Protocol: Ecdysteroid titers estimation by EIA (Enzyme ImmunoAssay)

Day one

1. Make IGG-coated plate.

- For one 96-well microtiter plate, usually need to make 10ml IGG solution with 1X PBS (360 X dilution) if all 96 wells are required. Otherwise, using Table. 1 to make desired volume.

Table.1 IGG dilution list

Total Volume (ml)	IGG (ul)
4	11.1
5	13.8
6	16.6
7	19.4
8	22.1
9	24.9
10	27.6
11	30.4
12	33.2

- Mix by inverting.
- Add 90ul per well of IGG using a multichannel pipette (8 tips/row) and a cell culture reservoir.
- Stand the plate at room temperature (RT), over night (O/N).

Day Two

1. Blocking the plate.

- Discard IGG, Tap the plate against paper towel to remove as much liquid as possible.
- Add 315ul of EIA blocking buffer using a multichannel pipette and a cell culture reservoir.
- Shake the plate for 1-2 hr at RT
- Go ahead to do next steps or store the plate at 4^0C for months.

2. Wash.

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

3. Add desired amount of Bovine Serum Albumin (BSA) to EIA buffer to make EIA/BSA buffer. For one 96-well plate, we add 25mg of BSA to 25ml of EIA buffer.

4. Estimate sample dilution factor.

- Based on the known ecdysteroid titer from close related species, you need estimate the ecdysteroid range of unknown samples (Unit: pg/insect). You will have to dilute your samples so that all your samples can fall within the assay range, which is 0.5 pg/insect to 40 pg/insect. If there is any sample that fall out of range, you have to dilute it again and perform the assay again until it is in the range (See the appendix IV).
- Add desired EIA/BSA buffer to dried samples (usually 200-400 ul), vortex 5 second twice to insure completely dissolving.

- Fill the plate template sheet (if necessary), so you can follow it and won't make any mistake when you add samples into 96-well plate (See the appendix III).

- Write down the sample name, EIA/BSA buffer and sample loading amount.

5. Standard and sample adding

- Add desired amount of samples and EIA/BSA buffer accordingly using a syringe (capacity: 100ul). Wash syringe with EIA/BSA buffer between each sample. Total 50ul /well.
- Add 100ul EIA/BSA buffer to the blank wells.
- Make ecdysteroid standards.
For standard I (0.25 pg/ul): Take 10ul of 50pg/ul 20-hydroxecdysteroid (20E) stock solution to glass culture tube, dry it out using speedvac in about 10 minutes (since 50pg/ul 20E was dissolved in methonal). Add 2 ml of EIA/BSA buffer to the tube. Store at 4^0C.
For standard II (1 pg/ul): Take 40ul of 50pg/ul 20E to glass culture tube, then follow above steps.
- Add ecdysteroid standards according to the plate template sheet (See the appendix III).

6. Antibody and conjugate
- Prepare antibody with 5 ul of anti-ecdysteroid antibody (100X) in 5 ml EIA/BSA buffer
- Prepare conjugate with 5 ul of peroxidase-labeled conjugated ecdysteroid (15X) in 5 ml EIA/BSA buffer.
- Add 50ul of anti-ecdysteroid antibody (100,000X) to each well except for the blanks. Add 50ul of peroxidase-labeled conjugated ecdysteroid (15,000X) to each well
- Shake for 5 minutes at RT and store the plate at 4^0C O/N. Cove the plate with plate sealer.

Day Three

1. Setup enzyme reaction

- Discard, tap plate and rinse three times with PBS-Tween, shaking 5 minutes after each wash.
- Add 100ul of substrate TMB to each well.

- Wait for 15 minutes at RT, and tap plate occasionally (You will see the blue color showing up).
- Add 100ul of 1M phosphoric acid to stop the enzyme reaction and blue color turns into yellow.
- Read plate at 450nm using a microplate reader. Data can be achieved by using sigma-plot method or linear regression.

