Chapter 9

Cell Culture for Production of Insecticidal Viruses

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Abstract

While large-scale culture of insect cells will need to be conducted using bioreactors up to 10,000 l scale, many of the main challenges for cell culture-based production of insecticidal viruses can be studied using small-scale (20–500 ml) shaker/spinner flasks, either in free suspension or using microcarrier-based systems. These challenges still relate to the development of appropriate cell lines, stability of virus strains in culture, enhancing virus yields per cell, and the development of serum-free media and feeds for the desired production systems. Hence this chapter presents mainly the methods required to work with and analyze effectively insect cell systems using small-scale cultures. Outlined are procedures for quantifying cells and virus and for establishing frozen cells and virus stocks. The approach for maintaining cell cultures and the multiplicity of infection (MOI) and time of infection (TOI) parameters that should be considered for conducting infections are discussed.

The methods described relate, in particular, to the suspension culture of *Helicoverpa zea* and *Spodoptera frugiperda* cell lines to produce the baculoviruses *Helicoverpa armigera* nucleopolyhedrovirus, HearNPV, and *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus, AgMNPV, respectively, and the production of the nonoccluded *Oryctes* nudivirus, OrNV, using an adherent coleopteran cell line.

Key words Insect cell technology, Suspension culture, Adherent culture, Insecticidal viruses, Bioreactors

1 Introduction

The major viruses considered for use as insecticides are baculoviruses due to their stability in the outside environment as a result of their natural occlusion by polyhedra and their inability to infect vertebrates [1, 2]. While a number of examples of successful programs to produce baculovirus biopesticide products using infected larvae have been documented over the past 40 years [2–4], a major limitation to their wider use is the lack of a cost-effective in vitro production technology [1, 5].

To be cost competitive with larvae production for the major baculovirus biopesticide markets, the manufacturing of baculoviruses using cell culture will need to be conducted using cells that display a doubling time of 24 h or less when grown in suspension

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culture using up to 10,000 l scale airlift or stirred tank bioreactors . Further, upon infection they will need to produce yields of at least 300–600 Occlusion Bodies per cell (OB/cell) at high cell densities ($5–20 \times 10^6$ cells/ml) [1]. This presents significant challenges but progress toward these production levels has been made with the three cell/baculovirus systems listed as follows.

The *H. zea* (HzAM1) cell line produced by Arthur McIntosh [6] has received a lot of attention for its ability to produce the *Helicoverpa armigera* nucleopolyhedrovirus, HearNPV, in vitro [7–9]. The Sf9 (*Spodoptera frugiperda*) cell line has been studied extensively in relation to its ability to be grown in suspension culture using serum-free media [10] and shows potential for the production of a *S. frugiperda* multicapsid nucleopolyhedrovirus, SfMNPV [11]. Finally a *S. frugiperda,* Sf21, and an *Anticarsia gemmatalis* cell line are available that show potential to produce an AgMNPV product [12, 13]. In this chapter, methods related to the suspension culture of *H. zea* and Sf21 cell lines to produce the HearNPV and AgMNPV viruses, respectively, will be provided.

However, insect cells that do not grow in suspension culture and that have slow growth rates (greater than 24 h doubling time) and nonoccluded viruses deserve attention as they may also meet future commercial opportunities. To cover the challenges of such cell lines and viruses, we include methods related to the production of a wild-type nonoccluded *Oryctes* nudivirus [14, 15], using a slow growing adherent coleopteran cell line, DSIR-HA-1179 [16].

The discovery and distribution of OrNV has been very successful in controlling the rhinoceros beetle pest of the palm oil industry [17]. Damage valued in millions of dollars annually is caused by the adult beetle which feeds on the growing shoots of palms often leading to death of the palm. However, control strategies based on the use of this virus suffer from difficulties in producing sufficient amounts of active virus, poor formulation of the virus to maintain its infectivity, and poor quality control in ensuring that only virulent strains of virus are released [18, 19]. The ability to produce OrNV in cell culture will overcome many current limitations in mounting an effective research program to more effectively use this virus as part of an Integrated Pest Management (IPM) strategy to control the beetle. While this virus will not need to be produced at as large scale as the HearNPV, SfMNPV, and AgMNPV viruses, it is likely that it will need to be produced using roller bottle or microcarrier-based systems and so these methods are outlined in this chapter using the DSIR-HA-1179 cell/OrNV system as a case study.

In this chapter there is insufficient space to address all the issues of insect virus production in cell culture. It is important to be aware that many of the issues of growing insect cells in bioreactors are similar to those faced for growing mammalian cells in culture. Hence the development of technology to maximize the production of monoclonal antibodies using CHO cells is relevant and of benefit

for the large-scale growth of insect cells. The shear sensitivity and oxygen consumption properties of insect cells are similar to those of CHO and other mammalian cells grown in suspension culture and so reactor/impellor and air/oxygen sparging systems developed for producing mammalian cell-based products can be utilized for insect cell suspension cultures. The formulation of serum-free media used for insect cell cultures and feeds for fed batch processes are also similar to those used in mammalian cell processes. Indeed insect cell cultures can be easier to culture than mammalian cells in some respects in that they normally do not require pH control during batch or fed-batch runs as they tend not to produce the high lactate levels seen by many mammalian cells in culture.

Thus, many of the methods and protocols developed for animal cell biotechnology in general are applicable to insect cell culture processes and readers are encouraged to review other books in the "Methods in Molecular Biology" series relevant to Animal Cell Biotechnology [20]. Similarly books in this series focusing on Baculovirus and Insect Cell Expression Protocols [21] are directly relevant to the topic of producing insecticidal viruses in culture. The use of insect cells, particularly Sf9 and High-Five (*Trichoplusia ni*,T. ni), cell lines have been studied extensively in relation to their ability to produce various vaccine, therapeutic, and gene therapy products using baculovirus expression systems [22, 23], and this work has much to teach us about producing virus insecticides.

While large-scale culture of insect cells is not easy and requires the involvement of experienced animal cell technology personnel, virus yields from bioreactor insect cell cultures are comparable to yields obtained in small-scale insect cell shaker cultures [24]. The main challenges for cell culture-based production of insecticidal viruses relate to the development of appropriate cell lines, managing problems related to stability of the virus strains in culture, enhancing virus yields per cell through an understanding of how the host cell responds to the infecting virus, and the development of chemically defined media and feeds for the desired production systems [1, 2]. All of these issues can be studied using small-scale (20–100 ml) shaker/spinner flasks, particularly given the fact that many insect cells growing in suspension culture do not alter the pH of their growth medium and so do not require online pH control. Such simple suspension culture systems can operate to high cell densities (up to 10^7 cells/ml) without showing signs of oxygen limitations [24, 25] and so there is no need to go to cumbersome bioreactor systems with pH and oxygen control to perform many of the required basic studies.

Hence this chapter presents mainly the methods required to work with and analyze effectively insect cell systems using small-scale suspension cultures. Outlined are procedures for quantifying cells and virus and for establishing frozen cell and virus stocks. The approach for maintaining stock cultures in good condition and the multiplicity of infection (MOI) and time of infection (TOI) parameters that

should be considered for conducting infections are discussed. As baculoviruses are produced in two forms, budded virus (BV) and occlusion bodies (OB), methods for quantifying both these virus forms are shown. BV is the virus form produced when virions bud from an infected cell, in order to spread an infection via hemolymph from one caterpillar cell to another. The BV is also the form of the virus normally used to set up infections in cell culture. OBs are the form in which baculoviruses spread from 1 host insect to another and are stable in the outside environment due to the polyhedral protein coat surrounding the virus. This is the version of the virus that is used as an insecticide. OBs occlude what are referred to as ODV, occlusion-derived virus. ODV and BV are genetically identical but vary in the lipid and protein content of their membranes. For some viruses such as HearNPV, very poor BV titers are obtained from cell culture infections [26], and it is desirable to infect cells using ODVs and so the methodology to extract infectious ODV from OBs is also outlined.

