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

<table>
<thead>
<tr>
<th>Total Volume (ml)</th>
<th>IGG (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>11.1</td>
</tr>
<tr>
<td>5</td>
<td>13.8</td>
</tr>
<tr>
<td>6</td>
<td>16.6</td>
</tr>
<tr>
<td>7</td>
<td>19.4</td>
</tr>
<tr>
<td>8</td>
<td>22.1</td>
</tr>
<tr>
<td>9</td>
<td>24.9</td>
</tr>
<tr>
<td>10</td>
<td>27.6</td>
</tr>
<tr>
<td>11</td>
<td>30.4</td>
</tr>
<tr>
<td>12</td>
<td>33.2</td>
</tr>
</tbody>
</table>

- 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°C 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°C.
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°C 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 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)
   Stock NaPO₄ buffer (see above)     100 ml
   NaCl                             8.76 g
   dH₂O               to 1 L

3. Blocking EIA buffer
   Stock NaPO₄ buffer     200 ml
   NaCl                   8.76 g
   NaEDTA · 2dH₂O        0.3722 g
   2% Na azide           1 ml
   BSA                   1 g
   dH₂O               to 1 L

4. PBS-Tween
   Stock 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)
   Stock 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. 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.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Estimated ecdysteroids level</th>
<th>EIA/BSA buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>10 pg/insect</td>
<td>200 ul</td>
<td>Pg/ul</td>
</tr>
<tr>
<td>S2</td>
<td>40 pg/insect</td>
<td>200 ul</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>200 pg/insect</td>
<td>200 ul</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Estimated ecdysteroids level</th>
<th>Total sample volume</th>
<th>Sample volume per well</th>
<th>EIA/BSA buffer volume per well</th>
<th>Total volume per well</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>10 pg/insect</td>
<td>200 ul</td>
<td>50 ul</td>
<td>0 ul</td>
<td>50 ul</td>
<td>4 X</td>
</tr>
<tr>
<td>S2</td>
<td>40 pg/insect</td>
<td>200 ul</td>
<td>20 ul</td>
<td>30 ul</td>
<td>50 ul</td>
<td>10 X</td>
</tr>
<tr>
<td>S3</td>
<td>200 pg/insect</td>
<td>200 ul</td>
<td>10 ul</td>
<td>40 ul</td>
<td>50 ul</td>
<td>20 X</td>
</tr>
</tbody>
</table>

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:


