ATP and Lactate Quantification

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[Abstract] Cells use glucose to generate energy by two different metabolic processes: lactic fermentation and aerobic respiration. In the first common series of reactions, glucose is converted into pyruvate. In anaerobic conditions, pyruvate is transformed into lactate, this process yields to 2 ATP molecules per glucose molecule. In the presence of oxygen, pyruvate is imported into mitochondria where it is used in the Krebs (or TCA) cycle and oxydative phosphorylation. The global process of oxydative phosphorylation yields to 32 ATP per glucose molecule. For reasons not fully understood, in some pathological cases like cancer, cells use anaerobic glycolysis even in the presence of oxygen, in which case the process is called aerobic glycolysis (or Warburg effect). This results in an increased uptake of glucose and lactate production. Measure of intracellular ATP content and lactate concentrations can provide a readout of aerobic glycolyis.

Materials and Reagents

A. For intracellular ATP content evaluation
   1. Murine Embrionic Fibroblasts (MEF)
   2. Dulbecco's Modified Eagle's Medium High glucose with L-glutamine (DMEM) (Gibco®, catalog number: 11965)
   3. Foetal Calf Serum (FCS) (Gibco®, catalog number: 16010)
   4. Penicillin/Streptamycin (Gibco®, catalog number: 15070)
   5. Phosphate Buffered Saline (PBS) (EuroClone, catalog number: E0B4004L)
   6. Protein Dye Reagent Concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
   7. Bovine Serum Albumine (BSA) (Sigma-Aldrich, catalog number: A4503)
   8. NaCl
   9. Na_4HPO_4/NaH_2PO_4
   10. Glycerol
   11. Triton X-100
   12. Complete protease inhibitors (Roche Diagnostics, catlalog number: 11697498001)
   13. Phosphatase inhibitors (1 mM final concentration of glycerophosphate, sodium orthovanadate and sodium fluoride)
   14. Complete protease inhibitors (Roche Diagnostics)

15. ATP détermination kit (Life Technologies, catalog number: A22066)
16. Lysis buffer (see Recipes)
17. Standard Reaction Solution (see Recipes)

B. For Lactate quantification
1. MEF
2. EnzyChrom™ L-lactate Assay Kit (BioAssay Systems, catalog number: ECLC-100)
3. Dulbecco's Modified Eagle's Medium High glucose with stable L-glutamine (DMEM) (Gibco®, catalog number: 11965)
4. Foetal Calf Serum (FCS) (Gibco®, catalog number: 16010)
5. Penicillin/Streptamycin (Gibco®, catalog number: 15070)
6. 0.4% Trypan Blue stain (Life Technologies, catalog number: T10282)

Equipment

A. For intracellular ATP content evaluation
1. 6 wells plates (Corning, Costar®, catalog number: CLS 3506)
2. Cell Scraper (Nunc®, catalog number: 179707)
3. Luminometer Gloomax 20/20
4. Spectrophotometer
5. 37 °C 5% CO_2 Cell culture incubator
6. Centrifuge spinning speed : 15682,186 G-force (13000 rpm, 8,3 cm radius)

B. For Lactate quantification
1. 37 °C 5% CO_2 Cell culture incubator
2. 6 wells plates (Corning, Costar®, catalog number: CLS 3506)
3. 96 wells plates (Corning, Costar®, catalog number: CLS 3596)
4. Countess automated cell counter with countess Cell Counting Chamber Slides (Life Technologies)
5. Plate reader at 565 nm

Procedure

A. For intracellular ATP content evaluation
1. MEF are plated at 60-70% cell confluency.
2. Cells are washed twice with PBS

3. Cells are lysed with a cell scraper in 35 µl of lysis buffer containing phosphatase inhibitors (1 mM final concentration of glycerophosphate, sodium orthovanadate and sodium fluoride) for 30 min at 4 °C.
4. Centrifuge at 13,000 rpm for 15 min at 4 °C.
5. Supernatant is isolated.
6. The sample is diluted at 1:1,000 (1 µl of sample is added to 1,000 µl of Biorad solution) in a Biorad solution (200 µl of Biorad and 800 µl of water).
7. Optical Density (OD) is read at the spectrophotometer at 595 nm.
8. The concentration of the sample is determined using a standard curve from BSA.
9. Samples are diluted at a final concentration of 25 ng/ml.
10. 250 ng of total proteins in parallel of the ATP standard curve (0 nM, 1 nM, 10 nM, 100 nM, 1,000 nM of ATP) in 10 µl is added to 90 µl the Standard Reaction Solution.
11. Luciferase activity is measured with a luminometer.
12. ATP is quantified for each sample using the ATP standard curve.

B. Lactate quantification

1. Cells are plated in 6 wells plates with 3 ml of medium (10% DMEM-FCS-1% Penicillin/Streptamycin).
2. The medium is changed when the cells reach 100% cell confluence.
3. The medium is collected after 24 h.
4. Cells are trypsinized and alive cells are counted with Trypan blue 1:1.
5. The medium collected is centrifuged at 13,000 rpm for 10 min.
6. The supernatant (clean medium) is diluted 1:10 in DMEM.
7. 20 µl of each sample and of the standard curve (0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mM of Lactate in DMEM) is put in wells of a 96 wells plate.
8. 80 µl of assay buffer from EnzyChrom L-Lactate Assay Kit is added in each well.
9. Mix well (Pipetting up and down).
10. D0 is absorbed 565 nm at the beginning of the incubation (t0) and after 20 min (t20).
11. The difference of absorbtion (delta OD) is determined for the samples and the standard curve.
12. The concentration of lactate for each sample is obtained from the standard curve.
13. The quantity of lactate is normalized by the final number of cells.

Recipes

1. Lysis buffer
   150 mM NaCl

20 mM Na_4HPO_4/NaH_2PO_4
10% glycerol
1% Triton X-100 (pH 7.2)
Complete protease inhibitors

2. Standard Reaction Solution
   1x Reaction Buffer
   0.1 mM DTT (dithiothreitol)
   0.5 ml 0.5 mM of D-luciferine
   2.5 µg/ml firefly luciferase and incubated at room temperature for 15 min (in the dark).

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References

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