As studies aimed at producing virus insecticides will eventually need to confirm that yields produced in shakers and spinner flasks can be reproduced in reactors, a basic method for growing insect cells in a stirred tank reactor (STR) and an airlift reactor (ALR) is included. Insect cells when infected by baculoviruses do become larger and develop a weakened cell membrane making them more susceptible to shear forces in a well-mixed bioreactor. Hence optimizing mixing conditions for large-scale cultures of infected insect cells may present some challenges beyond those met for mammalian cells. However, most current studies aimed at developing commercially viable processes involving insect cell cultures are based on systems biology approaches analyzing samples from small-scale suspension cultures [27–30].

2 Materials

2.1 Production of Insect Viruses in Suspension Cultures: Shake Flasks, Spinner Flasks, Stirred Tank, and Airlift Bioreactors

2.1.1 Cell Line and Virus

1. The HzAM1 (Hzea) cell line used was derived from the pupal ovarian tissue of *Helicoverpa zea* (Lepidoptera: Noctuidae) (*see* ref. 6). This cell line was obtained from CSIRO, Division of Entomology, Canberra, Australia at passage number 242.
2. *Spodoptera frugiperda* cell line Sf-21 (ATCC CRL 1711), saUFL-AG-286 cell line (*see* ref. 12).
3. Wild-type *Helicoverpa armigera* nucleopolyhedrovirus, HearNPV, was obtained as caterpillar occlusion bodies, strain H25EA1, an Australian isolate, from CSIRO (Entomology Division, Canberra, Australia), which was used to infect the HzAM1 cell line.
4. Wild-type *Anticarsia gemmatalis* multiple nucleopolyhedrosis virus (AgMNPV) (*see* ref. 31).

2.1.2 General Cell Culture Equipment and Consumables

1. Incubator maintained at 27–28 °C.
2. Biological Safety Cabinet (Class II certified).
3. Pipet gun (e.g., Pipet-Aid®, Drummond Scientific).
4. 1, 2, 5, 10, 25, and 50 ml plastic disposable sterile pipettes.
5. 96 well plates, tissue culture treated, flat bottomed.

2.1.3 Culture Medium and Supplements (See Note 1)

1. TC-100 insect basal medium with l-glutamine and sodium bicarbonate, liquid, sterile filtered (Sigma).
2. IPL-41: basal medium (e.g., Life Technologies).
3. Fetal bovine serum (FBS), supplement (Life Technologies).
4. Chemically Defined Lipid Concentrate, supplement (e.g., Life Technologies).
5. Yeastolate Ultrafiltrate 50×, supplement (e.g., Life Technologies).
6. Antifoam A, supplement (Sigma).
7. Pluronic F-68, supplement (Sigma).
8. Sf-900™ II: liquid, complete serum-free media (Life Technologies).
9. Sf-900™ III: liquid, complete serum-free media (Life Technologies).
10. Sodium dodecyl sulfate (SDS).

2.1.4 Bioreactors

1. Orbital shaker platform.
2. Shaker Flasks: Erlenmeyer flask with screw cap (autoclavable glass and/or disposable (e.g., polycarbonate)), 125 and 250 ml.
3. Spinner Flasks: 500 ml glass spinner flask with pendular magnetic agitator, mounted on magnetic stirrer base (Techne, UK).
4. Stirred Tank Reactor: 5 l Biostat® A glass fermentor (B. Braun Biotech, Melsungen, Germany).
5. Airlift Reactor: 1.0 l glass GAV-3 concentric tube airlift reactor.

2.1.5 Cell Counts/ Sampling

1. Manual cell counts: phase-contrast microscope (e.g., BX43 upright microscope, Olympus), improved-Neubauer hemocytometer, and 2-key manual cell counter (i.e., click counter).
2. Automated cell counts (e.g., Multisizer™ 4 Coulter Counter or ViCell® Cell Viability Analyzer, Beckman Coulter).
3. Diluent for cell counts (same medium as that used to propagate cells).
4. Trypan Blue 0.4% (w/v) solution.

2.1.6 Cell Cryopreservation Equipment

1. Cryogenic dewar (e.g., LD50, 50 l capacity, liquid nitrogen storage, Taylor-Wharton).
2. Ultralow temperature freezer (e.g., VIP™ MDF-U55V, –86 °C, Panasonic).

3. Freezing container (e.g., Mr Frosty™ Freezing Container, Thermo Fisher Scientific).
4. Cryogenic vials (e.g., 1 ml Nunc Cryotubes™, Thermo Fisher Scientific).
5. Cryogenic vial protection (e.g., Nunc Cryoflex™ Tube Wrap, Thermo Fisher Scientific).
6. Spirit lamp and scissor (hemostatic) clamps.
7. Isopropyl alcohol (≥99.7%).
8. Dimethyl sulfoxide (DMSO) (sterile-filtered, ≥99.7%, e.g., Hybri-Max™, Sigma-Aldrich) (*see* **Note 2**).

2.2 Production of Cells and Virus in Adherent Cell Cultures: T-Flasks, Roller Bottles, Microcarriers

2.2.1 Cell Line and Virus

1. The DSIR-HA-1179 coleopteran cell line originally was derived from embryonic tissues of the black beetle *Heteronychus arator* [16]. The cell line can be obtained from CAPE cell culture lab, University of Canterbury, New Zealand, where it is routinely maintained at 27 °C as adherent cultures (*see* ref. 32).
2. Wild-type *Oryctes* nudivirus (strain X2B) was produced in vitro in infected DSIR-HA-1179 cell cultures, harvested and stored at 4 °C. The X2B strain was originally isolated from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines in 1983 (*see* refs. 14, 15). OrNV stock may also be obtained from CAPE cell culture lab (*see* ref. 32).

2.2.2 Culture Medium, Solutions, and Microcarriers

1. TC-100 insect medium with l-glutamine and sodium bicarbonate, liquid, sterile filtered (Sigma) (*see* **Note 3**).
2. Fetal bovine serum (FBS) (Life Technologies).
3. TrypLE™ Express (Life Technologies).
4. Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma).
5. Cytodex-1 microcarriers (Sigma).
6. Trypan blue dye (0.4%) (Sigma).
7. Crystal violet lysis buffer: 0.9 g citric acid powder (Sigma) is dissolved in 100 ml distilled H_2O and mixed well. 0.01 g of crystal violet (Sigma) is added to the mixture and homogenously dispersed.

2.2.3 Bioreactors

1. T-Flasks: 25 and 75 cm^2 T-flasks with plug seal caps (Corning).
2. Roller Bottle System: (a) CELLROLL roller bottle system, consisting of two roller racks mounted with a drive unit (0.1–2 rpm) and connected to a control unit and drive supply (Integra Biosciences), (b) 490 cm^2 roller bottles with plug seal caps (Corning).
3. Spinner Flasks: 125 ml glass spinner flasks with pendular magnetic agitator, mounted on magnetic stirrer base (Techne).

3 Methods

3.1 Production of Insect Viruses in Suspension Cultures

As indicated in the Introduction, this section will concentrate on methods related to the production of the baculoviruses HearNPV and AgMNPV in suspension cultures (*see* **Note 4**).

