Total lactate dehydrogenase calorimetric enzyme activity measurement V.1

Renuka Sriram[1]

[1]University of California, San Francisco

Version 1

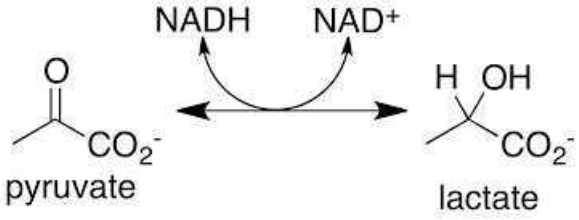
Jun 09, 2021

1 Works for me Share dx.doi.org/10.17504/protocols.io.bvhmn346

Renuka Sriram

ABSTRACT

Lactate Dehydrogenase is a key cytoplasmic enzyme that confers the observed Warburg effect in tumors. Its a tetrameric protein composed of combinations of one or two isomers. This protein catalyzes the reversible reaction



Source: Wikipedia

This reaction is monitored spectrophotometrically by observing the reduction in absorbance of NADH (which is maximal at 420nm) as pyruvate is converted to lactate.
This protocol, details the steps involved in such a calorimetric assay of cells and tissues.

DOI

dx.doi.org/10.17504/protocols.io.bvhmn346

PROTOCOL CITATION

Renuka Sriram 2021. Total lactate dehydrogenase calorimetric enzyme activity measurement. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bvhmn346

KEYWORDS

Lactate dehydrogenase, calorimetric, lactate, pyruvate, NADH

CREATED

Jun 02, 2021

LAST MODIFIED

Jun 09, 2021

PROTOCOL INTEGER ID

50445

MATERIALS TEXT

Material	Source	Catalogue No.	Storage
Cell Lysis Buffer (10X)	Cell Signaling Technology	9803S	−20˚C
NaCl	ThermoFisher	BP358-1	Room temperature
NADH (β-Nicotinamide adenine dinucleotide, reduced disodium salt)	Sigma	N6785-10VL	−20˚C
Sodium pyruvate (11mg/mL, 100x)	Sigma	S8636	2-8°C
Tris Base (Trizma Base)	Sigma	93362(BioUltra, for molecular biology, ≥99.8% (T)) or 93352 (≥99.0% (T))	Room temperature
Tris-HCl (Trizma hydrochloride)	Sigma	T6666-50G	Room temperature
DTT (1,4-Dithiothreitol)	Sigma	10197777001	2-8°C
EDTA (Ethylenediaminetetraacetic acid)	Sigma	1233508	
Bradford Assay Kit	ThermoFisher	23200	
Triton X-100	Thermofisher	28314	room temperature

Apparatus

1. If working with cells - Cell counter

We use Bio-Rad TC20™ Automated Cell Counter

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

or hemocytometer

hemocytometer

2. Stator-rotor homogenizer

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

or bead lyser

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

We use Qiagen TissueLyser LT

3. Plate reader

We use Tecan M200 Infinite

4. Multichannel pipette (20-200ul)
5. Micropipettes (10 - 1000 ul)
6. Stir plate
7. Water bath (@30C)
8. Centrifuge
9. Vortex mixer

Other consumables

1. 12 well reservoir

2. 96 well flat bottom clear plate
3. Pipette tips
4. 15 ml conical tubes
5. Eppendorf tubes (1.2 - 2 ml)

DISCLAIMER:

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

DISCLAIMER:

Pre-preparation

1 Reaction buffer – Tris buffer @ pH **7.2** , [M] **200 Milimolar (mM)** ☒ Sodium chloride **P212121** , [M] **200 Micromolar (µM)**

☒ β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) **Sigma Aldrich Catalog #N8129**

1.1 In a **200 mL** beaker, measure and add **140 mL** ☒ dH2O **Contributed by users**

1.2 Place magnetic stir bar in beaker, place onto stir plate and set to **200 rpm**

1.3 Measure and add the following:

i. **1.57 g** HCl Tris

ii. **0.15 g** Tris Base

iii. **1.64 g** of NaCl

iv. Use solution to dissolve NADH and add **20 mg** (10 vials of **2 mg** each)

1.4 Turn off stir plate and remove stir bar

1.5 Measure and record buffer pH (should be pH **7.2** , adjust with appropriate base/acid)

1.6 Store in **-20 °C** freezer as **12 mL** aliquots (for 12 dilutions of pyruvate). This solution will be stable for upto 6 months.

2 Lysis buffer – [M] **50 Molarity (m)** Tris buffer pH **8.2** , [M] **2 Milimolar (mM)** DTT, [M] **2 Milimolar (mM)** EDTA, 1% Triton x-100.

