

Chapter 14

Production of Virus-Like Particles for Vaccination

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Abstract

The ability to make a large variety of virus-like particles (VLPs) has been successfully achieved in the baculovirus expression vector system (BEVS)/insect cell system. The production and scale-up of these particles, which are mostly sought as vaccine candidates, are currently being addressed. Furthermore, these VLPs are being investigated as delivery agents for use as therapeutics. The use of host insect cells allows mass production of VLPs in a proven scalable system.

Key words Viral vector, Virus-like particle, VLP, Baculovirus, Insect cells

1 Introduction

Virus-like particles (VLPs) produced in insect cells have been the subject of research for nearly two decades for their potential use as vaccines. VLPs are structures that form as a result of the simple expression of viral structural proteins and resemble naturally occurring viruses without the nucleic acid content. These particles cannot self-replicate, making them ideal candidates as antigens or immunogens. The baculovirus expression vector system (BEVS) used with host insect cells can produce high levels of recombinant proteins and can perform most of the post-translational modifications of mammalian cells (*see* Chapter 18), thereby retaining the biological activity of the original protein; thus, it is natural to consider this system for the production of these particles. The BEVS is also very efficient at producing large quantities of VLPs and an increasing body of work focusing on the production and the process behind making VLPs has started to accumulate [1–3]. To briefly highlight, this includes work on bluetongue virus [4, 5], rotavirus [6–10], human [5, 11, 12] and porcine [13–16] parvoviruses, human immunodeficiency virus [17, 18], infectious bursal disease virus [19, 20], influenza virus [21, 22], and ebola virus [23].

VLPs can be composed of either a single or multiple virus proteins. VLPs composed of more than one structural protein can be produced using multiple baculoviruses, each carrying a gene, or with a single baculovirus carrying multiple genes. Sokolenko et al. discuss the benefits and drawbacks that come with working with either platform [24]. VLPs, like the viruses they are modeled after, can be either extracellular or intracellular, i.e., they are secreted into the medium or remain within the cells and must be released through cell lysis, respectively. Additional process considerations and downstream processing accompany the production of secreted VLPs in the BEVS because of the presence of budded recombinant baculovirus, which are often similar in size and morphology to the VLPs [25, 26]. Another important consideration and challenge for producing VLPs in cell culture is to ensure that they do not have any unwanted foreign DNA or RNA trapped inside the particle. One benefit of using the BEVS system to produce VLPs is that DNA from baculovirus and insect cells is either expressed minimally, or not at all, in mammalian systems [27].

Viral vectors produced in insect cells are a natural extension of VLP production. With the incorporation of DNA or RNA having a sequence coding for a transgene of interest, these VLPs gain the potential as a gene therapy agent [28]. Meghrou, Aucoin and their colleagues [29–31] investigated the process behind their production and scaled-up the system to a 20 L bioreactor.

This chapter describes a methodology for producing and monitoring VLPs and viral vectors using the BEVS with a host insect cell based on our experience producing AAV particles and influenza VLPs. These systems will be used as examples.

2 Materials

2.1 Cell Lines and Recombinant Viruses

1. *Spodoptera frugiperda* cell line Sf-9 (ATCC CRL1711) (*see Note 1*).
2. Recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (*see Note 2*).

2.2 Medium and Solutions

1. Insect cell culture medium (for Sf-9 cells): e.g., 9.5 kg H₂O, 384 g Sf-900 II SFM (Gibco® Cell Culture, Invitrogen), 8 mL Sf-900 II Supplement (Gibco® Cell Culture, Invitrogen), adjust pH to 5.9 with sodium hydroxide (NaOH) (10 N); 3.5 g sodium bicarbonate (NaHCO₃) solid, then add H₂O to 10 L. Final pH should be 6.2 ± 0.1. Adjust pH if necessary with HCl (12 N). Final osmolarity should be 350 ± 25 mOsm. Other serum-free media could be used with generally similar growth performances (*see Note 3* and Chapter 8).
2. Trypan blue solution (0.4 %).

2.3 Shake Flask and Bioreactor Culture

1. Shake flasks, e.g., Erlenmeyer filter top plastic flasks (*see Note 4*) (up to 500 mL working volume).
2. Temperature controlled incubator (able to maintain 27 °C, which usually requires a refrigerated incubator) equipped with a shaker table.
3. Bioreactors for larger working volumes, e.g., at mid-scale, a 3.5 L or 22 L Chemap bioreactor (Mannedorf, Switzerland) (*see Note 5* and Chapter 11).

2.4 Data Acquisition

On-line data acquisition is considered optional, but it can be used to understand the process and to check run reproducibility. A computer with data acquisition hardware/software that can record several signals simultaneously on-line should be installed, i.e., for temperature, DO, pH, gas flow rates, CO₂ levels pressure, capacitance, etc. (*see Chapter 11*).

