

Measurement of Intracellular ATP

This protocol was adapted from the manufactures protocol.

We've performed this assay on the following cell types:

- Dissociated Primary Cortical Neurons Plated at $0.2 - 1.0 \times 10^6$ cells/ml (100 μ l/well of 96 well plate; 1 ml/well of a 24 well plate; 4 ml/well of 6 well plate).
- Dissociated Primary Hypothalamic Neurons Plated at $0.4 - 0.6 \times 10^6$ cells/ml
- Differentiated 3T3-L1A Adipocytes

Drug Treatment:

- Perform drug/compound treatments in:
 - 100 μ l of conditioned culture media/well (entire plating volume) for 96 well plates
 - 500 μ l of conditioned culture media/well (half of the plating volume) for 24 well plates
 - 2 ml of conditioned culture media/well (half of the plating volume) to conserve drug
 - If it is necessary to remove and add back media to adjust the volume:
 - Remove media from 2-3 wells at a time to prevent the cells from drying out.
 - Allow cells to re-equilibrate in the new media at 37°C for 1 hr – over night before treatment.
- Treat cells with desired amount(s) of drug(s) for the desired amount of time at 37°C.
 - Include a no-treatment control and a vehicle control for each drug.

Preparation for Harvesting:

- 0.5 hr before the end of the treatment turn heat block on to 100°C and remove the ATP standard and Luciferase Reagent from –20°C freezer and thaw on ice or make fresh.
- Solubilize the Luciferase: add 10 ml of ddH₂O to one bottle of luciferase and allow to sit on ice for 5 min. Rotate to mix, do not shake. Light will reduce this reagents activity.
- Make ATP dilutions for the standard curve and keep on ice until use.
 - Make a 10 mg/ml (16.5 mM) stock of ATP in TE (100 mM Tris; 4 mM EDTA; pH = 7.75).
 - The optimum detection range of this kit is between 10^{-7} and 10^{-10} M ATP. Therefore, to make the standards, serially dilute the 16.5 mM stock, to 10^{-3} - 10^{-13} M ATP.

Harvesting:

- Following drug treatment, place plate on ice and remove media via vacuum. Be careful not to remove the cells.
- To lyse the cells, add TE (100 mM Tris + 4 mM EDTA, pH = 7.5) to each well
 - Amount depends on well size, cell density and cell type:
 - 96 well plate of CN = 200 μ l/well
 - 96 well plate of 3T3L1As = 300 μ l/well
 - 96 well plate of Hypothalamic Neurons = 200 μ l/well
 - 24 well plate of CN = 500 μ l of TE/well
 - 24 well plate of 3T3L1As = 1000 μ l of TE/well
 - ***Do only 3-4 wells at a time as stressing the cells by exposing them to air can change ATP levels, and move as quickly as possible***. Get all the wells into TE before taking

the

time to triturate and remove lysates from the wells

- Triturate the TE with the plate still on ice using a yellow (for 96 well plate) or blue tip (for 24 well tip) and get as many of the cells as possible into the TE solution, and transfer lysates to boil-proof ultracentrifuge tubes on ice.
- Heat tubes to at least 95°C for 7 minutes.
- Spin at 14000 RPM for 2-3 minutes to pellet cell debris.
- Remove 50 µl of each sample and each standard and transfer to a black 96 well plate.
- Quickly add 50 µl of luciferase reagent to each well use a multi-channel pipettor.
 - Triturate each set of wells 2 times quickly as you add luciferase.
 - Be careful not to generate bubbles - they interfere with the reading!
 - Extra luciferase can be kept at -20°C and reused within a week.
- Place black 96 well plate into Wallac microplate reader and read using the luminescence program
reading @ 0.1 sec/well.

Materials:

- ATP kit, Roche #1699695, \$446/kit.
- Boil Proof Tubes, Bio-express #C-3219-1, bag of 500.
- Microfluor Black Microtiter Plates, VWR # 62402-983, 50 plates.