3.1.1 Cell Density and Cell Viability Enumeration

Cell densities are usually determined via a counting chamber (Improved Neubauer hemocytometer) and a phase-contrast microscope, as follows:

1. Estimate the dilution required so that approximately 100 cells are counted on each side of the hemocytometer (each side being a counting grid with nine large squares, each being 1 mm in length and 0.1 mm in depth, with a cover slip).
2. Perform 1:1 serial dilutions of the culture sample with medium, but use 0.1 % (w/v) Trypan Blue (prepared in medium) as the diluent for the final dilution.
3. Load each side of the hemocytometer (with cover slip) with 10 μl of the diluted sample, and count the number of cells on each side with a click counter under the microscope (count unstained and stained cells separately).
4. Tally up the number of viable (unstained) and total cells (unstained + stained) from both sides of the hemocytometer (18 squares).
5. Calculate the viable and total cell density using the following formula: cell density (cells/ml) = (no. of cells in 18 squares × dilution factor)/0.0018 ml.
6. If the cell density is estimated from 3× hemocytometer counts (~600 cells counted from 54 large squares), then a relative error of approximately 15 % is obtained (*see* ref. 33).

Alternatively, viable and total cell densities may be determined using an automated imaging counting system, e.g., Cedex or ViCell (this can also determine the total cell density), and total cell densities may be determined using a Coulter Counter (e.g., Multisizer 4), according to the manufacturer's instructions. When using an imaging counting system, the analytical settings optimized for uninfected cells may not work well for infected cells (e.g., underestimated cell viabilities), in which case such settings should be reoptimized.

3.1.2 Cell Cryopreservation: Freezing/Thawing

Insect cells adapted to Serum-Free Media (SFM) can be frozen in liquid nitrogen for long-term storage using the following procedure, which works well for Sf-9, HzAM1, and Tn-5 cells in our laboratory. The general principle of animal cell cryopreservation is to freeze slowly and thaw quickly.

Freezing:

1. Prepare a 100 ml suspension culture, seeded at 5×10^5 cells/ml in a 250 ml shaker flask.
2. Prepare 15% (v/v) DMSO in fresh culture medium in a 10 ml centrifuge tube and store at 4 °C.
3. When the cells are at mid-exponential growth phase (e.g., 2×10^6 cells/ml, >95% viability, 24 h doubling time), aliquot 10 ml culture into a 10 ml centrifuge tube, centrifuge at $100 \times g$ for 5 min, transfer the supernatant into a new 10 ml tube and chill on ice (conditioned medium). Also chill the tube of 15% (v/v) DMSO in fresh medium on ice. Perform **step 3** at ~1 h before **step 5**.
4. Label 1 ml cryogenic vials with the designated freeze numbers.
5. Aliquot the remaining culture (90 ml) into 2× 50 ml centrifuge tubes, centrifuge at $100 \times g$ for 5 min, and discard the supernatant.
6. Resuspend the cell pellet in the required volume (9 ml in 10 ml tube) to obtain a cell density of 2×10^7 cells/ml (tenfold concentration), e.g., 4.5 ml ice-cold 15% (v/v) DMSO in fresh medium and approximately 4.5 ml ice-cold conditioned medium (use the pipette's graduation to estimate the final volume of 9 ml).
7. Aliquot 1 ml cell concentrate into each cryogenic vial, ensuring that the 10 ml tube is well mixed between each dispensing step, and chill on ice.
8. Insert each vial into a short length of Cryoflex™ Tube Wrap, and seal each end of the Cryoflex with the aid of a spirit lamp and scissor clamps.
9. Install the Cryoflexed vials in a 'Mr Frosty' freezing container filled with isopropyl alcohol, and place in an ultralow temperature freezer (−80 °C). The 'Mr Frosty' container ensures a slowed-down freezing rate of −1 °C/min.
10. On the next day, remove the vials from the 'Mr Frosty', install them on appropriate freezing canes, and store them under liquid nitrogen in a cryogenic dewar.

Thawing:

1. Remove a vial from the cryogenic dewar, and place it on dry ice for transport.
2. Rapidly thaw the frozen cells by placing the vial in a 28 °C water bath. Swirl the contents gently to speed up thawing.
3. In a BSC resuspend the thawed cells and transfer into a 10 ml centrifuge tube and add 5 ml fresh SFM.

4. Centrifuge the tube at 100 × *g* for 5 min, discard the supernatant, and resuspend the cell pellet in fresh SFM to a final volume of 25 ml (~8×10^5 cells/ml) in a 125 ml shaker flask (passage 1 after thaw).
5. Incubate the cells and monitor cell growth, viability, and sterility daily. Maintain using the cell passaging routine as described in Subheading 3.1.4 (*see* **Note 5**).

3.1.3 Aseptic Technique in the Biological Safety Cabinet (BSC)

1. Aseptic technique is critical for insect cell cultures as the risk of microbial contamination is high due to the richness of the growth media and the slow growth rate of insect cells in comparison to that of most microbes (24 h vs. ≤1 h doubling times). Insect cell culture experiments, uninfected or baculovirus infected, can take up to 2–3 weeks to complete depending on scale and mode (batch or fed batch).
2. The sterility of insect cell cultures is best managed inside a Class II BSC and by using presterilized single-use plastic accessories (e.g., culture flasks, serological pipettes, filters, bottles, and centrifuge tubes) as much as possible. If reusable items are employed, then these should be well cleaned, depyrogenized, and autoclave sterilized.
3. Before working in a BSC, the cabinet bench and any items placed on it should be surface sanitized to reduce the microbial load [e.g., by wiping with paper towels soaked in 70% (v/v) ethanol].
4. The laminar flow should be started at least 30 min prior to work.
5. When performing liquid handling procedures in a BSC, a high degree of attention to detail is required to maintain asepsis: (a) HEPA-filtered sterile air flows from the top to the bottom of the cabinet; thus, a sterile item (liquid or solid) remains sterile if care is taken not to pass a nonsterile item above it and physical contact between the sterile item and any nonsterile entity is avoided (solids, liquids, gases, or aerosols). (b) Good aseptic technique includes having a noncluttered work area, leaving sufficient empty space between sterile objects, and a spatial memory of where the sterile and nonsterile items are situated.
6. The BSC should be decontaminated and NATA tested annually to maintain optimal performance.

3.1.4 Culture of HearNPV in Shaker Flasks

Insect cell suspension cultures are initiated from cryopreserved stock cells. Once thawed, the cells are serially passaged regularly (e.g., twice weekly) in fresh Serum-Free Media (SFM) as Erlenmeyer shaker flask batch cultures. The following cell passaging procedure is suitable for Hzea cells grown in an optimized SFM such as

Sf-900 III. Small-scale 125 ml shaker flasks (20–50 ml working volume) are used to save on medium costs. If larger volumes are required, then 250 ml shaker flasks (50–100 ml working volume) can be used.

1. Hzea stock cells are ready for passaging when they have reached the mid-exponential growth phase (medium dependent). Hzea cultures grown in Sf-900 III reach a Peak Cell Density (PCD) of 8×10^6 cells/ml or better in our hands with a mid-exponential density of around 4×10^6 cells/ml with a cell viability of >95% (*see* **Note 6**).
2. On the passaging day set up a new 25 ml culture (in a 125 ml flask) at a seeding density of $4–5 \times 10^5$ cells/ml, which represents an approximate tenfold dilution of the stock cells with fresh Sf900III (e.g., 2.5 ml cells + 22.5 ml SFM).
3. Install the flask on an orbital shaker platform (120 rpm) in a refrigerated incubator (27–28 °C). Ensure that the screw cap is loosened (e.g., quarter turn anticlockwise) to allow for gas exchange.
4. Allow the new stock cells to reach mid-exponential growth phase and then repeat the cell passaging procedure.
5. For a twice-weekly passaging routine, the following schedule works well for us (assuming a 24 h cell doubling time): *Monday*: Set up stock cells at $2.5–3 \times 10^5$ cells/ml, *Friday*: Stock cells grown to $4–5 \times 10^6$ cells/ml (Passage N), set up new stock cells at 4×10^5 cells/ml, *Monday*: Stock cells grown to $3–4 \times 10^6$ cells/ml (Passage N + 1), set up new stock cells again at $2.5–3 \times 10^5$ cells/ml, and so forth.
6. The procedure used to setup experimental batch cultures is the same as that used for serial passaging of stock cells. The same agitation speed can be applied for both 125 ml and 250 ml shaker flasks (120 rpm).
7. If the batch cultures are to be infected with a baculovirus, then the settings of certain key infection parameters have to be optimized, including the infection cell density (ICD), the multiplicity of infection (MOI), and the Peak Cell Density (PCD).
8. For the production of HearNPV occlusion bodies (OBs) using Hzea cells in Sf900III, typical settings for ICD and MOI are $2–4 \times 10^6$ cells/ml and 5–10 PFU/cell, respectively. Maximum volumetric yields are obtained by infected cultures that reach PCDs of $3–4 \times 10^6$ cells/ml (*see* **Note 7**).