Off-line data acquisition primarily involves determining cell density and viability, e.g., with a hemacytometer (e.g., Hauser Scientific, Horshaw, PA) (*see Note 6*).

2.5 Post-Harvest

Downstream processing of VLPs should contain several steps that are completely dependent on the type of VLPs produced. The localization of the VLP at harvest time is the most critical factor. For secreted VLPs (e.g., influenza), localization will be in the cell culture supernatant, whereas for intracellular VLPs (e.g., AAV), particles need to be released from the cells prior to further processing. A second consideration regarding downstream processing is the possibility of VLP aggregation occurring during concentration and other downstream processing steps (*see Notes 7–10*).

The methods described below can be used for VLP detection. These methods are some of the most commonly used but do come with certain inherent drawbacks (*see Note 11*).

2.5.1 SDS-PAGE

1. 1.4 M dithiothreitol (*see Note 12*).
2. 5× concentrated tris-glycine running buffer: 800 mL ultrapure H₂O (18.2 MΩ), 15 g Tris, 72 g glycine, 5 g SDS; complete to 1 L with H₂O. Store at 4 °C.
3. Sample buffer: 6 mL 0.5 M Tris-HCl, pH 6.8, 5 mL glycerol, 6 mL 20 % w/v SDS, 0.6 mL 4 % bromophenol blue. Store at -20 °C.
4. Mini Protean® II system (Bio-Rad).
5. 4–15 % Tris-HCl Ready Gels (Bio-Rad).

2.5.2 Western Blotting

1. Gel blot paper.
2. Nitrocellulose membrane.
3. PBS (10×): 800 mL H₂O, 2 g KCl, 2 g KH₂PO₄, 80 g NaCl, 21.6 g Na₂HPO₄·7H₂O; complete to 1 L with H₂O. Filter through a 0.45 μm membrane filter.

4. PBS-T (0.1 %): 100 mL PBS (10×), 900 mL H₂O, 1 mL Tween-20.
5. 5 % Dried skim milk in PBS-T (0.1 %): 5 g Blotting grade blocker nonfat dry milk, 100 mL PBS-T (0.1 %); gently heat mixture while stirring until dissolved.
6. Towbin transfer buffer: 700 mL H₂O, 3.03 g Tris, 14.41 g glycine, 200 mL methanol; complete to 1 L with H₂O.
7. TRANS-BLOT® Semi-Dry Transfer Cell (Bio-Rad).
8. BM Chemiluminescence Blotting Substrate (e.g., Roche Diagnostic Corp).
9. Kodak Image Station.

2.5.3 Total Protein Analysis

1. RC DC™ Protein Assay (Bio-Rad) (*see Note 13*).
2. UV Spectrophotometer.

2.5.4 Electron Microscopy

1. TEN buffer: 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5.
2. Formvar TEM grid.
3. Centrifuge.
4. 3 % phosphotungstic acid pH 6.0.
5. Electron Microscope.

2.5.5 Other Equipment

1. Hot water bath or heating block.

3 Methods

3.1 Insect Cells

Verify cell quality prior to amplifying baculovirus stocks and producing VLP.

1. Seed shaker flask with Sf-9 cells at 5×10^5 cells/mL by diluting with fresh growth medium. Limit the working volume to 20 % of the total flask volume.
2. Determine the viable cell density at least once a day (twice daily would be preferred) until the cell density reaches $\sim 5 \times 10^6$ cells/mL. Viable and total cells can be counted using a hemacytometer (*see Note 6* and Chapter 11). Do not allow the viable cell density to exceed $5\text{--}6 \times 10^6$ cells/mL, i.e., maintain the cells in the exponential growth phase (*see Note 14* and Chapter 1) by transferring the appropriate amount of cells to another flask containing fresh medium.
3. Plot the viable cell density vs. time in culture to determine the population doubling time (PDT) (*see Chapter 1*). The PDT should be 24 h or less (i.e., a specific growth rate of $\sim 0.03 \text{ h}^{-1}$), which is consistent with healthy cells.

4. If a CEDEX cell counter (or equivalent equipment) is available, then it can be useful to confirm that the cell size distribution is as symmetric as possible and that the cell diameter is consistent with the cell diameter of non-infected cells.

3.2 *Baculovirus Stock Amplification*

Amplify baculovirus stocks using standard methods (e.g., *see* Chapter 11).

3.3 *VLP and Vector Production*

3.3.1 *Bioreactor Preparation*

Cells that are routinely transferred in fresh serum-free medium and maintained in the exponential growth phase should be used to inoculate the bioreactor at $3\text{--}5 \times 10^5$ cells/mL. It is recommended that the data acquisition system be ready to record as soon as the water, used for the sterilization of the bioreactor, is emptied from the bioreactor. The bioreactor should be maintained under a slight positive pressure at all times. Detailed information regarding bioreactor operation can be found in Chapter 11.