Appendix I. Reagents

1. Stock $NaPO_4$ Buffer (0.1M, pH 7.4)

Na_2HPO_4 (anhydrous)	11.925 g
$NaH_2PO_4 \cdot dH_2O$	2.253 g (or 2.208 g of anhydrous)
dH_2O	to 1 L

Note: Normally, no need to adjust pH.

2. Phosphate Buffered Saline (PBS)

0.1 M $NaPO_4$ buffer (see above)	100 ml
NaCl	8.76 g
dH_2O	to 1 L

3. Blocking EIA buffer

0.1 M $NaPO_4$ buffer	200 ml
NaCl	8.76 g
NaEDTA $\cdot 2dH_2O$	0.3722 g
2% Na azide	1 ml
BSA	1 g
dH_2O	to 1 L

4. PBS-Tween

0.1 M $NaPO_4$ buffer	100 ml
NaCl	8.76 g
5% Tween-20	10 ml
dH_2O	to 1 L

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

0.1 M $NaPO_4$ buffer	200 ml
NaCl	8.76 g
NaEDTA $\cdot 2dH_2O$	0.3722 g
dH_2O	to 1 L

6. 1 M Phosphoric acid

Conc. Phosphoric acid (14.7 M)	38.8 ml
dH_2O	to 500 ml

7. IGG (AffiniPure Goat anti-rabbit IgG, Fc Fragment Specific)
Company: Jackson ImmunoResearch Laboratories, INC.
872 W. Baltimore Pike, P.O. Box 9, West Grove, PA 19390.
http://www.jacksonimmuno.com/
800-367-5296, 610-869-4024, Fax: 610-869-0171

Code #: 111-005-008
Lot #: 60196

Size: 2.0 mg
Antibody concentration: 2.4 mg/ml

8. 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

9. Anti-ecdysteroid antibody
From Dale Gelman, made by Timothy G. Kingan

10. Peroxidase-labeled conjugated ecdysteroid
From Dale Gelman, made by Timothy G. Kingan

Appendix II. Equipment

1. 96-well EIA plate
EIA/RIA Plate
96 well Easywash™
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 $\lambda = 450$nm)

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

4. Orbital shaker and vortexer

Appendix III. Plate template sheet

See file: 96-well template-EIA.pdf

Appendix IV. Setup sample dilution

Assume that
(1). You have already added 200 ul EIA/BSA buffer to all the samples to dissolve ecdysteroids.

(2). Estimated ecdysteroids levels of each sample are showing as following table.

Sample number	**Estimated ecdysteroids level**	EIA/BSA buffer	**Concentration**
S1	10 pg/insect	200 ul	Pg/ul
S2	40 pg/insect	200 ul	
S3	200 pg/insect	200 ul	

Then you can decide your sample dilution based on above table.

Sample number	**Estimated ecdysteroids level**	**Total sample volume**	**Sample volume per well**	**EIA/BSA buffer volume per well**	**Total volume per well**	**Dilution factor**
S1	10 pg/insect	200 ul	50 ul	0 ul	50 ul	4 X
S2	40 pg/insect	200 ul	20 ul	30 ul	50 ul	10 X
S3	200 pg/insect	200 ul	10 ul	40 ul	50 ul	20 X

Note: You have to multiple your results with dilution factor to get the final ecdysteroid titer. Remember the assay detection range is 0.5 pg/insect to 40 pg/insect

Reference:

Kingan TG. A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids, and cyclic nucleotides. Anal Biochem. 1989;183(2):283-9.

Kingan TG, Zitnan D, Jaffe H, Beckage NE. Identification of neuropeptides in the midgut of parasitized insects: FLRFamides as candidate paracrines. Mol Cell Endocrinol. 1997 Sep 30;133(1):19-32.

Margam VM, Gelman DB, Palli SR. Ecdysteroid titers and developmental expression of ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae). J Insect Physiol. 2006;52(6):558-68.

Parthasarathy R, Tan A, Bai H, Palli SR. Transcription factor broad suppresses precocious development of adult structures during larval-pupal metamorphosis in the red flour beetle, *Tribolium castaneum*. Mech Dev. 2007, *in press*