

Protocol for fatty acid oxidation in fibroblasts

This protocol is designed for cells seeded in 24-well plates

1) Preparation of ion exchange resin columns. (Example for 30 columns)

You can start preparation of the columns day(s) in advance of the assay.

- Obtain 9" Pasteur pipet and seal the tips using Bunsen burner flame (sealing tip takes a few seconds only).
- Can store pipettes in a ziploc bag or wrap in saran wrap. Can also be stored in a beaker covered in saran wrap.
- Stuff/plug bottom of a Pasteur pipette using fiberglass. Plug only has to be approx. 1cm worth of fiberglass. It is best to stuff fiberglass into pipette using another pipette to push down. Try and stuff fiberglass as close to bottom of pipette (of the wider upper portion of the pipette).
- This can also be kept sealed in ziploc/saran wrap.

One day before the assay, the resin for columns is prepared.

- Prepare one column per well of cells that you plated.
- Place columns in make-shift Styrofoam "rack" so that columns are held in place in an upright position. Use Styrofoam from a 50ml conical tube rack.
- Weigh 19.9 gr. of resin (Sigma: DOWEX 1x2-400 catalog #217395)
- Add 54 ml of sterile Milli-Q water
- Stir with a magnetic bar 10 min
- Add 2.4 ml of resin per column, do not stop the stirring during this step.
- Make sure there are no bubbles after resin is poured into column. You can use another pipette to poke down into the resin to make sure of this.
- Let resin in column settle overnight.

2) Preparation of cell culture 24-well plates:

- Cultured Tritiated water release experiments are performed in triplicate (3 wells per sample/condition).
- The first three wells are blanks (i.e. wells without cells).
- Include duplicate wells/plates for all experimental conditions. These plates will not be treated with Palmitate/BSA and will not be assayed for FAO. These plates will only be used for BCA protein assay. But treat the cells the same as your actual "experimental" wells.

3) Preparation of the diluted Palmitate/BSA (stock solution 500mM- see recipe)

- Prepare on day of assay
- Thaw an aliquot of (previously prepared) Palm/BSA and make a 125 μ M solution by diluting the stock (dil $\frac{1}{4}$) in PBS Plus Ca-Mg
- The final volume should be sufficient for the number of wells assuming that the volume needed per wells is 200 μ l. The mix also contains 1mM carnitine (Sigma C0158)

Example: for an experiment of 12 wells; 12 x 200 μ l = 2.4ml

Mix: 0.6ml of stock Palm/BSA (500 μ M) + 24 μ l Carnitine (stock 100mM) + 1.8 ml of PBS.

For the calculation of the specific radioactivity (“**RDA**”) of the mix:

Prepare three (20ml) scintillation vials containing:

5 ml of Ultima Gold LLC (scint cocktail)

1.5 ml of sterile Milli-Q water

20 μ l of the Palm/BSA/carnitine mix

(If still cloudy, can add more scint cocktail)

4) Fatty acid oxidation (ONLY expt wells/plates, not wells/plates for protein)

- Cultured cell layers are washed 3 times with PBS minus Ca/Mg
 - Introduce 200 μ l of the Palm/BSA mix per well
 - Wrap the culture plate in foil to avoid evaporation
 - Incubation is carried out for 2h at 37 $^{\circ}$ C.
- During the 2 hours of incubation prepare 2 series of tubes
 - 1 tube/well containing 200 μ l of cold 10% TCA (Trichloroacetic acid, SIGMA, cat#76-03-9). Close the tubes and put them at +4 $^{\circ}$ C.
 - 1 tube/well containing 55 μ l of 6M NaOH
- 5 min before the end of the incubation snap off tip/end of the sealed Pasteur pipette columns and let the water flow out (can put chux underneath columns to soak up water).
- At the end of 2h incubation, remove the 200 μ l of mix and put in the tube containing 200 μ l 10% TCA. Close the tubes.
- Centrifuge at 3300 rpm (2,200g) 10 min at 4 $^{\circ}$ C in microfuge
- Remove 350 μ l of the supernatant and add to the 55 μ l of 6M NaOH to neutralize the TCA.
- Take the total volume (450 μ l) and apply to the ion-exchange resin.
- Let the liquid completely enter the column
- Then take each column and put it in a scintillation vial (1 vial/column)
- Elute each column with 1.7 ml of distilled water
- When the column is completely eluted remove the column and add 5ml of scintillation liquid
- Shake vigorously, and count. *Don't forget to count the 3 RDA vials.*

5) Protein measurement (Duplicate wells without radioactivity)

- Wash the wells 2 times with PBS-minus Ca/Mg
- Add 220 μ l of NaOH 1M
- Wrap the plate in foil and let it incubate at room temperature until the next day.

The total amount of protein per well should be determined using the Pierce BCA protocol. (see separate protocol)

6) Calculation

Palmitate oxidation rates in cells are expressed as nmol of ^3H fatty acid (^3H -FA) oxidized per hour per milligram of cell protein (nmol ^3H -FA h^{-1} mg^{-1} protein)

- Use the cpm counts
- Calculate the mean for the 3 RDA vials = mRDA
- Calculate the mean for the 3 blanks = mBl
- For each well calculate the $\text{cpm}-\text{mBl}=\Delta$

Results = $1429 \times \Delta/\text{mRDA} \times 1/\mu\text{g prot}$

7) Recipe for stock solution of Palmitate/BSA

- BSA: Sigma A7511 fatty acid free
 - H^3 -Palmitate: Perkin Elmer NET-043; 1mCi
 - Palmitate: Fluka catalog #76119
- a) Prepare a BSA solution at 2.5 mg/ml in PBS (Make 13ml)
- b) Prepare a solution of palmitate (reference 76119) at 50 mM
i.e.: weigh 12.8 mg, dissolve in 1 ml of 95% ethanol (heat at 60° to dissolve)
- c) Add 100 μ l of ^3H -Palmitate + 130 μ l of 50mM palmitate to a 50ml round-bottom plastic tube (kept in radioactive area, next to pipet rack)
- Evaporate under nitrogen gas (Ory-Schaffer lab) until no more ethanol is seen
 - During this period, it may be necessary to re-heat the tube (in order to prevent the solution from precipitating. It may also be necessary to sonicate the tube (1 to 3 min) to get rid of the precipitate and dissolve the palmitate back into solution. The Ory-Schaffer lab has a small sonicator/water bath that you can use. Also, the nitrogen gas apparatus that they have contains a water bath at the bottom, which can be set to 60° if the tube needs to be re-heated.
 - When the Palmitate appears dry (will look like white crusty film/layer) add 13 ml of BSA at 2.5mg/ml
 - Cover this tube with parafilm
 - Vortex vigorously and put in a 37°C bath to dissolve palmitate into BSA solution. Alternatively, can place in 37°C shaker O/N.
 - Make aliquots, **freeze** in -20°C

8) Product information

- PBS minus CaCl₂/MgCl₂: Sigma D1408 (Dilute to 1x before use)
- PBS plus CaCl₂/MgCl₂: Sigma catalog D1283 make 1 liter and adjust the Ph to 7.4 with NaOH.
- Ion exchange resin: Sigma: DOWEX 1x2-400 catalog # 217395
- BSA: Sigma A7511 fatty acid free
- H³-Palmitate: Perkin Elmer NET-043 1mCi/ml
- Palmitate: Fluka catalog #76119
- L-Carnitine: Sigma C0158
- Fiberglass (Glass Wool): VWR 32848-003

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Characterization of fatty acid oxidation in human muscle mitochondria and myoblasts.