Baculovirus methods:

Making virus stocks:

1. Seed a flask at the desired volume with Sf9 cells at 106/ml in ESF 921.

2. Infect the flask at an MOI of 0.1 (It is possible to generate a high titer stock using

an MOI of 0.01 but may not be as efficient).

3. Incubate flasks at 27º C with shaking for 3-5 days.

4. Beginning 3 days after setting up the culture, determine the viability of the

culture. Harvest the culture when the viability drops below 70%. There is no

advantage to letting the culture become less viable. The initial titers will be

similar but the cell debris associated with excessive cell death will negatively

impact storage.

5. Remove cells by centrifugation. Speed and duration increase with an increase in

volume.

6. Filter virus stock through a 0.2 micron, low protein-binding filter. This step is

important for virus titer stability. There is rarely a loss in titer due to the filtration

but there is often a loss of titer due to binding of virus to cell debris.

7. Titer stock. This typically yields >5x108 infectious units per ml.

8. Store stock at 4º C for up to 6 months.

http://www.expressionsystems.com/documents/High%20Titer%20Stock.pdf

Titering virus stocks:

**Plaque Assay Method no. 1**

I have generally found that plaques form better using Grace's medium and serum. Though this is certainly open for debate, here is a method that I use. There are many ways for virus to hide, and so this assay is usually an underestimate of the actual number of virus present. For instance, a pfu (plaque forming unit) may not find a cell by the time the attachment period is ended or it may stick to the plate where no cell is present and by the time the monolayer reaches it, it's too far behind to form a visible plaque.

**Materials:**

**Note:** I make specific reference to particular products not to indicate they are superior, but rather to indicate they are at least adequate for the task as I have used them. Substitutes may very well work just as well.

* Grace's Insect Medium, 2X (e.g. GIBCO #11667)
* Fetal Bovine Serum (Heat Inactivated), (e.g. GIBCO #16140)
* 3% SeaPlaque or other low-melting agarose in ddH2O
* Sterile water
* 50ml sterile conical screw-top tubes
* 37°C water bath
* microwave

**Protocol**

**Prepare infected monolayer of cells**

1. Grow a suspension culture of Sf9 cells to a density of less than 3x10E6.
2. Dilute this culture to to a density of 5 to 6x10E5.
3. For a 6-well culture dish, transfer 2 ml of this cell suspension to each well. For 6 cm dishes, double all volumes in this protocol. Scale by surface area.
	* This cell number will depend somewhat on the cell line and can be adjusted up or down according to your results.
	* If you don't have a confluent monolayer by day 3, increase the cell number. There should be space available on Day 2.
4. Let the cells settle for at least 30 min. Longer is just fine. Look at them and make sure they're stuck.
5. Meanwhile, dilute your virus to 1 ml aliquots at dilutions of 10E-4, 10E-5, 10E-6 and 10E-7.
	* Dilutions below this are only useful if you're trying to recover a dying culture.
	* Dilutions above this are helpful only if you have a very vigorous virus.
	* I usually don't use all four dilutions, but choose according to expectations and needs.
	* It is critical to use fresh tips when transfering each serial dilution to the next tube or you will overestimate titer.
6. After your cells have attached well to the plates, tip them up a bit to drain medium to one side, and aspirate off the media. It's not critical to remove all the medium at this point.
7. Quickly add 1 ml of diluted virus to each well of the 6-well plate. If you're using 6 cm dishes, don't try to do too many plates at once. It's easy to dry the cells and kill them. If you do this, you'll see a characteristic arc of dead cells on the edge of the plate that was tipped up to drain off medium.
8. Transfer the plates to a rocking platform and slowly rock for at least a couple hours, four hours is probably better, though after this the benefit diminishes substantially.

**Prepare your overlay agarose just before use.**

1. This is a bit tricky and the timing is critical. Low melt agarose will solidify at temperatures near or slightly below 37°C, but it's a bit easier on the cells if you can manage this.
2. The mix I use is as follows:
	* 1 part 2X Grace's Medium supplemented with 20% Fetal Calf Serum. Ignore the dilution due to FCS.
	* 1 part 3% SeaPlaque Agarose in ddH2O.
3. Melt the agarose completely in a microwave being careful to allow air to escape to avoid exploding glassware.
4. Allow the agarose to cool slightly, to near 70°C and then aliquot this 20 ml to each 50 ml conical tube.
5. Add 20 ml of room temp or warmer 2X Graces/FCS to each 20 ml aliquot of agarose, then place in a 35-37°C water bath.
6. In the hood I have a thermometer that is well scrubbed with 70% Isopropanol and standing in a 15 ml tube full of the same.
7. Remove the overlay agarose from the water bath one tube at a time and check the temperature. Let it cool to at least 38°C, but preferably less than 37C.

**Overlay agarose onto infected cell monolayer**

1. When ready, tip up the plates and let drain to one side. Aspirate all the medium off, and I usually go back a second time and aspirate each well a second time at the edge to get all medium out.
2. Return the plate to level and quickly add approximately 3 ml of molten overlay mix to each well by allowing it to slide down the far wall of the well and onto the plate.
3. This is the critical stage.
	* If you are too slow, your cells will dry out and you will see the arc of dead cells that I mentioned above on the high side of the plate.
	* If you leave too much medium on the plate, they will be sloppy and the plaques will spread or smear and won't be well defined.
	* If you put the overlay on too hot, you'll cook the cells especially at the point where the overlay first touches the plate.
	* If you tip the plate up on the near edge to drain away from you, and you slide the agarose down the far side of the well, you will be able to see easily which factors you need to adjust as different sides of the plate will be affected.
4. After overlaying the cells let the plates sit **level** in the hood for 30 or so minutes to dry a bit and solidify.
5. If your plaques are smearing, it is a good idea to crack open the lids for 20 minutes after they're poured to allow more moisture to escape.
6. Place in a 27°C, 98% humidity controlled incubator for at least 3 days. If you don't have a humidity controlled incubator, the plates can be sealed in a plastic bag or tupperware-type container with damp towels in the bottom, but this invites mold, and may limit oxygen somewhat.

**Staining the Plates**

1. Some say you can effectively count the plaques without staining, but I have always had considerable difficulty unless the plaques were polyhedra postive. I suggest you stain the plates.
2. Prepare a solution of 1% Neutral red. Ten mls of this will stain about 1000 assays.
3. Prepare an overlay agarose solution as above, but only prepare 1 ml for each assay.
4. Add 1/100th volume of the 1% Neutral Red solution to the molten agarose (e.g. 100 µliters to 10 ml).
5. Add approximately 1 ml of the Red Agarose to each well of a 6-well dish. Be sure the plate is level until the agarose sets up.
6. You only need to add enough Red Agarose to cover the surface evenly.
7. Return the plate to the incubator for at least 4 hours. After a couple hours the plaques will begin to appear as clear spots among stained cells.
8. I usually leave the plate overnight before counting. This makes it easier. If plaques aren't developing, and this depends somewhat on your cell line, leave the plates 4 or 5 days before staining, but this isn't usually necessary.
9. Do some controls and verify that longer incubations don't give higher titer results with your medium and cells.