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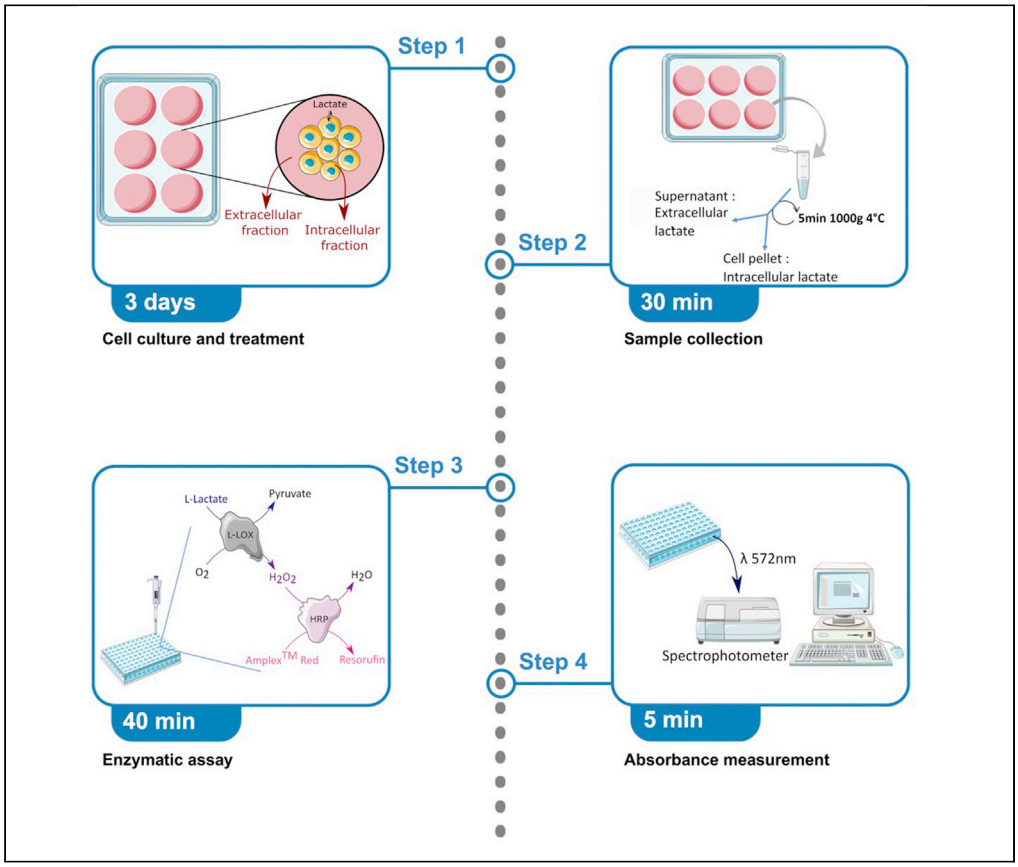
Protocol

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



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.

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Highlights

NADH-independent and highly sensitive enzymatic quantification of L-lactate

Specific quantification of intra- and extracellular L-lactate levels in cultured cells

Validation of specificity and sensitivity using lactate transport (MCT) inhibitors

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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 Martín et al., 2013; Vangrieken et al., 2021; Villa-Bellosta, 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 H_2O_2 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).

8×10^6–10^7 cells per well in non-adherent conditions and between 0.5×10^6 to 10^6 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.