3.1.5 ODV Extraction to Initiate HearNPV Infections in Suspension Culture

Baculoviruses are genetically unstable when passaged in culture due to the generation of defective interfering particles, DIPs (*see* ref. 34), and the rapid accumulation of few polyhedral, FP, mutants (*see* ref. 35). Normally to infect cells in culture with new wild-type

baculovirus isolates, infected caterpillars are harvested in nature, and extracted hemolymph is diluted with media, filter sterilized and the BV so obtained are used to infect cells in culture. However, this approach makes it difficult to establish master and working stocks of virus in sufficient volumes to support a large-scale manufacturing process, particularly if the BV yields in culture are low as is the case for HearNPV produced in culture (*see* ref. 26).

One solution to this problem is to maintain master and working stocks of baculoviruses as caterpillar generated OBs and extract the ODVs from the OBs to set up a BV stock in culture. However, ODV have been reported as only infecting cells in static cultures very poorly (*see* ref. 36). For HearNPV, we have found that ODVs extracted from OB infect Hzea cells in suspension culture efficiently and a commercially viable process is feasible that relies on the use of master and working stocks of OBs stored at 4 °C or frozen at −80 °C (*see* ref. 1). A method to extract ODVs for infecting cells in suspension culture is outlined as follows.

1. Take 500 µl of an OB stock generated in caterpillars and stored at a concentration of 10^{10} OB/ml in water or media at 4 °C or frozen at −80 °C.
2. Add 40 µl of an alkali solution (0.5 M Na_2CO_3 and 1.0 M NaCl) to the OBs in an eppendorf tube, vortex and incubate for 30 min at 28 °C.
3. Mix the digested OBs with 10 ml of Sf900III medium to neutralize the extract.
4. In a sterile cabinet use a 10 ml sterile syringe to suck up the ODV solution and filter it through a sterile 0.22 µm filter (Durapore® PVDF, Sartorius, Australia) into a sterile 10 ml tube (*see* **Note 8**).
5. Add this ODV extract (~9.5 ml) to a 90 ml culture (250 ml shaker flask) such that the final cell density at the time of infection is 5×10^5 cells/ml.
6. Incubate the infected culture at 28 °C and 120 rpm for 4 days to produce a P1 virus stock.
7. Use the P1 virus to immediately generate a P2, BV stock, by adding the P2 whole culture, 30% (v/v), to cells such that the final cell density at the time of infection is 1×10^6 cells/ml.
8. At 3 days postinfection harvest the P2, BV supernatant by centrifugation, $1000 \times g$ for 10 min at room temperature and store for 2–4 weeks at 4 °C or at −80 °C if to be stored for longer periods.
9. The P2, BV virus stock produced in this manner will typically have a titer of $2–5 \times 10^7$ PFU/ml (*see* **Note 9**).

10. All experiments aimed at optimizing yields ideally will be done using P2 virus stocks to initiate infections as excessive passaging of virus in culture can significantly affect the OB/cell yield (*see* ref. 35). A commercially viable process is feasible taking this approach whereby the virus will only be in cell culture for a total of three passages, counting the final fed batch production run (*see* ref. 1).

3.1.6 OB Count

1. Extract OB for counting by mixing an equal volume of 1% SDS to 0.2 ml of an infected cell suspension (*see* **Note 10**).
2. Incubate at 28 °C for 30 min to allow dissolution of the cell membrane and release of the polyhedra.
3. Dilute the OB extract serially with pure water to allow a count of 100–150 OB per small square of a hemocytometer.
4. A 0.1-mm-deep Improved Neubauer hemocytometer (Weber Scientific International Ltd., England) which consists of two chambers each divided into nine 1 mm squares is to be used for counts. The central square is further divided into 25 smaller squares which is the square used for this particular count. The volume of each 1 mm square, including the central square is equal to 10^{-4} ml.
5. Clean the counting chamber and cover-slip with 75% ethanol, dry with a paper wipe, and fix cover-slip in position.
6. A total of 5 small squares (out of the 25 smaller squares within the central square) were counted on each side of the hemocytometer (count OBs in the four corner plus the central small square). Each sample was counted using three hemocytometers, both sides, using an optical microscope (Olympus, Japan) at 400× magnification (including 10× eyepieces and 40× objective lens). Diluted samples were counted 10 min after loading to allow the polyhedra to settle on the base of the hemocytometer.
7. Each count consists of a tally of the number of occlusion bodies completely contained within a small square plus the number touching the left-hand and upper sides whereas occlusion bodies touching the bottom and right-hand sides are not counted.
8. The polyhedra concentration was then determined as follows (per side of each hemocytometer count:

$$\mathrm{OB}/\mathrm{ml}(\text{one side}) = \text{number counted in 5 small squares} \times 5 \times \text{dilution factor} \times 10^4.$$

9. The volumetric OB yield of each sample was the average of the OB/ml count from each side (six sides in total) of the hemocytometer.
10. A count conducted is this manner counts a minimum of 600 OBs and results in an acceptable random error of 15% (*see* ref. 33).

11. The cell-specific yield (polyhedra per cell) is obtained by dividing the volumetric yield (OB per ml) by the total peak cell density (*see* **Note 11**).

3.1.7 Culture of AgMNPV in Spinner Flask Bioreactors

1. Sterilize a 500 ml spinner flask and place it into the laminar flow cabinet.
2. Prepare the culture medium by adding fetal bovine serum to the TC-100 culture medium at a concentration of 10%.
3. Prewarm the culture medium in the incubator at 27 °C and aseptically transfer 50 ml of culture medium into the spinner flask.
4. Inoculate the spinner flask with an appropriate volume of IPLB-Sf-21 cells inoculum to give an initial cell density of 2×10^5 viable cells/ml.
5. Transfer the spinner flask to the magnetic stirrer base held within the incubator and adjust the speed of the magnetic stirrer to 60 rpm.
6. Withdraw 1 ml samples of the cell suspension in a BSC with a 1 ml pipette at regular intervals in order to assess viable cell density and culture viability.
7. Infect the culture, for example, during the early exponential growth phase by adding an appropriate volume of virus inoculum stock to obtain the desired MOI. Return the spinner flask to the incubator until 150 h postinfection when peak virus yields are obtained. Cell density at the moment of the infection, multiplicity of infection (MOI), and harvesting time could change according to experimental objectives (*see* ref. 31).
8. To assess budded virus titer, aseptically transfer 1 ml of an infected sample from the reactor into an Eppendorf tube, and centrifuge it at $4000 \times g$ for 10 min. The clear supernatant contains the budded virus progeny, which can either be stored at 4 °C or directly quantified according to the method in Subheading 3.2.6.
9. Polyhedra yields can be determined as described in Subheading 3.1.6.