1. Add medium, preheated to 27 °C, to the bioreactor.
2. Start the agitation at 110 rpm when using an axial pumping type of impeller such as a helical ribbon impeller or pitch blade impeller.
3. Adjust the DO set point to 30–60 % oxygen saturation (*see* **Note 15**).
4. Once the temperature has stabilized cells can be added to the bioreactor such that the initial cell density is in the range of $3\text{--}5 \times 10^5$ cells/mL.

The bioreactor should be operated at its working volume as indicated by the manufacturer to ensure functionality of probes and proper mixing and oxygen transfer through the headspace.

3.3.2 *Production Modes*

1. Grow the cells to $1.5\text{--}2.5 \times 10^6$ cells/mL prior to infecting, allowing the cells to be in the exponential growth phase (*see* Chapter 1).
2. Infect cells at an appropriate MOI (*see* **Note 16**).

Typical dynamics of VLP production of cells infected at an MOI of 5 are shown in Fig. 1.

3.3.3 *Culture Harvest and VLP Extraction*

The optimal time to harvest VLPs is between 48 and 96 h post-infection (h pi), before significant cell lysis occurs (*see* **Note 17**). The procedure to extract VLP from the cell culture is as follows.

1. Harvest the cells by centrifuging 15 min at $300 \times g$ and 4 °C.
2. Decant supernatant or lyse pellet to remove VLPs in the case where they were not secreted from the cell (*see* **Note 18**).
3. Purify VLPs either by chromatography or a combination of density gradient centrifugation and chromatography methods.

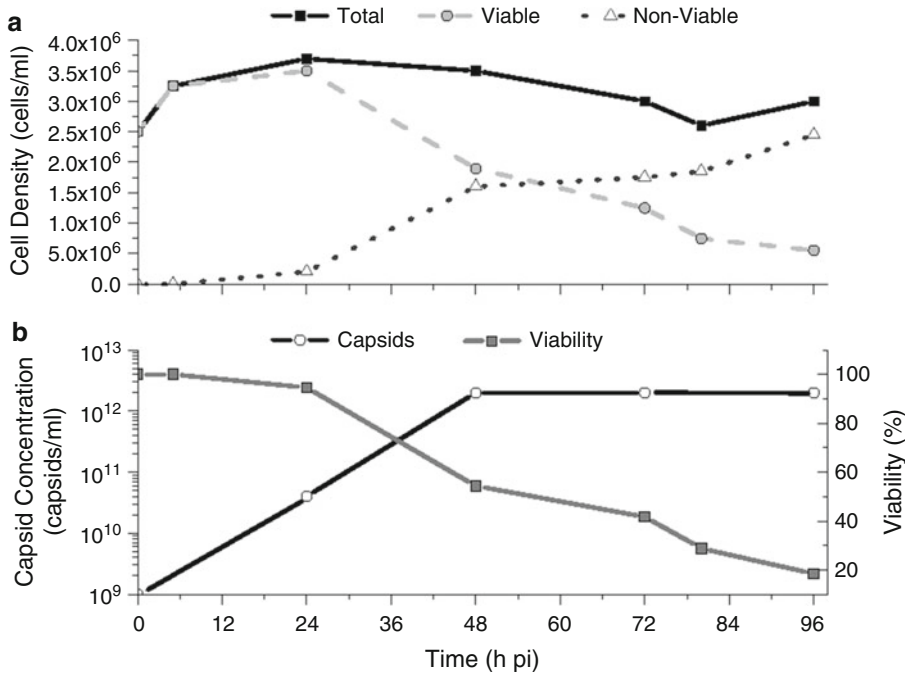


Fig. 1 Dynamics of adeno-associated virus-like particle production in Sf-9 cells infected at 2.5×10^6 cells/mL, with BacCap at a MOI of 5 in Sf-900 II medium

3.3.4 Culture Control

The following parameters can be controlled and/or monitored during cultivation:

1. Dissolved oxygen concentration.
2. Agitation rate.
3. Temperature (maintained at 27–28 °C).

Product yield can be affected if dissolved oxygen is too high or too low. We routinely use 40 % DO, but it can be anywhere from 30 to 60 % [32]. An example of a controlled bioreactor production is shown in Fig. 2. See Chapter 11 for more detail regarding DO control. An increased temperature can be used to increase the gene expression rate and reduce the time to harvest [31]. Other parameters that are not controlled but that are generally monitored include signals that can be collected online or measured offline. On-line parameters that are solely monitored may include signals from a fluorescence probe (e.g., if the GFP reporter protein is produced as discussed in Chapter 22) or a capacitance probe (to measure cell density). Off-line measurements may include cell density, cell viability, VLP concentration, specific protein concentration, and nutrient concentrations (e.g., glucose and glutamine).