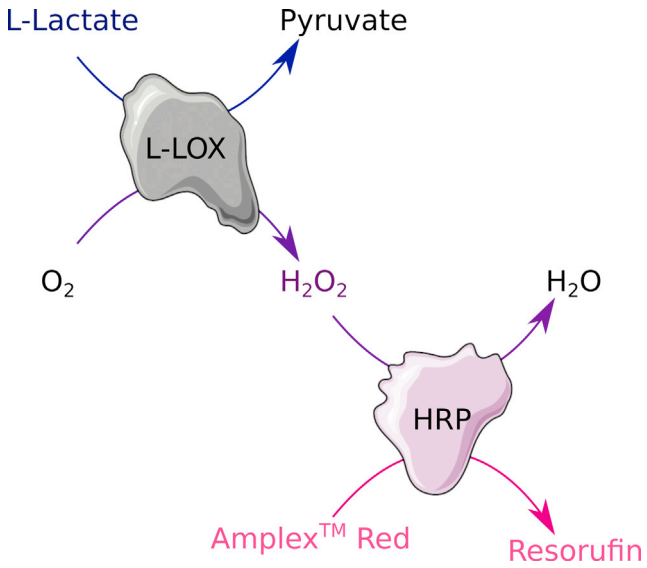

Figure 1. Representation of enzymatic reaction

L-lactate in samples is oxidized in pyruvate by L-Lactate Oxidase, this reaction forms H_2O_2 which reacts with peroxidase in presence of Amplex™ Red reagent to product resorufin. This component can be detected at 572 nm. L-LOX: L-Lactate Oxidase; HRP: Horse radish peroxidase.

⚠ 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# SML1908-5MG
AR-C155858	EMD Millipore	5.33436
KH_2PO_4	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*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEPES	Sigma-Aldrich	Cat# H0887-100ML
HCl	VWR	Cat# 20248.295
Ultrapure™ Water	Invitrogen	10977-035
DMEM	Dutscher	Cat# L0104-500
Neurobasal™	Gibco	Cat# 2110349
Pierce™ BCA Protein Assay kit	Thermo Scientific™	Cat# 23225
Experimental models: Cell lines		
P3 Glioblastome stem-like cells	BioDynaMit Lab (recommended range of passage numbers: P5 - 25)	Guyon et al. (2020)
HEK	BioDynaMit Lab (recommended range of passage numbers: P5 - 25)	(Bahri et al., 2021)
MEF	BioDynaMit (recommended range of passage numbers: P5 - 25)	Mourier et al. (2015)
Software and algorithms		
Microsoft Excel	microsoft.com	N/A
GraphPad Prism	https://www.graphpad.com	RRID: SCR_002798
Others		
Concentrator plus/Vacufuge® plus	Eppendorf	5305000304
CLARIOstar® Plus	BMG LABTECH	N/A
0.2 µm steril filter ClearLine	Dutscher	147842
Microplate 96-well F-bottom, Clear	Greiner Bio-One	655101
6-Well Ultra-Low Adherent Plate	STEMCELL Technologies, Inc	Cat# 38071
Centrifuge	Eppendorf	Micro Star 17/17R
pH meter (FiveEasy™ Plus)	METTLER TOLEDO® equipped with micro electrode	FP20

MATERIALS AND EQUIPMENT

Timing: 1 h 30 min

- Buffer composition.

Phosphate buffer		
Reagent	Final concentration	Amount
KH_2PO_4	50 mM	340 mg
H_2O	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
H_2O	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.

KOMO solution		
Reagent	Final concentration	Amount
KOH	2 M	11.2 g
MOPS	0.5 mM	10.45 g
H_2O	n/a	To make 100 mL
Total	**n/a**	**100 mL**

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

HEPES solution		
Reagent	Final concentration	Amount
HEPES	10 M	23.83 g
H_2O	n/a	To make 10 mL
Total	**n/a**	**10 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
H_2O	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).

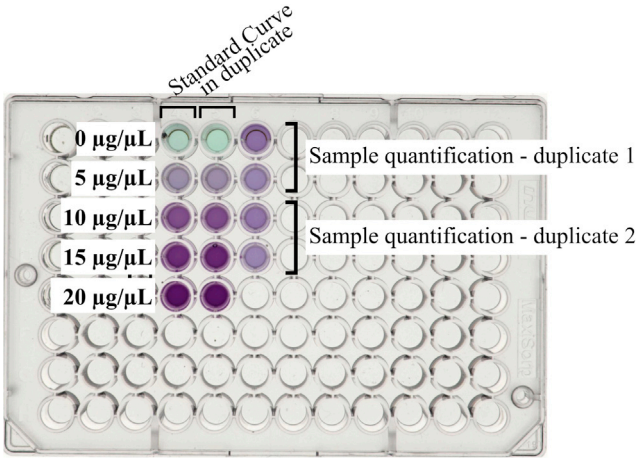

Figure 2. Protein quantification
Protein measurement is performed according to the Pierce™ BCA Protein Assay kit recommendations. First and second columns (4A to 4E and 5A to 5E) contain the BSA standard titration and third column (6A to 6D) contains intracellular (2A-B) and extracellular (2C-D) samples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

