

## INFORMATION

pg. 1 Protocol from cell cultures

pg. 2 (continue)

pg. 3. Experimental Considerations

### **Preparation of Iridovirus Inoculum from cell cultures**

**Wayne Hunter**

**Xiomara Sinisterra**

**January 30, 2007**

#### Objectives:

The idea behind this work was to provide high quality, high concentration Iridovirus inoculum to collaborators, to be used as inoculations of a variety of insect species (specifically a beetle (june bugs)).

Starting inoculum was made from IIV injected samples of Diaprepes larvae which had been previously inoculated [in 2003, kept at 4°C, in dark, tissues were ground in buffer Tris-HCL 0.1M, pH 6.5, and kept at 4-10°C in 1.5 mL aliquots] were used as initial material to be inoculated onto SF9 cells for fresh virus propagation.

#### To make crude inoculum from infected insects:

- One larvae (depends on size may use more) is homogenized in 10 µL of buffer in mortar/pestle, small ~3" ceramic.
- The solution is aliquoted into 2 mL centrifuge tubes.
- Three or four passes on Eppendorf table-top centrifuge.
- Spin setting 300xg to pellet debris, transfer supernatant to new tube and repeat 2-3 more times (depending on lipid layers in samples).
- The final transfer of supernatant is to clean tube and -
- Pass the solution through a 0.45 micron syringe filters. (note may have to use two or three filters depending on how much protein is still suspended in supernatant).
- Pass this filtrate through a 0.22 micron syringe filter.
- This filtrate should be free of bacteria and is used to inoculate cell cultures, or to inject into propagation insect hosts, such as beetle grubs.

#### **Protocol Propagate and Harvesting of Iridovirus from SF9 cell cultures.**

- 1- Grow SF-9 cells to 80 – 100% confluence, 25 cm<sup>2</sup> flasks.
- 2- Dilute the crude larvae macerate **1:1** in buffer Tris-HCL 0.1M, pH 6.5
- 3 -Pass the dilute through 0.4 micron syringe filters. (note may have to use two or three filters depending on how much protein is still suspended in supernatant)

Following steps: **INSIDE STERILE FLOW HOOD - to inoculate insect cell cultures**

- 4- Inside sterile laminar flow cabinet pass the dilute through 0.2 micron syringe filters and collect the filtrate in a sterile tube.

- 5- Remove the media from the SF-9 cell flasks, leaving only a residue covering cells.
- 6- Very quickly but gently (drop by drop) apply a minimum of 1 mL of filtrate on the cell layer, rotate flask to cover across all the cells, put lid on flask and lay flat in hood floor.
- 7- Close the flasks and allow to incubate for a period of 30 – 40 min
- 8- Add new cell culture media (4-5 mL) to each flask and place them in the incubator at 25°C-27°C for five days. (either in an incubator, or on lab counter top if temperatures are right, but cover and keep flasks in dark).
- 9- After the five day incubation period, (Back in Sterile Hood) Remove and collect the media (under sterile conditions) from the inoculated flasks.  
Add fresh media (4-5 mL) and initiate a new five day incubation period.
- 10- Collect media with floating cells from the inoculated cell cultures, put into 15 mL tube, centrifuge for 20 min at 9Xg, (we use an IEC clinical centrifuge).
- 11- Discharge the supernatant, Keep the cells which are now pelleted,  
Re-suspend the cells in (3-7 mL) of buffer Tris-HCL 0.1M, pH 6.5.  
(NOTE: this will depend on the size of the pellet)
- 13- Disrupt the cells by several passes through syringe needle gauge 22 and 30<sup>1/2</sup> respectively.
- 14- Centrifuge the lysate at 5000 rpm for 3 minutes, (Eppendorf table top centrifuge)  
KEEP the supernatant and discard the pellet (repeat twice transferring to new tubes)
- 15- Centrifuge the final lysate at setting 14 for 14 min; at the end of this cycle you should see a slightly fluorescent blue pellet that is characteristic of Iridovirus accumulation.  
This bluish pellet is the virus.
- 16- Discard the Supernatant, and Keep the pellet, dilute the pellet in fresh buffer Tris-HCL 0.1M, pH 6.5 at the desired ratio. (Note: We used ~1.5 – 2 mL). This will be your STOCK inoculum.

We are sending you a STOCK tube made in this manner.  
Store wrapped in foil, in refrigerator, NOT freezer.

NOTE: freeze/thawing of IIV, is estimated to loss infectivity by about half each time thawed and refrozen, so virus may be lost easily if too many times in and out of freezer.

Use the inoculum as is, to inject 10-40 µL into your insects (Larvae or Adults) using a hypodermic syringe to create fresh Stock inoculum. This will also depend on size of

insect, for leafhoppers this amount would be 5-10  $\mu\text{L}$  of inoculum, and I have successfully injected 100  $\mu\text{L}$  in Beetle larvae and they survive.

After you do your first trial and we find out if the virus infects your insect then the inoculum may be reduced to 1:10, Stock- to -Buffer (10  $\mu\text{L}$  stock into 90  $\mu\text{L}$  buffer for use as inoculum).

If you want an inexpensive syringe get disposable individually wrapped, sterilized, 1 mL syringes. On the 1 mL syringe, push the plunger down until you see liquid emerging from the needle. Then inject and move the plunger down from one line increment to the next (Look at the side with larger numbers these are cc units, (numbers appear 4, 6, 8, 10...each incremental line is  $\sim$ 20-40  $\mu\text{L}$ ). If you are in doubt if the inoculum was introduced push the plunger down two increments, as sometimes the syringes get air bubbles in them.

Don't discard any left over inoculum in the syringe but cap the syringe, wrap in foil, and store at 4°C in the chill cabinet or refrigerator.

## **Experimental Considerations:**

When you do your injections, please inject the 'Buffer Only' into your 'Control group', and *then* inject your 'Treated' group with the 'Virus inoculum'.

The virus has only been found to infect Insects, but you will still want to use safety precautions, wear latex gloves, safety glasses, etc...

If you have enough insects of the same age/gender, you can inject 10 or 20 individuals for each treatment (20+ would be better statistically).

Monitor activity, mortality, longevity, and fecundity when females, and when they die send to us for virus detection, or if you have your PCR machine email me and I'll send the protocol and primers to you.

If you do the partial virus concentration and you get a bluish pellet then we will know you are getting virus infection/replication in your insects.

When you inject adult females, if possible measure and record:

- the number of eggs laid on average if easy to observe?,
- the number hatch, and of the ones which survive we can test for virus.
- You can also collect some of the egg masses from treated and control, and analyze them for virus presence (This can be done over time, or after several weeks so the virus has time to replicate, usually 7 days post inoculation).

Insects Injected or Feeding, with IIV normally take 7 days to be positively detected.

You can do injections of:

- 1) Larvae, and/or adult males vs. females,
- 2) Monitor egg production in injected females vs control females
- 3) Treat food with virus and measure ability to infect orally on a bait? diet? leaf sprayed

(In Weevils we used 10% sucrose plus virus inoculum under a tender leaf disk for the weevils to eat, they could be observed drinking the liquid and then ate the leaf disk).

- 4) If you have a micro spray system, you can try to do a spray treatments of leaves or surfaces which might be attractive to insects, such as surface areas under lights at night.

In weevils the virus was observed in TEM sections to be infecting the cells around the trachea, suggesting the virus was infecting as an aerosol particle, and insects become infected walking around on virus contaminated surfaces.