

Measurement of Beta-oxidation in primary mouse hepatocytes using 3H-labeled palmitate

-Includes protocol for coupling of sodium-FFA with BSA

Materials

- A. Columns:
1. Dowex 1x2-400 resin; Sigma #217935
 2. 1mL unfiltered pipette tips; extended length preferred
 3. Polyester batting/polyfill/fiberglass fiber
 4. Razer blade/X-acto knife
 5. Thin dowel/rod/9" glass pipette, diameter less than that of pipette tips
 6. Rack for scintillation vials w/corresponding support for columns
- B. FFA coupling:
7. Two waterbaths, @50°C and 70-80°C
 8. Sterile syringe and 0.22um syringe filters (optional)
- C. 3H counting:
9. Scintillation vials & caps; PerkinElmer #6000292 recommended
 10. Scintillation fluid; EcoLume 0188247001 recommended

Reagents

1. Fatty acid-free BSA; 2mM solution in compatible media, sterile filtered
2. [9, 10-³H]-palmitic acid; Perkin-Elmer #NET043001MC, 5mCi/mL
3. Na-Palmitate, FW 278.41; Sigma P9767
4. Trichloroacetic acid, 100%
5. NaOH; 6N and 0.01N
6. L-Carnitine Inner Salt (Sigma 0158) FW 161.2; make 100mM stock in H₂O (Optional)
7. (±)Etomoxir Sodium Hydrate (Sigma E1905) FW 338.75 ; make 10mM stock in H₂O (Optional)

Principle

This assay measures beta-oxidation by quantitation of ^3H -labeled H_2O generated at the acyl-CoA dehydrogenase step. For further details, consult reference #4.

Media containing radiolabel and cold substrate is added to the cells, and after a period of incubation, the media is removed. Protein is precipitated using TCA, and the deproteinated supernatant is alkalized prior to loading onto an ion-exchange column. The column binds the majority of radioactivity, and the radiolabeled H_2O is eluted and counted.

There is a fraction within the eluate that is not specific for beta-oxidation; the source of this radioactivity is unknown, but it can be accounted for by taking media that has not been added to cells, and processing it in the same fashion as one would for the experimental samples. The nonspecific radioactivity is simply subtracted from the final values. The contribution of this nonspecific fraction is generally 10-25% of the total radioactivity (after elution), and varies from batch-to-batch (at least with radiolabel from Perkin-Elmer).

Protocol

I. Stock solutions

A. 2mM BSA

1. Weigh out the quantity of BSA you will need to make a sufficient volume of 2mM solution. The formula weight of BSA is 66000.
2. Add BSA to ~80% of the target volume; the quantity of BSA you need to add to make a 2mM solution makes a significant contribution to the final volume.

Example: To make 10mL of 2mM BSA, you will need 1.32g in 10mL. Weigh out 1.32g, add it to 8mL of solution, allow to dissolve, and add additional solution to reach 10mL.

3. BSA takes some time to go into solution. You may wish to keep the mixture at 4°C overnight to slowly dissolve. Alternatively, you may keep it at room temperature or 37°C to speed up dissolution. Mild centrifugation aids in dissolution.
4. Once in solution, sterile filter in the hood and store at 4°C, away from light. The solution is stable for many months at 4°C.

B. 20mM Na-Palmitate

1. Determine the quantity of Na-Palmitate you will need.
2. Mix Na-Palmitate powder and the appropriate volume of 0.01N NaOH in a tube. The contribution of palmitate powder to the final volume is negligible. DO NOT vortex.
3. Allow the mixture to solubilize in a 70-80°C waterbath.
4. Store at -20°C; the solution is stable for many months. Re-heat in a 70°C waterbath before each use.

II. Coupling of Na-FFA with BSA

Overview: BSA must be used to keep FFA from forming toxic micelles; each molecule of BSA can bind anywhere from 4-7 FFA moieties, depending on whether the binding sites are filled (hence the recommendation to use fatty acid-free BSA). The recommended molar ratio of BSA:FFA ranges from 1:1 to 1:7. The majority of research uses 1:2 to 1:3 ratios.