3.1.8 Culture of HearNPV in Stirred Tank Bioreactors

1. The 5 l bioreactor was prepared for cell culture by cleaning and depyrogenization using 10 g/L Terg-a-zyme® detergent (Alconox, White Plains, NY) and 0.1 M NaOH, respectively (overnight soaking), followed by rinsing with deionized water, assembly and autoclave sterilization for 45 min (121 °C, 100 kPa). Hydrophobic filters (0.2 μm Millex FG-50, Millipore) were installed for gas addition or venting.
2. The bioreactor was operated at an agitation speed of 160–220 rpm, to account for changes in liquid volume during fed-batch processes, while maintaining a constant ungassed power/volume (P/V) ratio of 16.3 W m^{-3}. The power consumption

was estimated by using a well-established correlation with agitation speed (*see* ref. 37) and a power number of 1.7 for pitched blade 'elephant ear' impellers (*see* ref. 38).

3. Bioreactor cultures were maintained at a temperature set point of 28 °C via the micro DCU-400 control system (B. Braun Biotech).
4. The dissolved oxygen tension (DOT) was controlled at 50 % of air saturation using the Wheaton Control Tower® (Wheaton Science Products, Millville, NJ) with pure O_2 sparging. In addition, a low flowrate of air was introduced into the headspace gas inlet.
5. The liquid addition/withdrawal device was either a glass bottle or a fernbach flask, adapted with a vented screw-cap lid (fitted with a 0.2 μm hydrophobic filter) and a glass spigot at the base (fitted with a length of silicone tubing and ending with a polypropylene Y-piece connector). Similar tube /Y-piece assemblies were also fitted to the bioreactor's addition/withdrawal ports. During autoclaving, the tubes were sealed using gate clamps just upstream from the Y-piece. Two lengths of tubing were connected aseptically by fitting one arm of each Y-piece together with tubing and connecting the other arm to either a live steam source or a steam trap. The Y-piece assembly was then steamed for 30 min (180 kPa supply pressure), then the steam and condensate lines were clamped, and the connection was cooled prior to use.

3.1.9 Culture of AgMNPV in Airlift Bioreactors

1. Sterilize a 1.0 L glass concentric airlift reactor and place it into the laminar flow cabinet.
2. Fill it partially (~50 % of its working volume) with prewarmed at 27 °C TC-100 culture medium supplemented with 10 % fetal bovine serum, 200 ppm silicone antifoam and 0.20 % w/v of Pluronic F-68.
3. Inoculate the reactor with an appropriate volume of IPLB-Sf-21 cells inoculum grown in a spinner flask to give an initial cell density of 2×10^5 viable cells/ml.
4. Complete reactor working volume by adding culture medium as described in Subheading 3.1.9 **step 2** making sure its level goes over the reactor's riser (internal draft tube).
5. Check outlet of reactor is open and protected by a 0.2 μm air filter.
6. Connect the reactor to the air supply source and sparge air filtered with 0.2 μm filter into the airlift reactor at a superficial gas velocity, J_G, ranging from 0.09 to 0.1 cm/min.
7. Withdraw 1 ml samples of the cell suspension. The system used for this task varies according to the airlift reactor design. Usually, a sample is collected from a secondary port which has a vial

attached by exerting a slightly positive pressure inside the reactor (or negative pressure from the vial). Once the sample is taken, the vial is replaced with a new clean one for the next sample.

8. Infect the culture, for example, during the early exponential growth phase by adding an appropriate volume of virus inoculum stock to obtain the desired MOI. Cell density at the moment of the infection, multiplicity of infection (MOI), and harvesting time could change according to experimental design and objectives (*see* refs. 12, 39).
9. To assess budded virus titer, aseptically transfer 1 ml of infected sample from the reactor into an Eppendorf tube, and centrifuge it at 4000 × *g* for 10 min. The clear supernatant contains the budded virus progeny, which can either be stored at 4 °C or directly quantified according to the method in Subheading 3.2.6.
10. Polyhedra yields can be determined as described in Subheading 3.1.6.

3.2 Production of Insect Viruses Using Adherent Cultures: DSIR-HA-1179-OrNV System as an Example

3.2.1 Preparation of Culture Medium

1. Ensure that the culture medium is free of adventitious agents. It must always be stored in sterile, capped bottles. Glassware must be soaked overnight in a solution of 1% Virkon, rinsed well with MilliQ water and autoclaved (121 °C for 20 min) prior to use.
2. The DSIR-HA-1179 cell line requires 10% FBS for optimal growth. Therefore, prepare appropriate volumes of TC-100 culture medium supplemented with 10% FBS (*see* **Note 12**).
3. Prepare culture medium ahead of use. Incubate it at 27 °C for 24 h to check for microbial contamination prior to use.

3.2.2 Preparation of Cell Inoculum

1. Prepare cell inoculum to seed bioreactors from T-flask cultures in which the cell monolayer is 80–90% confluent.
2. Pipette out spent culture medium from the T-flask and discard.
3. Add 2 ml of D-PBS free of calcium and magnesium per 25 cm^2 of flask surface area. Rock the flask gently to evenly coat the surface for 2 min. Pipette out the spent D-PBS and discard.
4. Add 1 ml of TrypLE™ Express (prewarmed to 27 °C) per 25 cm^2 of flask surface area. Rock the flask gently to evenly coat the surface. Transfer flask to the incubator and incubate for 30 min at 27 °C.
5. Observe culture under microscope to confirm cells have detached. Add an appropriate volume of prewarmed culture medium to the flask and pipette gently to break up any cell clumps and to create a homogenous single cell suspension (*see* **Note 13**).
6. Transfer 1 ml of the cell suspension to a 1.5 ml microcentrifuge tube to make a cell count. A sample of the cell suspension is

stained with trypan blue and loaded on the hemocytometer. Both total and viable cells are counted in duplicate in order to estimate viable cell density and culture viability (*see* refs. 40, 41).

7. Based on viable cell count, use the appropriate volume of culture to inoculate the bioreactor.

3.2.3 OrNV Production in T-Flasks

1. Inoculate a 25 cm^2 T-flask at an initial DSIR-HA-1179 cell density of 2×10^5 viable cells/ml in a culture volume of 5 ml (*see* **Note 14**).
2. Incubate the culture at 27 °C until early exponential growth phase (cell density of ~5×10^5 viable cells/ml).
3. Infect the culture by adding to it the appropriate volume of OrNV stock to achieve the desired MOI (*see* **Note 15**).
4. Incubate the infected culture at 27 °C until day 6 postinfection (*see* **Note 16**).
5. In order to harvest virus, transfer the entire contents of the infected culture into a 15 ml centrifuge tube and centrifuge at 4000 × *g* for 10 min (*see* ref. 32).
6. Aseptically transfer the supernatant (containing virus) to a fresh, sterile centrifuge tube and store at 4 °C.

3.2.4 OrNV Production in the Roller Bottle System

1. Add 25 ml of fresh culture medium to the roller bottle and roll at 0.1 rpm for 24 h in the incubator at 27 °C to precondition the surface of the roller bottle (*see* **Note 17**).
2. Inoculate the roller bottle with an appropriate volume of cell inoculum to obtain a cell density ~4×10^4 cells/cm^2. Incubate roller bottle at 0.1 rpm for a further 24 h. The inoculation is done in a reduced culture volume (25 ml) in order to facilitate better adhesion of the cells to the roller bottle surface and to form an even cell monolayer (*see* ref. 42).
3. Adjust the final culture volume to 60 ml by adding fresh culture medium to the roller bottle and continue incubation under the same conditions.
4. Cell growth is assessed by harvesting the full content of the roller bottle. It involves the dissociation of the cell monolayer with TrypLE™ Express enzyme using a slightly modified method to that described in Subheading 3.2.2, as follows: Pipette the spent culture medium out of the roller bottle and add 20 ml of D-PBS free of calcium and magnesium to the cell monolayer. Return roller bottle to incubator and roll at 0.1 rpm for 10 min. At the end of this period, remove the spent D-PBS and add 20 ml of TrypLE™ Express to the roller bottle. Return the bottle to the incubator and roll at 0.1 rpm for 30 min until cells have detached from the monolayer. Add an appropriate volume of prewarmed culture medium supplemented with 10%

FBS, and gently aspirate the cell suspension with a 25 ml pipette to break up any cell aggregates. Aseptically transfer a 1 ml sample of the cell suspension into a microcentrifuge tube and estimate cell density and culture viability.