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

2.1 In a **50 mL** conical tube, measure and add **20 mL** of dH2O

2.2 Measure and add the following:

i. **0.71 g** HCl Tris

ii. **0.67 g** Tris Base

iii. **0.15 g** EDTA

iv. **0.06 g** (or 59.326 uL) of DTT

v. **2 mL** Triton

2.3 Store as **200 µl** l aliquots in **-20 °C** C freezer (is stable for up to a year). This is 10 times concentrated, so for the assay add **1800 µl** dH_2O.

3 Store **500 µl** aliquots of sodium pyruvate (**11 mg/ml**) in the - **20 °C** freezer. This solution is stable for up to a year when frozen.

Set-up

4 Take from the freezer, one tube each of the pyruvate and reaction buffer and place in **30 °C** water bath.Thaw the lysis buffer and add **1800 µl** of dH_2O and place on ice

5 Prepare 12 pyruvate concentrations using pyruvate and reaction buffer by carefully pipeting into pre-labeled conical tubes

5.1 Example of dilutions (can be subject to change based on sample type and amount of buffer needed):

Pyruvate Concentration (mM)	Vol. of Pyruvate stock sol'n to be added (uL)	Vol of Reaction Buffer to be added (uL)
0.05	1.25	2498.75
0.1	2.5	2497.5
0.2	5	2495
0.3	7.5	2492.5
0.4	10	2490
0.5	12.5	2487.5
0.6	15	2485
0.7	17.5	2482.5
0.8	20	2480
0.9	22.5	2477.5
1	25	2475
2	50	2450
TOTAL	188.75	29811.25

Pyruvate Dilution table

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

5.2 Calculate the amount of pyruvate + reaction buffer dilution needed for the entire assay - **147 µl** x 3 (triplicate) x (# samples)

5.3 Pipette calculated amount + **150 µl** (to ensure enough is available) into each corresponding 12 well multichannel reservoir- from lowest to highest pyruvate concentration.

5.4 Keep the multi-well reservoir in the **30 °C** water bath

10m

6 TECAN spectrophotometer on and set temperature at **30 °C** .
a. Ensure the sample plate map of the appropriate method is set for the assay.
b. Set-up the protocol for a dynamic **00:10:00** readout at 420 nm and for the appropriate 96 well plate

7 Set-up the bead homogenizer in the cold room

8 Set -up buckets of ice and dry-ice as needed

Cell preparation

9 Trypsinize and collect cells:

9.1 Aspirate the medium from cell culture vessel, and rinse with PBS. Aspirate the PBS, and add 0.25% trypsin in PBS.

9.2 Place the culture vessel in the incubator (4-10 minutes based on cell type), and after the cells detach, add media (containing serum to inactivate the trypsin).

9.3 Transfer the cells to a **15 mL** tube and keep some in an eppendorf tube for cell counting.

5m

9.4 Centrifuge **15 mL** tubes at **300 x g** for **00:05:00** at **4 °C** .

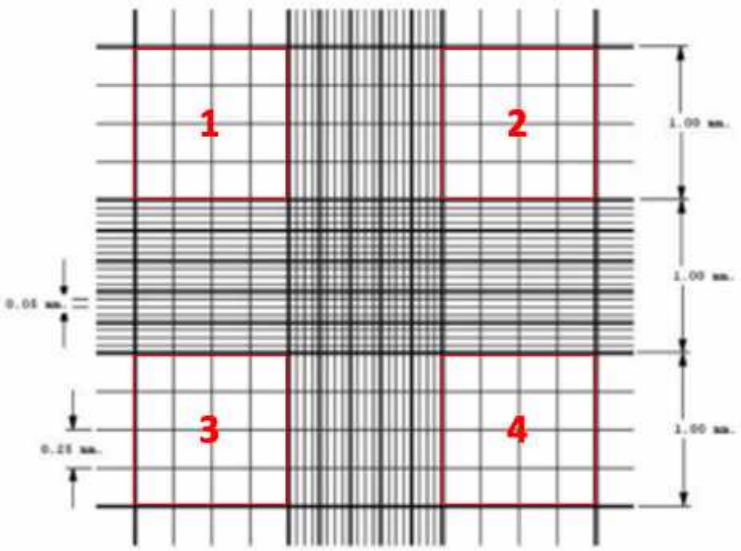
10 Cell Counting

If using a cell counter, use the average of three measurements to determine the number of cells.

