Free Cholesterol Assay

This assay does not involve sonicating the samples and thus measures primarily free sterols. Sonication is needed to release and emulsify the cholesterol esters stored in lipid droplets. Although cholesterol esterase is included in this assay, it makes little difference in the final result.

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, 200 U/ml cholesterol oxidase in 1x reaction buffer, and 200 U/ml cholesterol esterase in 1x reaction buffer. These can be stored at -20°C for later use.

2) Collect samples in 1.5 ml microfuge tubes (75 embryos, 30 first instar larvae, 5 adult male flies). Rinse several times with cold PBS to remove all traces of food that might be attached to the outside of the animal. Embryos should be dechorionated following standard procedures and subsequently transferred to a 1.5 ml microfuge tube. Larvae can be washed in a 1.5 ml microfuge tube, but adults should be rinsed in a 9-well glass plate. Transfer adult flies to a 1.5 ml microfuge tube. Carefully remove all liquid. For larvae, centrifuge at 3,000 x g and remove all remnants of PBS.

3) Homogenize animals in 100 µl of 1x reaction buffer with a pellet pestle (Kontes; 749521-1500) on ice. A motor can be used to facilitate homogenization (Kontes; 749540-0000).

4) Centrifuge homogenate for 5 min at 2,300 x g at room temperature. Transfer the supernatant to a fresh 1.5 ml microfuge tube, including the upper "fatty" layer and mix gently. Remove 10 µl of homogenized sample to measure protein content with a Bradford assay. Keep samples on ice and do not heat-treat. Protein samples can be frozen and stored at -80ºC for later analysis.

5) Prepare 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.

6) 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 row, add 50 µl of each sample into individual wells.

7) 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
1 µl 200 U/ml cholesterol esterase in 1x reaction buffer
+ 1494 µl 1x reaction buffer

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

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