Glycogen Assay (GO kit)

1) Collect samples (25 mid-second instar larvae; 5 adult flies).

2) Rinse several times with 1 ml cold PBS to remove all traces of food that might be attached to the outside of the animal. 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. Snap freeze animals in liquid nitrogen for later homogenization or add 100 µl cold PBS.

3) Rapidly homogenize animals in PBS with a pellet pestle (Kontes; 749521-1500) on ice. A motor can be used to facilitate homogenization (Kontes; 749540-0000). Frozen samples should be kept on dry ice until addition of PBS. If samples are not kept cold, glycogen and trehalose will be enzymatically degraded into free glucose by endogenous enzymes and skew the final analysis.

4) 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) Heat supernatant for 10 min at 70ºC, then centrifuge for 3 min at maximum speed in a refrigerated tabletop centrifuge that has been prechilled to 4ºC.

6) Pipette the resulting supernatant into a new 1.5 ml microfuge tube. At this time, the heat-inactivated sample can be frozen and stored at -80°C, if desired.

7) Set up glucose and glycogen standards. Using 1 mg/ml glucose or glycogen stock solutions, make a dilution series of glucose or glycogen standards in PBS, both in the range of 0 – 0.16 mg/ml, as outlined in glucose assay protocol. Glycogen stock solutions can be stored at -20ºC or 4ºC.

8) Add 30 µl of each glycogen standard (including PBS blank) to the top row of a clear-bottom 96 well plate. Similarly, add 30 µl of each glucose standard in the next row down.

9) Dilute heat-treated fly samples for glycogen measurement 1:5 in PBS (the required dilution may range from 1:5 to 1:20 depending on experimental conditions) and load 30 µl into each well. Samples should be loaded in duplicate rows such that one row will be used to measure glycogen + glucose (treated with amyloglucosidase) and the adjacent row will be used to measure glucose alone (no amyloglucosidase).

10) Prepare the GO reagent (see glucose assay protocol) for your glycogen measurements by adding 1 µl amyloglucosidase (Sigma A1602; 25mg) per 1 ml of GO reagent.

11) Using a multichannel pipette, add 100 µl of GO reagent + amyloglucosidase to the glycogen standards and the first row of each set of duplicate samples. Then add 100 µl of GO reagent alone (without amyloglucosidase) to the glucose standards and the remaining samples.

12) Seal the wells with parafilm to prevent evaporation and incubate the plate at 37°C for 30-60 minutes. Briefly centrifuge the plate in an appropriate swing-bucket rotor to clear condensate from the sides of the wells and remove any air bubbles present in the samples. Then add 100 µl of 12 N sulfuric acid and measure absorbance at 540 nm using a plate reader.

13) Free glucose concentration in each sample is calculated based on the glucose standard curve. For glycogen measurements, first subtract the absorbance measured for free glucose in the untreated samples from the absorbance of the samples that have been digested with amyloglucosidase. The glycogen content in each sample is then calculated based on the glycogen standard curve. This method is linear from 0.01 to 0.16 mg/ml glycogen.

Glycogen Assay (HK kit)

1) Collect samples (25 mid-second instar larvae; 5 adult flies).

2) Rinse several times with 1 ml cold PBS to remove all traces of food that might be attached to the outside of the animal. 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. Snap freeze animals in liquid nitrogen for later homogenization or add 100 µl cold PBS.

3) Rapidly homogenize animals in PBS with a pellet pestle (Kontes; 749521-1500) on ice. A motor can be used to facilitate homogenization (Kontes; 749540-0000). Frozen samples should be kept on dry ice until addition of PBS. If samples are not kept cold, glycogen and trehalose will be enzymatically degraded into free glucose by endogenous enzymes and skew the final analysis.

4) 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) Heat supernatant for 10 min at 70ºC, then centrifuge for 3 min at maximum speed in a refrigerated tabletop centrifuge that has been prechilled to 4ºC.

6) Pipette the resulting supernatant into a new 1.5 ml microfuge tube. At this time, the heat-inactivated sample can be frozen and stored at -80°C, if desired.

7) Prepare amyloglucosidase stock (AS) solution by adding 1.5 µl amyloglucosidase (Sigma A1602; 25mg) to 1 ml of PBS.

8) Set up glycogen standards by diluting 16 µl of glycogen stock (1 mg/ml) with 50 µl of AS solution and 34 µl of PBS (100 µl final volume) for 0.16 mg/ml standard. Do four 2-fold serial dilutions into 1:1 mix of AS and 1xPBS (50 µl 0.16 mg/ml + 50 µl AS+PBS for 0.08 mg/ml standard, etc.) to generate 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml glycogen standards. Glucose standards should be diluted as outlined in the glucose assay protocol (no amyloglucosidase added).

9) In 1.5 ml microfuge tubes, dilute the heat-treated fly samples 1:3 in PBS (this dilution can range from 1:2 to 1:5 depending on experimental conditions) by combining 20 µl of sample with 40 µl PBS. Aliquot 20 µl of these diluted samples into two separate tubes, one containing 20 µl of AS solution, and the other containing 20 µl of PBS (final sample dilution will equal 1:6).

10) Add 30 µl of each glycogen standard (including PBS blank) to one row at the top of the plate. Add 30 µl of the glucose standards (and PBS blank) to the next row.

11) Load 30 µl of each diluted sample to the plate. Samples should be loaded in duplicate rows such that one row will be used to measure glycogen + glucose (amyloglucosidase treated) and the adjacent row will be used to measure glucose alone (no amyloglucosidase).

12) Seal the wells with parafilm and incubate the plate at 37°C for 60 minutes to digest the glycogen. Spin briefly to clear the condensate from the sides of the wells.

13) Add 100 µl of HK reagent to each well with a multichannel pipette and incubate at room temperature for 15 minutes. Measure absorbance at 340 nm using a plate reader.

14) Free glucose concentration in each sample is calculated based on the glucose standard curve. For glycogen measurements, first subtract the absorbance measured for free glucose in the untreated samples from the absorbance of the samples that have been digested with amyloglucosidase. The glycogen content in each sample is then calculated based on the glycogen standard curve. This method is linear from 0.01 to 0.16 mg/ml glycogen.