Lactate is a central metabolite in energy metabolism and is also involved in cell signaling and epigenetic regulations. Here, we describe an NADH-independent enzymatic assay allowing rapid, selective, and sensitive quantification of L-lactate down to the pmol range. We detail lactate extraction from intracellular and extracellular fractions, followed by total protein amount determination and enzymatic assay. This approach allows quantification of intracellular and extracellular L-lactate levels, validated by treating adherent and non-adherent cells with inhibitors of lactate transporters (MCT).

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

NADH-independent enzymatic assay to quantify extracellular and intracellular L-lactate levels

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SUMMARY

Lactate is a central metabolite in energy metabolism and is also involved in cell signaling and epigenetic regulations. Here, we describe an NADH-independent enzymatic assay allowing rapid, selective, and sensitive quantification of L-lactate down to the pmol range. We detail lactate extraction from intracellular and extracellular fractions, followed by total protein amount determination and enzymatic assay. This approach allows quantification of intracellular and extracellular L-lactate levels, validated by treating adherent and non-adherent cells with inhibitors of lactate transporters (MCT).

BEFORE YOU BEGIN

We hereby present a new method for the quantification of intra- and extracellular lactate using a NADH/NAD⁺ independent enzymatic assay. The vast majority of assays used to detect and quantify lactate are based on lactate dehydrogenase (LDH) enzymatic system (Harrison et al., 2021; San Martin et al., 2013; Vangrieken et al., 2021; Villa-Bellosa, 2020). These enzymes allow the reversible conversion of lactate into pyruvate by using NADH/NAD⁺ as redox cofactors. As these enzymatic systems are working close to the thermodynamic equilibrium, pyruvate present in the extracts can bias the results thus NAD⁺/NADH ratio has to be adjusted to favor the conversion of lactate into pyruvate (Markert, 1984). The use of L-Lactate Oxidase allowing the transformation of lactate into pyruvate via the production of H₂O₂ overcomes the problems previously described (Figure 1).

Cell culture

© Timing: 3 days

This protocol has been developed using adherent (HEK, MEF) and non-adherent cells (P3 Glioblastome stem-like cells) (Guyon et al., 2020; Mourier et al., 2015).

8 × 10⁶–10⁷ cells per well in non-adherent conditions and between 0.5 × 10⁶ to 10⁶ cells per well in adherent conditions are seeded in a 6-well plate with 2 mL of cell culture medium (DMEM for HEK and MEF; Neurobasal™ for P3 cells). The treatment is applied during the plating and metabolites are extracted after 3 days of culture.

The protocol below describes the specific steps for measurement in 6-well plates. However, it can be adapted to other plate formats or dishes.
CRITICAL: Allowing the cells to grow during 3 days before extracting L-lactate is justified by the low proliferative rate of the cell type used here as P3 (doubling time of 4 days). Therefore, this delay should be adjusted to the proliferative rate of the specific cell type used proliferative rate and metabolic activity, and avoiding cell confluency.

Abbreviations list
MOPS: 3-(N-morpholino)propanesulfonic acid; KOH: Potassium hydroxide; H2O: Water; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KOMO: KOH/MOPS buffer; HCl: hydrogen chloride; PCA: Perchlorid acid; PBS: Phosphate buffer saline; BSA: Bovine serum albumin; RT: Room temperature.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Peroxidase from horseradish | Sigma-Aldrich | Cat# 77332-100MG |
| L-Lactate Oxidase from Aerococcus viridans | Merck | Cat# L9795 |
| Amplex™ Red reagent | Thermo Scientific | Cat# A12222 |
| L-Sodium Lactate | Sigma-Aldrich | Cat# 71718-10G |
| D-Sodium Lactate | Sigma-Aldrich | Cat# 71716-5G |
| Syrosyngopine | Sigma-Aldrich | Cat# SML1908-SMG |
| AR-C155858 | EMD Millipore | 5.33436 |
| KH₂PO₄ | EUROMEDEX | Cat# 2018-A |
| Tris base | EUROMEDEX | Cat# 200923-A |
| KOH | VWR | Cat# 26670.294 |
| MOPS | VWR | Cat# 0670-500G |
| DMSO | Sigma-Aldrich | Cat# D8418-100ML |
| Perchloric acid w/o EDTA (PCA) | Merck | Cat# 1.00519.2501 |

(Continued on next page)
MATERIALS AND EQUIPMENT

© Timing: 1 h 30 min

- Buffer composition.