5. Infect the roller bottle culture with OrNV at, for example, the early exponential growth phase (approximately 5×10^5 viable cells/ml) by adding an appropriate volume of virus inoculum stock to obtain the desired MOI. Return the roller bottle to the incubator and roll at 0.1 rpm until day 8 postinfection.
6. Harvest virus from the infected roller bottle culture on day 8 postinfection as peak virus yields are reached at this time point (*see* ref. 43). Gently transfer the entire content (60 ml) of the infected roller bottle culture and aliquot into equal volumes of 15 ml in four 15 ml centrifuge tubes. Centrifuge the tubes at $4000 \times g$ for 10 min. Aseptically transfer out the supernatant containing the virus into new sterile tubes and store at 4 °C.

3.2.5 OrNV Production in Microcarriers

1. Prepare a Cytodex-1 microcarrier stock solution of 10 g/l. Weigh 1 g of dry Cytodex-1 microcarriers and add it to 100 ml of D-PBS free of calcium and magnesium in a Schott-duran bottle. Gently swirl the mixture for 5 min to evenly disperse the microcarriers. Incubate the mixture at 27 °C for 5 h to hydrate the microcarriers.
2. Decant the supernatant and wash the microcarriers twice, with two changes of 50 ml of fresh D-PBS free of calcium and magnesium.
3. Autoclave the microcarriers in 50 ml of fresh D-PBS at 121 °C and 15 psi for 20 min. Store at 4 °C until time of use (*see* refs. 44, 45).
4. Transfer to the laminar cabinet and decant the D-PBS solution from the bottle. Wash the microcarriers with two exchanges of 50 ml serum-free TC-100 culture medium, prewarmed to 27 °C. Finally, resuspend microcarriers in 10 % FBS-supplemented TC-100 culture medium.
5. For the DSIR-HA-1179 cell line, a procedure of initially inoculating cells on microcarriers on a flat surface of a 75 cm^2 T-flask in a reduced culture volume of 20 ml is used, followed by a 12 h period under static conditions, which has been found to improve cell attachment. Following this, the culture is transferred to a spinner flask with a final culture volume of 60 ml.
6. Hence: Transfer an appropriate volume of microcarriers to give a concentration of 1 g/l for a final culture volume of 60 ml into a 75 cm^2 T-flask. Adjust the volume of the flask to 20 ml by adding fresh culture medium. Inoculate accordingly to obtain 30 cells per bead (microcarrier). Incubate the culture at 27 °C for 12 h under static conditions (*see* **Note 18**).

7. At the end of the 12 h attachment period, microscopically observe cultures to check that all cells have attached to microcarrier beads. Aseptically transfer the 20 ml culture out of the T-flask and into a sterile 125 ml spinner flask, and adjust the final culture volume by adding 40 ml of prewarmed culture medium.
8. Place the spinner flask onto the stirrer base within a 27 °C incubator. Adjust the magnetic stirrer speed to 40 rpm.
9. Total cell density in microcarrier cultures is evaluated at periodic intervals over batch growth by nuclei counting (*see* refs. 46, 47). Aseptically remove 1 ml samples of microcarrier culture into 1.5 ml microcentrifuge tubes and centrifuge the tubes at 12,000 × *g* for 5 min to separate cell-bound microcarriers from the culture supernatant. Remove the culture supernatant and add 1 ml of crystal violet lysis buffer to the cell-microcarrier pellet. Vortex the mixture for 1 min, and then incubate it overnight at 27 °C. Citric acid does not affect the microcarriers, and only lyses the cells' plasma and nuclear membranes to release their nuclei, which are stained by crystal violet. The individually stained nuclei are counted in duplicate using a Neubauer hemocytometer in order to assess total cell density (*see* ref. 40).
10. Infect the microcarrier culture with OrNV in the early exponential growth phase by adding an appropriate volume of virus inoculum stock to obtain the desired MOI. Return the spinner flask to the incubator until day 4 postinfection when peak virus yields are obtained.
11. To harvest the virus, aseptically transfer the entire 60 ml infected culture as 15 ml aliquots in four centrifuge tubes and centrifuge at 4000 × *g* for 10 min.
12. The cells and microcarriers will have pelleted at the bottom of each tube leaving a clear supernatant fluid containing the virus. Aseptically transfer the viral supernatant out into new sterile tubes and store at 4 °C.

3.2.6 Quantification of Infectious Virus Titer by Endpoint Dilution (See Note 19)

1. Prepare a single cell suspension of DSIR-HA-1179 cells from a parent T-flask culture using TrypLE™ Express treatment, as described in Subheading 3.2.2.
2. Dilute the culture with fresh culture medium to a concentration of 2.5×10^5 viable cells/ml.
3. Inoculate the wells in each of 5 columns of a 96-well plate with 50 µl of the cell suspension per well (*see* **Note 20**).
4. Set up a tenfold dilution series (10^{-1}–10^{-9}) of the virus sample in culture medium in microcentrifuge tubes.
5. From each dilution of viral supernatant, add 50 µl of the respective supernatant to each of five replicate wells in the 96-well plate.

6. Place the plates in a humidified, disinfected plastic container and incubate at 27 °C for 11–14 days until the cytopathic effect is well developed (*see* **Note 16**), and the plates can be reliably scored for infection. The incubation length to assess cytopathic effect changes with the insect cell line and virus used.
7. The $TCID_{50}$ value is calculated according to the method of Reed and Muench (*see* ref. 48).

4 Notes

1. The culture media described in Subheading 2.1.3 were used to culture the cell lines described in the section; however, the methodology applied is of general use for any insect cell line to be cultivated under the same conditions.
2. Dimethyl sulfoxide (DMSO) may be sourced from a different supplier.
3. Alternatively TC-100 dry powder medium (Sigma or Invitrogen) may be purchased and prepared for use according to the manufacturer's instructions.
4. While a case was made in the introduction that the current challenges for producing baculovirus insecticides in culture (yield/media/feed improvements) can be addressed at small scale—the small-scale work referred to is 20–100 ml suspension shaker/spinner cultures. Research in static cultures is not as relevant to large-scale suspension processes. Static cultures are not mixed and oxygen limitation becomes an issue once cells get above $1–2 \times 10^6$ cells/ml. In addition, adherent cells most likely have a different protein expression profile for many proteins compared to that for cells in suspension and it is very difficult to accurately quantify cells in static cultures leading to inaccurate quantification of cell-specific virus yields.
5. Cryopreserved cells should have recovered (i.e., normal cell growth rate and viability) by 2–3 passages after thaw. If not, then discard the cells and thaw out another vial from the cell bank. If there is a low success rate of recovering cells from a particular cryopreserved batch, then the freezing procedure may not have been carried out properly. In this case, repeat the cryopreservation procedure with a new batch of cells.
6. Insect cells will obviously exhibit different growth characteristics depending on the medium used. One of the most important initial tasks in insect cell culture is to establish a cell growth curve (cell density and viability over time) for a particular medium, from which the cell doubling time, mid-exponential growth phase, and PCD can be determined. This information is used to set the cell density and temporal parameters for passaging events.

7. Peak cell-specific yields of HearNPV of 400–500 OB/cell are obtained only for infections at $0.5–1 \times 10^6$ cells/ml. The cause of the drop off in peak cell specific yields seen for infections conducted at higher cell densities is due to the so-called cell density effect (*see* ref. 49). Cell-specific and volumetric yields at higher cell densities can be improved by the use of fed-batch processes (*see* refs. 24, 25). It is also possible to develop low cost media that give similar yields to those obtained with commercial media such as Sf900III (*see* refs. 50, 51). Low cost media for insect cells are typically based on the basal medium formulation of IPL41, plus a lipid emulsion additive containing cholesterol and a yeast extract. Other hydrolysates are often required if the addition of expensive purified amino acids is to be avoided (*see* ref. 50). Feeds for insect cells are based on concentrates of the ingredients used in the media. Quality assurance is a significant problem in producing low cost media if they are to perform in a reproducible manner, due to the challenges of producing good quality lipid emulsions and variability in the quality of the hydrolysates and the yeast extracts used.
8. Typically it requires two disposable filters to sterilize the 10 ml ODV extract.
9. The P2, BV titer can be determined using a plaque assay, an endpoint titration assay (*see* Subheading 3.2.6), or by a relatively simple suspension culture-based assay (*see* ref. 52). BV can be stored at 4 °C for short periods (up to 1–3 months) but for longer term storage they should be frozen quickly and stored at −80°°C or in liquid nitrogen (*see* ref. 53). BV should not be stored at −20 °C as at this temperature the BV freeze too slowly and are damaged (*see* ref. 53). Long-term storage at 4°C leads to loss of BV activity due to clumping of the virus according to an excellent study by Jorio et al. (*see* ref. 53). The work by Jorio et al. was done with a rAcMNPV virus but we believe BV clumping is even more problematic for HearNPV BV. Due to the study of Jorio et al we also store OB at 4 or −80 °C. OB are stable for years at 4 °C but they are nonsterile and they also clump after storage for 2 years or more. If glycerol is added to help preserve them, then they are very hard to quantify and remove from the glycerol after a few years storage and tend to clump even more.
10. HearNPV OB counts are best done with cultures harvested at 6–7 days postinfection (dpi). For synchronous infections conducted at a high MOI of 3–5 PFU/cell, OB yields typically peak by 3–4 dpi, but the OBs continue to mature and increase in size and are easier to count if harvested at 6–7 dpi.
11. OB counts are difficult to determine accurately and only by counting a large number will a reasonably accurate value be

obtained. Even then it can be subjective at times to differentiate OBs from other cellular debris of a similar size. For this reason, we also quantify OB yields via SDS gels using densitometry of the polyhedra band. When high OB/ml yields are obtained, the resulting SDS gels show a very clear large polyhedra band that is quite distinct from other protein bands on the gel.

12. While work without antibiotics is recommended, antibiotics may be supplemented to the culture medium [for example, gentamycin (Sigma) at 50 μg/ml] to maintain asepsis.
13. It is important that the culture medium used for resuspension contains 10% FBS, as FBS acts as a protease inhibitor against the action of TrypLE™ Express on cells.
14. For 75 cm^2 T-flasks, final culture volume is 15 ml.
15. In general, if infections are carried out for the purpose of producing working stocks infect cultures at a low MOI (i.e., MOI 0.1) to reduce the likelihood of formation of defective interfering particles. Experimental/production cultures may be infected at MOIs > 5 to ensure synchronous infection. Cytopathic effect will appear earlier in synchronous infected cultures.
16. Observe infected cultures under the microscope for the typical OrNV cytopathic effect which includes cellular hypertrophy, rounding up of infected cells, and appearance of small 'vesicle-like' structures around infected cells.
17. The need for a preconditioning period is likely to be dictated by the attachment characteristics of the individual cell line as well as the substrate material. A similar procedure for Sf-21 cells in glass roller bottles is described by Vaughn et al. (*see* ref. 42).
18. An alternative is to directly inoculate cells on microcarriers within a reduced culture medium volume of 20 ml in a spinner flask, with intermittent stirring of the culture (i.e., 3 min every 30 min) at 40 rpm for the first 12 h of culture. Following this, the culture volume is adjusted to 60 ml with fresh medium and the culture is continuously stirred at 40 rpm for the rest of the batch growth period. This method produces 91% of cell attachment to microcarriers within the first 12 h of culture.
19. OrNV does not form plaques with DSIR-HA-1179 cells, therefore the method used so far for quantification of infectious virus titer has been the $TCID_{50}$ assay based on the 50% endpoint dilution technique (*see* ref. 48).
20. The minimum number of replicates to be used with this method is 3. Accuracy of the method improves with the number of replicates. Only a noneven number of replicates can be used.

References

1. Reid S, Chan LCL, Van Oers MM (2014) Production of entomopathoginic viruses. In: Morales-Ramos JA, Rojas MG, Shapiro-Ilan DI (eds) Mass production of beneficial organisms Invertebrates and entomopathogens. Elsevier, Amsterdam. ISBN 978-0-12-391453-8
2. Harrison R, Hoover K (2012) Baculoviruses and other occluded insect viruses. In: Vega F, Kaya H (eds) Insect pathology, 2nd edn. Elsevier, Amsterdam, pp 73–131
3. Buerger P, Hauxwell C, Murray D (2007) Nucleopolyhedrovirus introduction in Australia. Virol Sin 22:173–179
4. Ignoffo CM (1973) Development of a viral insecticide – concept to commercialization. Exp Parasitol 33:380–406
5. Black BC, Brennan LA, Dierks PM, Gard IE (1997) Commercialisation of baculoviral insecticides. In: Miller LK (ed) The baculoviruses. Plenum, New York, NY, pp 341–388
6. McIntosh AH, Ignoffo CM (1981) Replication and infectivity of the single-embedded nuclear polyhedrosis-virus, Baculovirus-heliothis, in homologous cell-lines. J Invertebr Pathol 37:258–264
7. Lua LHL, Reid S (2000) Virus morphogenesis of *Helicoverpa armigera* nucleopolyhedrovirus in *Helicoverpa zea* serum-free suspension culture. J Gen Virol 81:2531–2543
8. Nguyen Q, Qi YM, Wu Y, Chan LCL, Nielsen LK, Reid S (2011) In vitro production of *Helicoverpa baculovirus* biopesticides—automated selection of insect cell clones for manufacturing and systems biology studies. J Virol Methods 175:197–205
9. Pedrini MRS, Reid S, Nielsen L, Chan LCL (2011) Kinetic characterization of the Group II *Helicoverpa armigera* nucleopolyhedrovirus propagated in suspension cell cultures: Implications for development of a biopesticides production process. Biotechnol Prog 27:614–624
10. Mena JA, Kamen AA (2011) Insect cell technology is a versatile and robust vaccine manufacturing platform. Expert Rev Vaccines 10:1063–1081
11. Almeida AF, Macedo GR, Chan LCL, Pedrini MRS (2010) Kinetic analysis of in vitro production of wild-type *Spodoptera frugiperda* nucleopolyhedrovirus. Braz Arch Biol Technol 53:285–291
12. Micheloud GA, Gioria VV, Eberhardtb I, Visnovsky G, Claus J (2011) Production of the *Anticarsia gemmatalis* multiple nucleopolyhedrovirus in serum-free suspension cultures of the saUFL-AG-286 cell line in stirred reactor and airlift reactor. J Virol Methods 178:106–116
13. Micheloud GA, Gioria VV, Perez G, Claus JD (2009) Production of occlusion bodies of *Anticarsia gemmatalis* multiple nucleopolyhedrovirus in serum-free suspension cultures of the saUFL-AG-286 cell line: influence of infection conditions and statistical optimization. J Virol Methods 162:258–266
14. Crawford AM, Zelazny B, Alfiler RA (1986) Genotypic variation in geographical isolates of *Oryctes baculovirus*. J Gen Virol 67:949–952
15. Zelazny B, Lolong A, Crawford AM (1990) Introduction and field comparison of baculovirus strains against *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) in the Maldives. Environ Entomol 19:1115–1121
16. Crawford AM (1982) A coleopteran cell line derived from *Heteronychus arator* (Coleoptera: Scarabaeidae). In Vitro 18:813–816
17. Jackson TA, Crawford AM, Glare TR (2005) *Oryctes virus*—time for a new look at a useful biocontrol agent. J Invertebr Pathol 89:91–94
18. Bedford GO (2014) Advances in the control of rhinoceros beetle, *Oryctes rhinoceros*. In oil palm—review article. J Oil Palm Res 26:183–194
19. Manjeri GR, Muhamad R, Tan SG (2014) *Oryctes rhinoceros* beetles, an oil palm pest in Malaysia. Ann Res Rev Biol 4:3429–3439
20. Portner R (2014) Animal cell biotechnology, 3rd edn. Humana, New York, NY. ISBN 978-1-62703-732-7
21. Murhammer DW (2007) Baculovirus and insect cell expression protocols, 2nd edn. Humana, Totowa, NJ. ISBN 978-1-59745-457-5
22. Drugmand J-C, Schneider Y-J, Agathos SN (2012) Insect cells as factories for biomanufacturing. Biotechnol Adv 30:1140–1157
23. Felberbaum RS (2015) The baculovirus expression vector system: a commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10:702–714
24. Meghrous J, Mahmoud W, Jacob D, Chubet R, Cox M, Kamen AA (2010) Development of a simple and high-yielding fed-batch process for the production of influenza vaccines. Vaccine 28:309–316
25. Chan LCL, Greenfield PF, Reid S (1998) Optimising fed-batch production of recombinant proteins using the baculovirus expression vector system. Biotech Bioeng 59:178–188
26. Matindoost L, Hu H, Chan LCL, Nielsen LK, Reid S (2014) The effect of cell line, phylogenetics and medium on baculovirus budded virus yield and quality. Arch Virol 159:91–102
27. Tran TTB, Dietmair S, Chan LCL, Huynh HT, Nielsen LK, Reid S (2012) Development of quenching and washing protocols for

