Cholesterol ester assay

This assay uses sonication of the animal homogenates to release and emulsify the cholesterol esters stored in lipid droplets. Each sample is then divided into two tubes, one of which is treated with cholesterol esterase. The amount of cholesterol esters is determined by subtracting the background level of free cholesterol in the untreated sample from the total amount of esterified and free cholesterol present in the sample treated with cholesterol esterase.

1) Follow the Amplex Red Cholesterol Assay Kit (Invitrogen; A12216) instructions to prepare aliquots of the 20 mM Amplex Red reagent in DMSO, 200 U/ml HRP in 1x reaction buffer, and 200 U/ml cholesterol oxidase in 1x reaction buffer. These can be stored at -20°C for later use.

2) Collect samples in 1.5 ml microfuge tubes (30 adult male flies). Rinse animals several times with cold PBS to remove all traces of food. Carefully remove all liquid and homogenize animals in 250 µl of cold PBS + 0.05% Tween 20 (PBST) with a pellet pestle (Kontes; 749521-1500) on ice. A motor can be used to facilitate homogenization (Kontes; 749540-0000). Remove 10 µl of homogenized sample to measure protein content with a Bradford assay. Protein samples can be frozen and stored at -80ºC for later analysis.

3) Add 650 µl of cold PBST to each sample and sonicate on ice using a tip sonicator, three times for 30 seconds each, at a medium setting to prevent foaming.

4) Split each sample into two 1.5 ml microfuge tubes and add 10 µl of 200 U/ml cholesterol esterase (either Sigma C1403 or C3766) to one set of tubes.

5) Incubate both sets of tubes overnight (> 16 hrs) at 37°C with occasional vortexing.

6) Extract all tubes with 900 µl 2:1 chloroform:methanol by shaking the tubes several times vigorously over a period of about 3 minutes. Centrifuge to separate the phases, remove the lower organic phase, and transfer to a fresh 1.5 ml microfuge tube. Use a centrifugal vacuum concentrator to remove all of the organic solvent, leaving an oily lipid residue (approx. 30 minutes).

7) Solubilize lipids in 500 µl PBST by vortexing and sonicating as above.

8) Prepare free cholesterol standards in 1x reaction buffer using the 2 mg/ml cholesterol reference standard provided in the kit. We prepare a dilution series, ending up with at least 50 ml each of 0, 2, 4, 6, 8 mg/ml cholesterol.

9) Add 50 µl of the cholesterol standards and a 1x reaction buffer blank to the first row of a black 96 well plate (Optiplate-96 Perkin Elmer; 6005270). In the next rows, add a 1:1 dilution of each sample with 1x reaction buffer (25 µl of each sample + 25 µl 1x reaction buffer) to each well.

10) Prepare the reaction mix using stored aliquots:

15 µl 20 mM Amplex Red reagent in DMSO
10 µl 200 U/ml HRP in 1x reaction buffer
10 µl 200 U/ml cholesterol oxidase in 1x reaction buffer
+ 1495 µl 1x reaction buffer

11) Add 90 µl of reaction mix to each well with a multichannel pipette. Be sure to keep in the dark and incubate for at least 30 min at 37°C.

12) Measure immediately using a fluorescence plate reader with excitation at 530 nm and emission at 590 nm.

13) Determine the amount of cholesterol esters by subtracting the measurements performed on samples that were not treated with cholesterol esterase from those that were treated with the enzyme. Each data point resulting from the assay should represent an average of at least three collections of animals, and the assay should be repeated at least three times.