1. This mixture will contain radiolabel; it is therefore recommended that you only make enough for a single experiment.
2. Using the accompanying quick-calculation Excel spreadsheet, determine the volume of 20mM Na-Palmitate and 2mM BSA you will need to make a 4mM palmitate-BSA solution. For most experiments, you should be able to perform the coupling step in a 1.7-2.0mL microfuge tube.
3. Set up two waterbaths: one at 70°C (for the Na-Palmitate) and one at 50°C (for the BSA and coupling).
4. Dissolve your 20mM stock of Na-Palmitate in a 70°C waterbath. Quickly pipette the required volume of 20mM Na-Palmitate into a microfuge tube, and keep the microfuge tube at 70°C.
5. Add the desired quantity of radiolabel to the aliquot of 20mM Na-Palmitate at 70°C.
6. In a separate microfuge tube, aliquot the required volume of 2mM BSA into a separate microfuge tube. Keep at 50°C.
7. Quickly and carefully add the required quantity of (now radiolabeled) 20mM Na-Palmitate to the aliquot of BSA. You will be transferring a 70°C solution into a 50°C solution. Pipette up and down quickly, repeat several times, cap the tube, and vortex gently for a few seconds. Return the mixture to the 50°C waterbath. Allow to couple for 10-15 minutes with occasional vortexing.

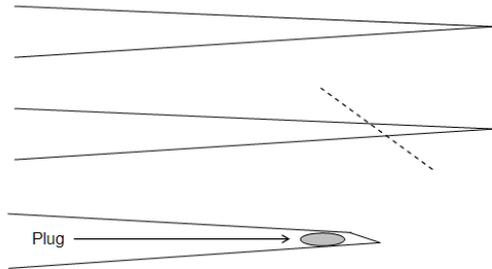
If properly coupled, the solution will appear perfectly clear.

Note: When working with (physically) hot solutions, it is highly recommended that you use a positive displacement pipette for accuracy.

III. Preparation of columns and racks

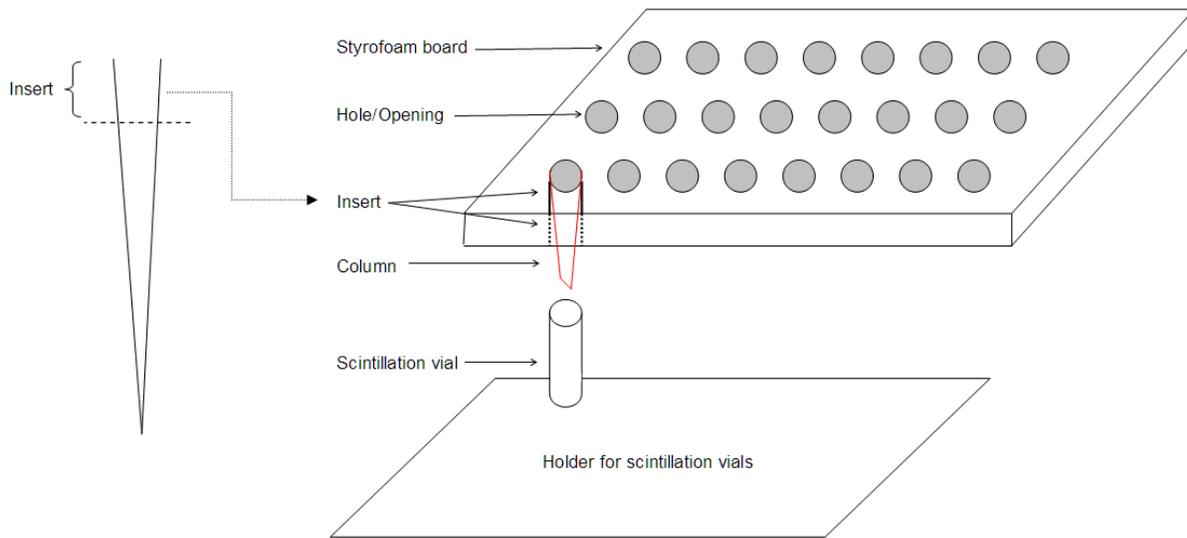
Overview: 1mL pipette tips cut at an angle and plugged with polyester batting (or fiberglass- but polyfill works just as well is much less irritating to work with) are filled with Dowex ion-exchange resin to capture $^3\text{H}_2\text{O}$, the beta-oxidation product, while allowing the majority of nonspecific radioactivity to pass through.