Phosphate buffer

| Reagent        | Final concentration | Amount  |
|----------------|---------------------|---------|
| KH₂PO₄         | 50 mM               | 340 mg  |
| H₂O            | n/a                 | 50 mL   |
| Total          | n/a                 | 50 mL   |

*Note: Final pH 6.8–7.*

Tris-HCl buffer

| Reagent        | Final concentration | Amount  |
|----------------|---------------------|---------|
| Tris base      | 50 mM               | 302 mg  |
| H₂O            | n/a                 | 50 mL   |
| Total          | n/a                 | 50 mL   |

*Note: Final pH 6.8–7 with HCl.*

*Note: Buffers have to be filtered (with 0.2 µm filter ClearLine) and can be stored at RT or at +4°C for months.*
Note: This solution can be stored at RT for months.

### KOMO solution

| Reagent    | Final concentration | Amount  |
|------------|---------------------|---------|
| KOH        | 2 M                 | 11.2 g  |
| MOPS       | 0.5 mM              | 10.45 g |
| H₂O        | n/a                 | To make 100 mL |
| Total      | n/a                 | 100 mL  |

Note: This solution can be stored at RT for months.

- Dilution and storage of enzymes and reagents:

For HRP Peroxidase: the enzyme is diluted in the phosphate buffer to a final concentration of 5,000 U/mL, then aliquoted in small volumes (from 50 to 100 μL) and stored frozen at −20°C for months.

For L-Lactate Oxidase: the enzyme is diluted in the phosphate buffer to 100 U/mL, then aliquoted in small volumes of 20 μL/tube. Aliquots are desiccated using speed vacuum before being stored frozen at −20°C for months. Dried aliquots should be freshly prepared. Dilution of the enzyme in phosphate buffer to a final concentration of 0.5 U/mL.

For Amplex™ Red reagent: the reagent is diluted to 5 mM in DMSO and stored frozen at −20°C for months.

- Enzymatic master mix (for 20 samples).

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Tris-HCl buffer          | N/A                 | For total 4 mL |
| Peroxidase               | 5 U/mL              | 4 μL    |
| Amplex™ Red reagent      | 2.5 μM              | 2 μL    |
| Lactate Oxidase          | 0.5 U/mL            | 20 μL   |
| Total                    | n/a                 | 4 mL    |

Note: This mix is stable for 2 h on ice.

- L-Lactate standard solution.

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| L-sodium lactate | 2 M                 | 224 mg  |
| H₂O          | n/a                 | To make 1 mL |
| Total        | n/a                 | 1 mL    |

Note: Final pH 7 (The pH of small volume solutions are determined using the METTLER TOLEDO™ Micro pH Electrodes).
Note: This solution can be stored at RT for months.

- Syrosyngopine solution.

| Reagent     | Final concentration | Amount   |
|-------------|---------------------|----------|
| Syrosyngopine | 10 mM               | 6.66 mg  |
| DMSO        | n/a                 | To make 1 mL |
| Total       | n/a                 | 1 mL     |

Note: Syrosyngopine is stored to −20°C and aliquoted into 100 μL tubes, for months.

- AR-C155858 solution.

| Reagent     | Final concentration | Amount   |
|-------------|---------------------|----------|
| AR-C155858  | 100 μM              | 0.46 mg  |
| DMSO        | n/a                 | To make 10 mL |
| Total       | n/a                 | 10 mL    |

Note: AR-C155858 is stored to −20°C and aliquoted into 100 μL tubes, for months.

