Protocol: Hemolymph insulin-like peptide (DILP) measurement by ELISA

Day one

1. Coat EIA plate
- Dilute 0.5 µl to 1 µl of hemolymph samples with 50 µl 1X PBS (Notice that Pierre Leopold's lab has a better protocol by acid-treatment of hemolymph to improved DILP detection), and coat onto one well of 96-well microtiter EIA/RIA plate (Corning Inc.), usually I perform a duplicate for each test sample.
- Stand the plate at room temperature (RT) over night (O/N).

Day Two

1. Blocking the plate
- Discard coated protein samples, Tap the plate against paper towel to remove as much liquid as possible.
- Add 300 µl of EIA blocking buffer containing 1% BSA (BSA freshly added) using a multichannel pipette and a cell culture reservoir.
- Shake the plate for 1-2 hr at RT.

2. Wash
- Discard EIA blocking buffer, tap plate.
-Rinse plate two times with 200 µl-300 µl PBS-Tween. Shake 5 minutes using orbital shaker after adding PBS-Tween.

3. Primary antibody
- Dilute primary antibody (anti-DILP2 or anti-DILP5 antibody) 1: 2000 in EIA-BSA buffer.
- Add 100 µl of primary antibody solution to each well except for blank well (Blank well contains only PBS without any test samples).
- Shake 2 minute. Then incubate at RT for 1-2 hr.

4. Wash
- Discard primary antibody, tap plate.
-Rinse plate three times with 200 µl-300 µl PBS-Tween and shake 5 minutes.

5. Secondary antibody
- Dilute secondary antibody (anti-rabbit/rat IgG horse reddish peroxidase linked antibody from donkey) 1: 2500 in EIA-BSA buffer.
- Add 100 µl of primary antibody to each well.
- Shake 2 minute. Then incubate at RT for 1 hr.

6. Wash
- Discard secondary antibody, tap plate.
-Rinse plate three times with 200 µl-300 µl PBS-Tween and shake 5 minutes.

7. Setup enzyme reaction
- Add 100 µl of substrate TMB to each well.

- Wait for 10-15 minutes at RT (You will see the blue color showing up).
- Add 100 µl of 1M phosphoric acid to stop the enzyme reaction and blue color turns into yellow.
- Read plate at 450 nm using a plate reader. Absorbance of each sample (Blank reading is subtracted from each sample) is used from data analysis.

Note: If necessary, test each coated sample with and without adding primary antibody (secondary antibody added for both).Then absorbance for well with no primary antibody is subtracted from well containing primary antibody to eliminate the effects of non-specific binding to secondary antibody.

Appendix I. Reagents

1. Stock NaPO4 Buffer (0.1M, pH 7.4)

Na2HPO4 (anhydrous) 11.925 g

NaH2PO4 ·dH2O 2.253 g (or 2.208 g of anhydrous)

dH2O to 1 L

Note: Normally, no need to adjust pH.

2. Phosphate Buffered Saline (PBS)

0.1 M NaPO4 buffer (see above) 100 ml

NaCl 8.76 g

dH2O to 1 L

3. Blocking EIA buffer

0.1 M NaPO4 buffer 200 ml

NaCl 8.76 g

NaEDTA ·2dH2O 0.3722 g 2%

Na azide 1 ml

BSA 10 g

dH2O to 1 L

4. PBS-Tween

0.1 M NaPO4 buffer 100 ml

NaCl 8.76 g

5% Tween-20 10 ml

dH2O to 1 L

5. EIA buffer without BSA (add BSA before use)

0.1 M NaPO4 buffer 200 ml

NaCl 8.76 g

NaEDTA ·2dH2O 0.3722 g

dH2O to 1 L

6. 1 M Phosphoric acid Conc.

Phosphoric acid (14.7 M) 38.8 ml

dH2O to 500 ml

7. TMB (3,3',5,5'-teramethylbenzidine) solube-one step solution

Company: American Qualex antibodies, 920-A Calle Negocio, San Clemente, California 92673. 949-492-8298, 800-772-1776 Fax: 949-492-6790 Email: americanqualex@yahoo.com, www.aqsp.com Catalog #: C5801-250 ml Lot #: 5A04991

8. Anti-DILP2 and anti-DILP5 antibody, gift from Pierre Leopold.

9. Anti-rabbit/rat IgG horse reddish peroxidase linked antibody (Jackson Immuno).

Appendix II. Equipment

1. 96-well EIA plate EIA/RIA Plate 96 well EasywashTM No lid, High Binding Certified Polystyrene 25/Pack, 100/Case Non-Sterile Company: Corning Incorporated. Corning, NY 14831
www.corning.com/lifesciences Catalog #: 3369 Lot #: 33905028

2. Microplate reader (with λ = 450nm)

3. Multiple channel pipette, HPLC syringe and cell culture reservoir

4. Orbital shaker and vortexer