1. Cut tips and plug as so:



2. Prepare ion-exchange resin by making a 0.2g/mL solution in H_2O . Pipette 1 mL of resin per column; shake the stock solution between columns to maintain consistency. Store excess resin sealed at 4°C for >6 months.

3. Make a holder for the scintillation vials, and a rack for the columns, as so:



The Styrofoam containers for disposable cuvettes work nicely as scintillation vial holders. Keep in mind that most scintillation cocktails dissolve Styrofoam.

Make sure you have enough holes for the number of samples + 4. The extra four holes are for nonspecificity controls.

IV. Performing the assay

Overview: Cells will use the radiolabeled FFA and generate $^3\text{H}_2\text{O}$ as the final beta-oxidation product, which is released into the media.

1. Determine the final concentration of FFA you wish to use, and make the appropriate dilutions in your media/buffer. Make extra media for nonspecificity controls (3-4 extra wells' worth).
2. Keep the remaining media to use as your standard(s).
- 2a. It is recommended you use L-Carnitine as a positive control* (promotes beta-oxidation), and Etomoxir as a negative control (inhibits beta-oxidation). L-Carnitine may be added at the beginning of the assay, at 200-1000uM final concentration. For Etomoxir, it is recommended you pre-incubate your cells with 1-10uM for an hour, and maintain the Etomoxir concentration for the duration of the assay. *Note: L-carnitine may not be a suitable positive control for healthy cells, where native carnitine levels are preserved- this will, of course, vary depending on mouse strain, experimental conditions, and carnitine concentration. A simple time-course may be a better option.
3. Add media to cells. Use a minimum volume that will cover the cells. 300uL/well is recommended for 12-well plates.
4. Allow the assay to proceed for 15 minutes to 4 hours, depending on cell type, number, and what you wish to observe.
6. At the end of the assay, pipette all, or a fixed fraction of your media into eppendorf tubes. Add 100% TCA to reach a final concentration of 5-10%. Vortex and allow to precipitate at 4°C for 20-60 minutes. Periodically check on tubes and agitate them gently to facilitate precipitation. Precipitation is complete when you see a fluffy white precipitate and a clear supernatant. For nonspecificity controls, treat the extra media samples exactly as you did your experimental samples (i.e. pipette same volume of media into eppendorf tubes, add same volume of 100% TCA, and precipitate).
7. Centrifuge at 4°C at max speed for 10 min to pellet protein.
8. Transfer all or a fixed fraction of supernatant to a new set of tubes. Add 6N NaOH to reach a final concentration of 0.8-1.0N. The solution **MUST** be basic to bind to the ion-exchange columns properly. Test with pH paper if necessary.
9. Prepare columns by adding the resin. 1mL of resin (0.2g/mL) per column is sufficient. Allow excess water to flow through- columns should not be dry, but should have stopped dripping water, before loading.
10. Load all, or a fixed fraction of the alkaline supernatant to columns. Discard run-through (treat as radioactive, even though there should be no or minimal radioactivity coming out of the columns at this time).
11. Once columns have stopped dripping, place columns directly over scintillation vials and elute columns with milli-Q H_2O into scintillation vials. Use 2-3 column volumes in total.
12. Add scintillation fluid to vials. 4-5mL should be sufficient; actual volume depends on the capacity of the scintillation vial. Make sure your scintillation fluid is compatible with aqueous solutions- EcoLume is recommended.
13. Make standards/blanks in same volume of H_2O /scintillation fluid.
14. Vortex and read in scintillation counter. Subtract nonspecificity controls as background.

References

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