**STEP-BY-STEP METHOD DETAILS**

**Sample collection**

© Timing: 30 min

Lactate extraction from intracellular and extracellular fractions is a key step. To this end, proper quenching of interfering enzymatic reactions and removal of endogenous enzymes is achieved using acidic extraction.

Two methods are proposed according to cell types:

Non-adherent cells:

1. Collect 2 mL cells in their culture medium in a 2 mL Eppendorf tube kept on ice for 5 min and centrifuged at 1,000 g and 4°C, for 5 min.
2. Collect the supernatant containing cell culture medium (extracellular lactate fraction) in a new tube.
3. Wash the cell pellet with PBS and centrifuge as performed in step 1.
4. Collect the cell pellet (intracellular lactate fraction) quenched in 100 μL of 14% PCA w/o EDTA and incubate 5 min on ice.
5. Centrifuge at 20,000 g and 4°C for 1 min and collect the supernatant containing intracellular lactate.
6. Neutralize the supernatant to pH 7.4 with KOMO solution.
7. For extracellular lactate quantification, check the pH of cell culture medium collected in step 2 and if required, adjust with HEPES solution. The final volume is 450 μL.

Adherent cells:

8. Collect the medium (extracellular lactate fraction) and wash cells with PBS.
9. Scrape cells in PBS, and collect them in a 15 mL Falcon tube kept on ice.
10. Centrifuge at 1,000 g and 4°C, during 5 min.
11. Remove the supernatant and collect the pellet (intracellular lactate fraction) quenched in 100 µL of 14% PCA w/o EDTA.
12. Centrifuge at 20,000 g for 1 min and collect the supernatant containing intracellular lactate.
13. Neutralize the supernatant to pH 7.4 with KOMO solution.
14. For extracellular lactate quantification, check the pH of cell culture medium and if required, adjust with HEPES solution. The final volume is 450 µL.

**Note:** After acidic precipitation and neutralization, samples can be stored at –20°C, and lactate levels are stable over several months.

**Note:** Initial volume of medium in the culture dish or well should be reported and used to calculate the final concentration of extracellular fraction.

**Note:** Resuspension volume as well as PCA and KOMO volume used during acidic extraction of the pellet should be reported and used to calculate the final concentration of lactate in the intracellular fraction.

**Determination of the total protein content in extracted samples using the BCA kit protocol**

© Timing: 40 min

The intracellular L-lactate level is normalized to the amount of protein recovered in the pellet after protein precipitation with PCA. In this protocol, the Pierce™ BCA Protein Assay kit is used in 96-well plate.

15. Prepare mix following BCA assay protocol.
16. In a 96-well plate, add the mix in 5 wells with a defined quantity of BSA: 0, 5, 10, 15, 20 µg (200 µL final/well).
17. Add 190 µL of mix in wells and add 10 µL of sample (intracellular fraction only) per well.
18. Incubate the plate at 37°C during 30 min.
19. Measure absorbance at 562 nm (Figure 2).

**Figure 2. Protein quantification**

Protein measurement is performed according to the Pierce™ BCA Protein Assay kit recommendations. First and second columns (4A to 4E and 5A to 5E) contain the BSA standard titration and third column (6A to 6D) contains intracellular (2A-B) and extracellular (2C-D) samples in duplicate.
Measurement

© Timing: 40 min

To quantify the absorbance signal and validate that the absorbance values are comprised within a range where absorbance is proportional to the L-lactate level, a standard curve is systematically performed. Two independent measures were performed for each sample. Moreover, to further validate the absence of interfering reaction in the extract, an internal control (50 pmol of L-lactate standard) was added at the end of each reaction. Absorbance measurement is performed at 572 nm, using 22 flashes (0.5 s) per well.