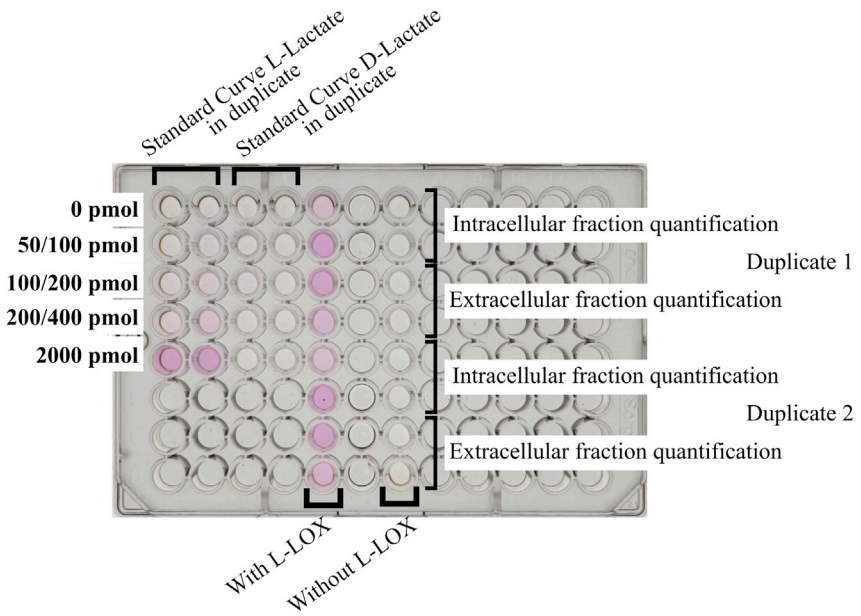

Figure 3. Lactate measurement with one or four additions of samples

The first and second columns (1A to 1E and 2A to 2E) contain standard curve with L-lactate. The third and fourth columns (3A to 3E and 4A to 4E) is standard curve with D-lactate. In the first and the third columns, lactate amount in wells are 0, 50, 100, 200, and 2000 pmol. In the second and the fourth columns, lactate amount in wells are 0, 100, 200, 400 and 2,000 pmol. The fifth column (5A to 5F) is sample quantification in this order: measurement of intracellular for control (5A and 5E) and treated with MCTs' inhibitors (5B and 5F) and extracellular fraction for control (5C and 5G) and treated with MCTs' inhibitors (5D and 5F), in duplicate. The seventh column (7A to 7F) is identical to column 5 but does not contain L-LOX in the master mix. Samples volume is 10 µL per well.

Step-by-step procedure to use MCT inhibitor in cell culture

28. Seeding.

Seed 8 × 10^6–10^7 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 syrosyngopine and 100 nM final for AR-C155858).

30. Incubation.

Table 1. Example of Lactate standard curve measured on a plate reader (CLARIOstar® Plus)

Standard curve: Lactate initial quantity in pmol	Raw data			
	L-lactate		D-lactate	
Duplicate	#1	#2	#1	#2
0	0.043	0.042	0.043	0.043
50	0.052	–	0.044	–
100	0.065	0.063	0.051	0.047
200	0.080	0.079	0.050	0.049
400	–	0.107	–	0.049
2,000	0.283	0.283	0.050	0.050

