J_V = dc_B/dt \cdot \nu_B^{-1} \qquad (5)$$

where ν_B is the stoichiometric number of compound B (Gnaiger, 1993), which is equal to 1 in the reactions (1) and (3).

5.2 Specific enzyme activity: reaction rate per unit sample

The specific enzyme activity is proportional to the experimental reaction flux (Eq. 5) and inversely proportional to the dilution factor, $V_{sample}/V_{cuvette}$ and to the mass concentration, ρ [mg·cm^{-3}] or cell density [10^6·cm^{-3}] in the sample, V_{sample}. The specific enzyme activity, v, is the velocity of the enzyme-catalyzed step per unit of sample, measured under experimental incubation conditions with saturating substrate. Combining Eq.(4) and (5),

$$\text{Specific activity}: \qquad v = \frac{r_A}{l \cdot \varepsilon_B \cdot \nu_B} \cdot \frac{V_{cuvette}}{V_{sample} \cdot \rho} \qquad (5)$$

v Specific activity of the enzyme is expressed per mg protein or per million cells [IU/mg protein or IU/10^6 cells], depending on ρ. Enzyme activity is frequently expressed in

	international units, IU [µmol/min]. 1 IU of LDH forms 1 µmol of lactate (or oxidizes 1 µM of NADH) per min. Note that the minute is used here as the unit of time (although the second is the preferred *SI* base unit; Gnaiger, 1993).
$r_A = dA/dt$	Rate of absorbance change [min^{-1}] (Eq. 4).
l	Optical path length (= 1 cm).
ε_B	Extinction coefficient of NADH at 340 nm and pH 7.1 (= 6.22 $mM^{-1} \cdot cm^{-1}$).
ν_B	Stoichiometric number of B (NADH) in the reaction (Eq. 3) (= 1).
$V_{cuvette}$	Volume of solution in the cuvette (= 1000 µl).
V_{sample}	Volume of samle added to cuvette (200 µl, 100 µl, 25 µl, 5 µl).
ρ	Mass concentration or density of biological material in the sample, V_{sample} (protein concentration: $mg \cdot cm^{-3}$; cell density: $10^6 \cdot cm^{-3}$).

6 References

Bergmeier HU (1970) Methoden der enzymatischen Analyse. 2. Auflage, Akademie Verlag, Berlin.

Gnaiger E (1993) Nonequilibrium thermodynamics of energy transformations. *Pure & Applied Chem.* 65: 1983-2002.

Renner K, Amberger A, Konwalinka G, Kofler R, Gnaiger E (2003) Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. *Biochim. Biophys. Acta* 1642: 115-23.