20. Prepare enzyme master mix and keep it on ice.

Standard curve

21. In a 96-well plate, add the master mix in 5 wells with a defined quantity of L-lactate: 0, 50, 100, 200, 2,000 pmol (200 μL final/well).
22. Step 21 is repeated to replicate measurement of standard curve with 0, 100, 200, 400, 2,000 pmol.
23. Homogenize quickly by flushing 2–3 times with a P200 pipette and incubate 5 min at RT before the measurement (Figure 3 and Table 1).

L-lactate quantification (simultaneously)

24. Add 190 μL of master mix and 10 μL of sample per well. Inhibitor cocktail of lactate transporters are used to challenge this quantification during cell culture step 29 (Table 2).
25. Step 24 is repeated to replicate measurement of each sample.
26. Homogenize quickly by flushing 2–3 times with a P200 pipette and incubate 5 min at RT before the measurement (Figure 3).

Internal control quantification

27. Add 50 pmol of L-lactate standard in well with samples.

Note: As presented above, D-lactate was used to verify the specificity of the L-Lactate Oxidase from Aerococcus viridans for L-lactate. Our results demonstrated that this enzyme is highly specific for L-lactate as Amplex red is not converted in resorufin in the presence of D-lactate (Figure 3). The standard curve as well as the samples reads should be performed at least in duplicates.

△ CRITICAL: In order to keep the values of sample measurement from extracellular fraction in a standard range, a 1/100 dilution is carried out.

Note: This protocol is described for a measurement in a 96-well plate but can be adapted to other spectrophotometric fluorometric measurement methods.

Control section

© Timing: 3 days

Syrosyngopine (10 μM) and AR-C155858 (100 nM) are well validated inhibitors of lactate transporters (MCT1, MCT2 and MCT4 (Benjamin et al., 2018; Nancolas et al., 2015; Ovens et al., 2010).
**Step-by-step procedure to use MCT inhibitor in cell culture**

28. **Seeding.**

Seed 8 × 10^3–10^7 cells per well in non-adherent conditions and between 0.5 × 10^6 to 10^6 cells per well in adherent conditions in a 6-well plate with 2 mL of cell culture medium.

29. **Treatment.**

Directly in the well, add inhibitor under the indicated conditions (10 μM final for syrosynogopine and 100 nM final for AR-C155858).

30. **Incubation.**
Treated cells are incubated at 37°C, under 5% CO₂ for 3 days before collecting cell to perform experiments.

Note: Our results showed that, as expected, MCT inhibitors increase intracellular L-lactate and decrease extracellular lactate levels, validating that these inhibitors counteract the L-lactate secretion in the extracellular compartment (Table 2).

EXPECTED OUTCOMES
The method presented herein allows L-lactate quantification with high sensitivity. Results presented in the Figure 4 demonstrate that as expected, cells treated with MCT inhibitor exhibit a burst of intracellular L-lactate level (Left graph) while reducing the extracellular lactate (right graph). This method allows quantification of intracellular lactate levels but can be adapted to quantify L-lactate levels in any biological samples.

QUANTIFICATION AND STATISTICAL ANALYSIS
We strongly recommend to systematically perform a standard curve with increasing concentration of L-lactate to determine (i) the linearity range between absorbance and lactate concentration and (ii) to determine the molar attenuation coefficient to determine lactate concentration from absorbance values (Table 3 and Figure 5). To this end, the results presented below show that this method can specifically measure L-lactate with a range between 50 pmol/well to 2,000 pmol/well (i.e.,

![Figure 4. Linear standard curve obtained with L-lactate titration](Image)

Standard curve correlating optic density measured at 572 nm (AU) to L-lactate amount (pmol), y = 0.0001x + 0.0085 and R² = 0.9948.
0.25 μM–10 μM). Using the L-Lactate Oxidase from Aerococcus viridans allows a more sensitive quantification than most of lactate quantification kits commercialized by Abcam (L-Lactate assay kit ab65331, range 0.02 mM–10 mM; L-Lactate assay ab65330, range 0.001 mM–10 mM) or Sigma-Aldrich (Lactate Assay Kit II, range 0.02–10 mM).

