VERSION 1

JUN 09, 2021

OPEN ACCESS**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>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol and it's working

Created: Jun 02, 2021**Last Modified:** Jun 09, 2021

PROTOCOL integer ID:
50445

Keywords: Lactate dehydrogenase, calorimetric, lactate, pyruvate, NADH

Total lactate dehydrogenase calorimetric enzyme activity measurement V.1

Renuka Sriram¹¹University of California, San Francisco

Renuka Sriram

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to [protocols.io](#) is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with [protocols.io](#), can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

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.

MATERIALS

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

Material	Source	Catalogue No.	Storage
Tris Base (Trizma Base)	Sigma	93362(BioUltra, for molecular biology, $\geq 99.8\%$ (T)) or 93352 ($\geq 99.0\%$ (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](#)

or

hemocytometer

hemocytometer

2. Stator-rotor homogenizer

We use Qiagen TissueLyser LT
or bead lyser

We use Tecan M200 Infinite

3. Plate reader
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)

Pre-preparation

- 1 Reaction buffer – Tris buffer @ $\text{pH } 7.2$, $[\text{M}] 200 \text{ millimolar (mM)}$
 $\otimes$ Sodium chloride **P212121**, $[\text{M}] 200 \text{ micromolar } (\mu\text{M})$
 $\otimes$ β -Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) **Merck**
MilliporeSigma (Sigma-Aldrich) Catalog #N8129

- 1.1 In a 200 mL beaker, measure and add 140 mL $\otimes$ 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 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 – 50 Molarity (m) Tris buffer 8.2, 2 millimolar (mM) DTT, 2 millimolar (mM) EDTA, 1% Triton x-100.

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 aliquots in -20 °C freezer (is stable for up to a year). This is 10 times concentrated, so for the assay add 1800 µL dH₂O.

- 3 Store 500 μL aliquots of sodium pyruvate (11 mg/mL) in the $-20\text{ }^{\circ}\text{C}$ 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 $30\text{ }^{\circ}\text{C}$ 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 (μL)	Vol of Reaction Buffer to be added (μL)
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

- 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 $^{\circ}\text{C}$ water bath

6 TECAN spectrophotometer on and set temperature at 30 $^{\circ}\text{C}$. 10m
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.

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

5m

10 Cell Counting

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

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

Hemocytometer

10.3 Total cells = cells/ml × dilution (if used) × 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 (compatible with the bead homogenizer) and place on ice.

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

13 Two options for homogenizing tissue

2m 50s

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

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.
- Use **1mM 0.6 millimolar (mM)** pyruvate concentration and plate triplicates of different sample dilutions.
 - In a 96-well plate, add **3 µL** of each TL:LB dilution in triplicate.
 - Add **147 µL** of the **1mM 0.6 millimolar (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 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 activity is calculated as $\mu\text{M NADH/min} = \text{OD/min (slope)} * \text{Solution Volume in each well (} \text{150 } \mu\text{L)} / (\text{volume of sample lysate (} \text{3 } \mu\text{L)} * e)$
 where $e = \text{extinction coeff} = 0.00622 \text{ uM}^{-1}\text{cm}^{-1}$
 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 $R_{sq} > 0.9$
- 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. $K_M = \text{slope} / \text{intercept}$; [mM]
 b. $V_{max} = 1 / \text{intercept}$; $\mu\text{M NADH/min}$
- Use Bradford assay kit
 (<https://www.thermofisher.com/order/catalog/product/23200#/23200>) to determine protein concentration to express V_{max} (LDH activity) in $\mu\text{M NADH/min/mg}$ of protein