Table 2. Example of raw data measured on a plate reader

		Raw data			
		w/ L-LOX		w/o L-LOX	
Duplicate	Condition	#1	#2	#1	#2
Intracellular fraction	Control	0.109	0.112	0.040	0.042
	Syrosyngopine + AR-C155858	0.220	0.226	0.042	0.045
Extracellular fraction	Control	0.213	0.216	0.040	0.042
	Syrosyngopine + AR-C155858	0.139	0.143	0.038	0.039

Treated cells are incubated at 37°C, under 5% CO_2 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.,

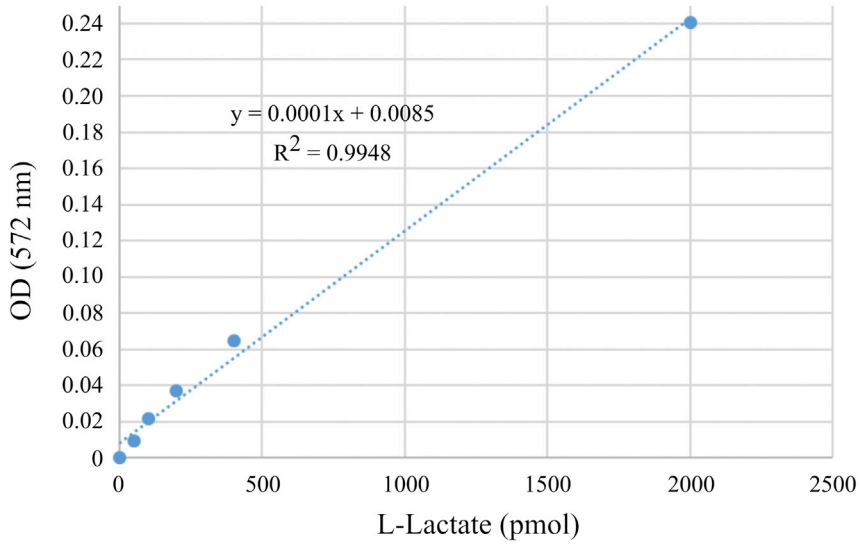

Figure 4. Linear standard curve obtained with L-lactate titration

Standard curve correlating optic density measured at 572 nm (AU) to L-lactate amount (pmol), y = 0.0001x + 0.0085 and R^2 = 0.9948.

Table 3. Example of standard curve calculation

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

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 H_2O_2. 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).

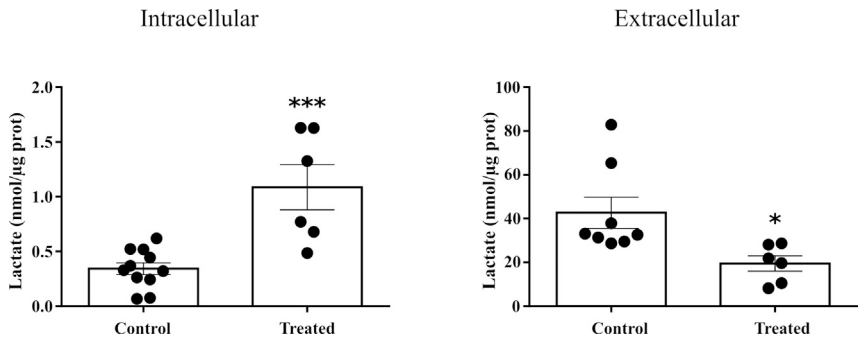

Figure 5. Intra- and extracellular L-lactate quantification in different conditions

L-lactate quantification in intracellular fraction (*left, n=11 for control sample and n=6 for treated sample*) or extracellular fraction (*right, n=8 for control sample and n=6 for treated sample*) measured after or not a 3-days treatment of inhibitors lactate transporter cocktail (Syrosyngopine and AR-C155858). The bar graph shows the mean ± SEM. *, $p < 0.05$; ***, $p < 0.001$.

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

Problem 2

It is important to validate that the resorufin absorbance originates from L-lactate and that H_2O_2 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 H_2O_2 present in the extracted samples, we suggest performing the measurement in absence of L-LOX to quantify the background associated with H_2O_2 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.

ACKNOWLEDGMENTS

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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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