The different calculation steps determining the amount of lactate normalized to the protein content in extracted samples from the absorbance values are further detailed in Table 4.

**Note:** “Raw data-blank” is calculated with blank of the standard curve; “pmol” is calculated with equation of standard curve; “Initial sample volume” is resuspension volume of pellet for intracellular fraction and initial volume of dish or well for extracellular L-lactate with dilution factor.

**LIMITATIONS**

The main limitations of this protocol are related to the production and storage of the master mix. Therefore, it is essential to keep the L-Lactate Oxidase dried and frozen and to prepare the master mix just before use.

**TROUBLESHOOTING**

**Problem 1**

Resuspended L-Lactate Oxidase can be contaminated by a spontaneous production of H₂O₂. This production drastically increases the noise.

**Potential solution**

To preserve the L-LOX under optimal condition, the L-LOX should be aliquoted and dried before storage (see Dilution and storage of enzymes and reagents).

| L-lactate (pmol) | Absorbance | Absorbance - blank |
|------------------|------------|-------------------|
| 0                | 0.0425     | 0                 |
| 50               | 0.052      | 0.0095            |
| 100              | 0.064      | 0.0215            |
| 200              | 0.0795     | 0.037             |
| 400              | 0.107      | 0.0645            |
| 2,000            | 0.283      | 0.2405            |
Problem 2
It is important to validate that the resorufin absorbance originates from L-lactate and that H₂O₂ potentially contaminating the extracts or in the buffers used for the enzymatic quantification is not interfering with the L-lactate quantification.

Potential solution
To make sure that the resorufin fluorescent signal originates from the L-LOX activity and not from contaminating H₂O₂ present in the extracted samples, we suggest performing the measurement in absence of L-LOX to quantify the background associated with H₂O₂ contaminations (step 20, preparation of master mix without L-LOX).

Problem 3
The master mix has a low stability over time, that can trigger a wrong absorbance measurement.

Potential solution
The master mix has to be prepared extemporaneously and kept on ice to allow a longer stability (step 20. If stored between different series of measurements, the stability of the blank value can reflect the quality of the master mix.

Problem 4
The use of inhibitors on cells can affect enzymatic activities of the L-Lactate Oxidase (step 29).

Potential solution
The first time that the new treatment is used, it is important to verify the absence of interreference between the added inhibitor and the enzymatic reaction catalyzed by L-Lactate Oxidase and HRP peroxidase. To this end, the L-lactate standard curve is performed in presence of this new inhibitor.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Arnaud Mourier (arnaud.mourier@ibgc.cnrs.fr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This protocol includes all datasets generated or analyzed during this study.

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Table 4. Example of result quantification

| Samples _ intracellular fractions | Duplicate | Raw data | Raw data - blank | /Volume in well | pmol | *Initial sample volume | nmol/µg prot. |
|----------------------------------|-----------|----------|------------------|----------------|------|----------------------|---------------|
| Control                          | #1        | 0.109    | 0.0665           | 0.00665        | 66.5 | 29925                | 0.204         |
|                                  | #2        | 0.112    | 0.0695           | 0.00695        | 69.5 | 31275                | 0.214         |
| Inhibitors                       | #1        | 0.220    | 0.1775           | 0.01775        | 177.5| 79875                | 0.654         |
|                                  | #2        | 0.226    | 0.1835           | 0.01835        | 183.5| 82575                | 0.676         |
AUTHOR CONTRIBUTIONS
A.M. developed the technique. C.L.B. designed and performed all experiments. C.L.B., A.M., and T.D. discussed the results and wrote the manuscript. T.D. supervised the work of C.L.B. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS
There is no conflict of interests.

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