quantitative intracellular metabolite analysis of uninfected and baculovirus-infected insectcells. Methods 56:396–407
28. Nguyen Q, Nielsen LK, Reid S (2013) Genome scale transcriptomics of baculovirus-insect interactions. Viruses 5:2721–2747. doi:10.3390/v5112721
29. Monteiro F, Carinhas N, Carrondo MJ, Bernal V, Alves PM (2012) Toward system-level understanding of baculovirus-host cell interactions: from molecular fundamental studies to large-scale proteomics approaches. Front Microbiol 3:391
30. Chen YR, Zhong S, Fei Z, Gao S, Zhang S, Li Z, Wang P, Blissard GW (2014) Transcriptome responses of the host *Trichoplusia ni* to infection by the baculovirus *Autographa californica* multiple nucleopolyhedrovirus. J Virol 88:13781–13797
31. Visnovsky G, Claus J (1994) Influence of the time and multiplicity of infection on the batch production of *Anticarsia gemmatalis* nuclear polyhedrosis virus in lepidopteran insect cell cultures. Adv Bioproc Eng 1994:123–128
32. Pushparajan C, Claus JD, Marshall SDG, Visnovsky G (2013) Characterization of growth and *Oryctes rhinoceros* nudivirus production in attached cultures of the DSIR-HA-1179 coleopteran insect cell line. Cytotechnology 65:1003–1016
33. Nielsen LK, Smyth GK, Greenfield PF (1991) Hemacytometer cell count distributions-implications of non-poisson behavior. Biotechnol Prog 7:560–563
34. Pijlman GP, Van Schijndel JE, Vlak JM (2003) Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells. J Gen Virol 84:2669–2678
35. Lua LHL, Pedrini MRS, Reid S, Robertson A, Tribe DE (2002) Phenotypic and genotypic analysis of *Helicoverpa armigera* nucleopolyhedrovirus serially passaged in cell culture. J Gen Virol 83:945–955
36. Lynn DE (1994) Enhanced infectivity of occluded virions of the gypsy-moth nuclear polyhedrosis-virus for cell-cultures. J Invertebr Pathol 63:268–274
37. Nielsen J, Villadsen J, Liden G (2003) Bioreaction engineering principles. Kluwer, New York, NY
38. Zhu H, Nienow AW, Bujalski W, Simmons MJH (2009) Mixing studies in a model aerated bioreactor equipped with an up- or a down-pumping 'Elephant Ear' agitator: power, hold-up and aerated flow field measurements. Chem Eng Res Des 87:307–317
39. Visnovsky G, Claus J, Merchuk JC (2003) Airlift reactors as a tool for insect cells and baculovirus mass production. LA Appl Res 33:117–121
40. Louis KS, Seigel AC (2011) Cell viability analysis using Trypan blue: manual and automated methods. Mammalian cell viability. Methods Mol Biol 740:7–12
41. Phillips HJ (1973) Dye exclusion tests for cell viability. In: Kruse PF, Patterson MK (eds) Tissue culture. Academic, London, pp 406–408
42. Vaughn JL (1976) The production of nuclear polyhedrosis viruses in large-volume cell cultures. J Invertebr Pathol 28:233–237
43. Pushparajan C (2015) Development and optimization of an in vitro process for the production of *Oryctes nudivirus* in insect cell cultures. Ph.D. thesis, University of Canterbury, Christchurch
44. Ikonomou L, Drugmand J-C, Bastin G, Schneider Y-J, Agathos SN (2002) Microcarrier culture of lepidopteran cell lines: implications for growth and recombinant protein production. Biotechnol Prog 18:1345–1355
45. Lazar A, Silberstein L, Reuveny S, Mizrahi A (1987) Microcarriers as a culturing system of insect cells and insect viruses. Dev Biol Stand 66:315–323
46. Sandford KK, Earle WR, Evans JE, Waltz HK, Shannon JE (1951) The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J Natl Cancer Inst 11:773–795
47. Van Wezel AL (1973) Microcarrier cultures of animal cells. In: Kruse PF, Patterson MK (eds) Tissue culture: methods and applications. Academic, New York, NY, p 372
48. Reed LJ, Muench H (1938) A simple method of estimating 50% endpoints. Am J Epidemiol 27:493–497
49. Huynh HT, Tran TTB, Chan LCL, Nielsen LK, Reid S (2013) Decline in baculovirus-expressed recombinant protein production with increasing cell density is strongly correlated to impairment of virus replication and mRNA expression. Appl Microbiol Biotech 97:5245–5257
50. Huynh HT, Chan LCL, Tran TTB, Nielsen LK, Reid S (2012) Improving the robustness of a low-cost insect cell medium for baculovirus biopesticides production, via hydrolysate streamlining using a tube bioreactor-based statistical optimization routine. Biotechnol Prog 28:788–802
51. Agathos SN (2007) Development of serum free media for lepidopteran insect cell lines. In: Murhammer DW (ed) Baculovirus and insect cell expression protocols, 2nd edn. Humana, Totowa, NJ. ISBN 978-1-59745-457-5
52. Matindoost L, Chan LCL, Qi YM, Nielsen LK, Reid S (2012) Suspension culture titration: a simple method for measuring baculovirus titers. J Virol Methods 183:201–209
53. Jorio H, Tran R, Kamen A (2006) Stability of serum-free and purified baculovirus stocks under various storage conditions. Biotechnol Prog 22:319–325