Protocol for Protein Assay

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Equipment needed:

10 mL pipette and two tips
10-100 μL pipette and box of tips
200 μL pipette and box of tips
Ice bucket with small amount of ice (for the standards)
Samples hosts (e.g., *Drosophila* or *Daphnia*)

Before you begin:

(*a*) Turn on the plate reader in room 313 (double check this)

(*b*) Open the Brewer Protocol and set temperature to 25°C
*(it takes a bit to arrive at the correct temperature).

(c) Prepare your samples: see Sample Prep Protocol.

Remember:

This is a colorimetric assay, i.e., once you mix in the standards etc., it is light sensitive, so keep it covered in aluminum foil and try to move efficiently; ** it is important to prep everything and know your steps before you begin to pipette into the 96 well plate.**

Overview of protocol (detailed steps outlined below):

(1) Make Reagent
(2) Label centrifuge tubes A-H (for the 8 stocks)
(3) Make the 8 stocks (using the serial-dilution table below)
(4) Use multi-channel pipette to distribute 15 μL of isolation buffer (DDI H_20) into each well.
(5) Distribute 10μL of each stock into the 96 well plate (with 2 technical replicates); total of 18 wells
(6) Distribute **35 μL** of your samples
(7) Use multi-channel pipette to distribute 80 μL glycerol reagent to each well
(8) Cover the plate with aluminum foil and incubate at 37°C for 30 min
(9) While the plate is in the oven, prepare the plate reader
(10) Read Plate
(11) Use multi-channel pipette to distribute 20 μL triglyceride reagent into each well
(12) Cover the plate with aluminum foil and incubate at 37°C for 30 min
(13) Read Plate
(14) Analyze data

Now that you have a brief overview of the assay, we will go through each step in detail.

Step 1. Make Reagent

Sigma Free Glycerol Reagent (F6428) | Sigma Trigylceride Reagent (T2449)

**The reagents are shipped in a powdered form. Simply add ddH_2O to the darkened glass bottles to reconstitute:*

40 mL (ddH_2O) to the free glycerol reagent

20 mL (ddH_2O) to the triglyceride reagent

Swirl gently to mix and look in the bottom to be sure that all the solid has gone into solution before using.

The reagents are stable at 4 ^oC for several weeks.

Step 2.

Label 8 centrifuge tubes: A,B,C,D,E,F,G, H

These will be for your standards.

KEEP ALL STANDARDS ON ICE

USE A DIFFERENT PIPPETTE EACH TIME

Vortex after each mixture and before you pipette it into the 96 well plate

To make the serial dilution, we will follow this formula, which has been tested to span the entire range of protein that is usually found in both *Drosophila* and *Daphnia*.

Glycerol Standards	Vol. glycerol stock (0.26 mg/mL) (0.26 mg/mL stock glycerol is Sigma G7793)	Vol. ddH_2O	Final Vol.
A: 0.0 µg glycerol/10 µl	0.0 µl	500 µl	500 µl
B: 0.2 µg glycerol/10 µl	38.46 µl	461.54 µl	500 µl
C: 0.4 µg glycerol/10 µl	76.93 µl	423.08 µl	500 µl
D: 0.8 µg glycerol/10 µl	153.85 µl	346.16 µl	500 µl
E: 1.2 µg glycerol/10 µl	230.77 µl	269.23 µl	500 µl
F: 1.6 µg glycerol/10 µl	307.7 µl	192.31 µl	500 µl
G: 2.0 µg glycerol/10 µl	384.62 µl	115.39 µl	500 µl
H. 2.4 µg glycerol/10 µl	461.54 µl	38.46 µl	500 µl

Step 3.

Use Multi-channel pipette to distribute 15 µL of isolation buffer (in our case, DDI H_20) into each well.

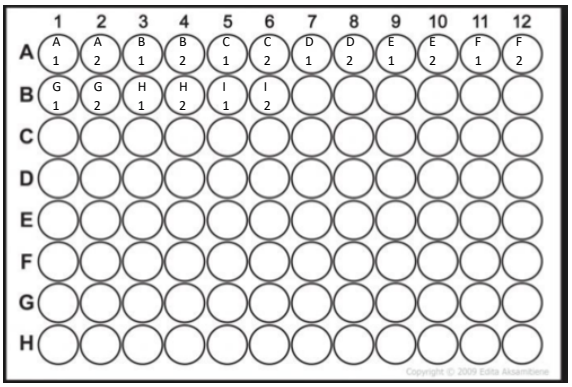
Step 4. Distribute 10µL of each standard into the 96 well plate in duplicate**

** Remember, you have 2 technical replicates, so you will use 18 wells.

Gently mix everything in the well each time before moving on.

Distribute this way (the plate reader we will use later is also set up this way):

USE A DIFFERENT PIPPETTE TIP FOR EACH STANDARD !!!



Step 5.
Add 35 µL of your prepared sample (i.e., the homogenate of *Daphnia* or *Drosophila*) to the next set of empty wells (see protocol on Sample Prep).

Step 6.
Gently swirl the free glycerol reagent to mix: Distribute 80 µL into each well (use the multi-channel pipette).

Step 7.
Cover the plate with foil and incubate at 37°C for 30 minutes. Prepare plate reader.

Step 11.
Remove foil cover and take reading at 540 nm for the "background glycerol" reading.

Step 12.
Pipet 20 µL triglyceride reagent into each well using the multi-channel pipettor.

Step 13.
Cover plate with foil and incubate @ 37°C for 30 minutes.

Step 14.
Remove cover and take reading at 540 nm for the "30 min lipase" reading

Step 15.
Repeat incubate/read sequence to get 60 min lipase, 90 min lipase, 2 hr lipase, 3 hr lipase, and 4 hr lipase readings

Step 16.
- Plot on x/y graph the TRI standards vs. corresponding "background glycerol" absorbance values.
- Fit a linear regression line to the standards.
- For each sample, subtract the "background glycerol" absorbance from the final 4 hr absorbance reading; use the resulting value to plug into the regression line to calculate the µg of TRI for each sample.