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

10.1

10.2 If using a hemocytometer (count the cells in 1,2,3 and 4 squares in both chambers and take the average). cells/ml = average count per square × 10^4



Hemocytometer

10.3 Total cells = cells/ml × dilution (if used) x total original volume of cell suspension from which sample was taken)

10.4 Re-suspend the cells in lysis buffer to obtain a concentration of 10^7 cells/ml

Tissue homogenization

11 Weigh and record tissue sample (**6-10 mg** is sufficient) carefully using sterile forceps (keep frozen).
a. Record any additional information about the sample (sample name/description, locus of tissue taken, date, etc.).
b. Place tissue into a screw cap vial (compaitble with the bead homogenizer) and place on ice.

12 Add **500 µl** of lysis buffer into the vial with tissue and place on ice

2m 50s

13 Two options for homogenizing tissue
Option 1 - Using bead homogenizer -
a. Place a bead (3-5 mm stainless steel) into the bead homogenizer compatible vial (from step 11b)
b. Operate the machine for **00:02:00** at 20–30 Hz

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

Option 2 - Using stator-rotor -
a. Label plastic tubes EtOH and 0.1% DMPC (dimethyl pyrocarbonate) , one set for each sample) for cleaning.
b. Place homogenizer into EtOH for **00:00:10** ; wipe excess with Kimwipe.
c. Place homogenizer into DMPC H2O for **00:00:10** and wipe excess with Kimwipe.
d. Use homogenizer to grind tissue at medium to high settings for approximately **00:00:30** or until tissue is fully homogenized.
e. repeat steps b and c before moving to the next sample to clean the homogenizer

14 Centrifuge homogenate at **4 °C** for **00:05:00** at **7000 rpm** 5m

15 Transfer supernatant and place into a separate eppendorf tube labeled with sample name.

Estimation of tissue homogenate dilution

16 Prepare varying Tissue/Cell lysate : Lysis Buffer (TL:LB) dilutions (subject to change with varying sample type):
a.2X (**100 µl** TL : **100 µl** LB)
b.5X (**40 µl** TL : **160 µl** LB)
c.10X (**10 µl** TL : **190 µl** LB)
Keep tissue lysate on ice until ready to pipette into 96 well plate.

17 Determine appropriate tissue/cell dilution to be used for the assay.
a. Use **[M]0.6 Milimolar (mM)** pyruvate concentration and plate triplicates of different sample dilutions.
b. In a 96-well plate, add **3 µl** of each TL:LB dilution in triplicate.
c. Add **147 µl** of the **[M]0.6 Milimolar (mM)** pyruvate concentration to each well.

18 Place the 96-well plate in the spectrophotometer and measure immediately.

19 **147 µl** Once the measurement has completed in **00:10:00** , Calculate a linear regression for each well to obtain the kinetic output slope (OD/min) and determine the correct dilution by identifying the dilution with the most linear slope. 10m

LDH activity measurement

20 

Pipette **3 µl** of appropriate TL:LB dilution into three rows (technical replicates) and 12 columns (corresponding to the 12 pyruvate dilutions) on the 96 micro well plate.
Note: Ensure that there are no bubbles

21 

Using a **200 µl** multichannel pipettor, carefully pipette **147 µl** of each 12 pyruvate dilutions in triplicate; assay

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346

immediately.
Note: Ensure that there are no bubbles

22 Place the 96-well plate in the spectrophotometer and measure immediately.

23 Once the read has completed in **00:10:00** , click Export to excel and save the file. Eject the plate and clean up 10m

Data Analysis

24 Do linear regression for each well (change in absorbance over the 10 minutes) to obtain the slope OD/min. (Note: the TECAN Infinity200M accounts automatically for the **1 cm** path length. So the measurement is actually OD/min/cm)

25 LDH enzyme ctivity is calculated as uM NADH/min =
OD/min (slope) * Solution Volume in each well (**150 µl**) / (volume of sample lysate (**3 µl**) * e)
where e=extinction coeff=0.00622 $uM^{-1}cm^{-1}$
Note: take the absolute value of OD/min (omit the negative sign)

26 Take the average (of the three technical replicates) activity per pyruvate concentration subject to a threshold of Rsq > 0.9

27 Plot Tissue Activity **Michaelis-Menten** curve
a. X = Pyruvate Concentration (mM)
b. Y = Average Activity

28 For simpler linear curve

28.1 plot Lineweaver-Burke:
a. X = 1/Pyruvate Concentration
b. Y = 1/Average Activity

28.2 Obtain trend-line linear fit in the Excel program and calculate
a. KM = slope / intercept ; [mM]
b. Vmax = 1/ intercept ; uM NADH/min

Use Bradford assay kit (https://www.thermofisher.com/order/catalog/product/23200#/23200) to determine protein concentration to express Vmax (LDH activity) in umols NADH/min/mg of protein

Citation: Renuka Sriram (06/09/2021). Total lactate dehydrogenase calorimetric enzyme activity measurement. https://dx.doi.org/10.17504/protocols.io.bvhmn346