3.3.5 VLP Downstream Processing

As discussed in Notes 7–10, different methods can be used to purify VLPs. After clarification of some VLPs, e.g., influenza or triple layered rotavirus, are concentrated/semi purified with

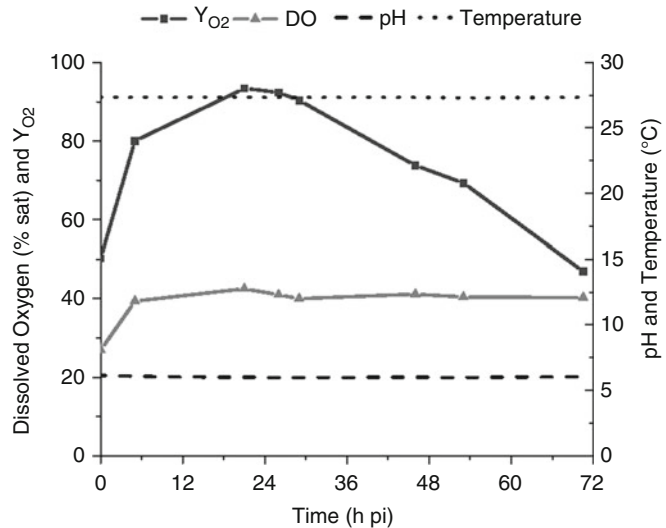


Fig. 2 Controlled (DO, temperature) and monitored (pH) viral stock production of recombinant baculovirus expressing influenza Neuraminidase protein (NA) in a 3.5 L bioreactor with Sf-9 cells at an MOI of 0.2

ultracentrifugation [22, 33, 34], while others, e.g., parvovirus, can be precipitated [15]. Additives such as protease inhibitors, nucleases and chelating agents may be added (*see Note 19*) during these steps or during production. Finally, VLPs must undergo a polishing step in order to be considered clinical grade material, which can be done using chromatography steps based on affinity or ion-exchange mechanisms [1].

3.4 VLP Detection

3.4.1 SDS PAGE/Western Analysis

To assess the composition of the particles, individual viral proteins can be assessed using specific antibodies (*see Note 20*).

1. Subject cell samples to three freeze/thaw cycles.
2. The quantity of proteins in the samples can be determined using the Bradford method (RC DC™ is used here). 450–600 ng of protein should be loaded for detection.
3. Add 36 μ L dithiothreitol (DTT) to 300 μ L of sample buffer.
4. Dilute culture samples in sample buffer with reducing agent (*see Note 21*).
5. Heat samples in a boiling water bath for 5 min.
6. Centrifuge samples at 16,000 $\times g$ for 2 min.
7. Load supernatant on gel.
8. Run gel at 200 V for 60 min.
9. Stain with a Coomassie blue or silver stain solution (*see Note 22*) or continue to **step 10** for Western blot analysis.

10. Rinse gel in transfer buffer (keep the equilibration time short to prevent diffusion of low molecular weight proteins out of the gel) by changing the buffer three times in 5 min.
11. Prior to the end of the electrophoresis run, soak the blot paper and nitrocellulose (NC) membrane in one container with Towbin Transfer buffer for 15–30 min (keep it at 4 °C), in the following order: (a) 2× Gel blot Paper, (b) 1× nitrocellulose membrane (NC) and (c) 2× Gel blot Paper.
12. Place in the following order, on top of the anode of the TRANS-BLOT Semi-Dry Transfer Cell. Carefully roll out bubbles with a test tube after each layer is laid down (except on the gel): (a) 2× Gel blot paper, (b) 1× NC membrane, (c) gel (avoid moving the gel against the NC membrane once it is laid down) and (d) 2× Gel blot Paper.
13. Carefully place the cathode assembly onto the stack.
14. Run at 10 V for 60 min.
15. Once the transfer is complete remove the NC membrane and dry for 5 min.
16. Block in 5 % dried skim milk/PBS-T (0.1 %) for 1 h at room temperature with shaking.
17. Wash the membrane with PBS-T (0.1 %): (a) 1×15 min and (b) 2×5 min.
18. Add monoclonal against the desired protein, typically diluted 1:1000 in PBS-T (0.1 %).
19. Incubate overnight with shaking.
20. Wash the NC membrane with PBS-T (0.1 %) with shaking: (a) 1×15 min and (b) 2×5 min.
21. Add the conjugated secondary antibody in PBS-T (0.1 %) and incubate for 1 h at room temperature with shaking.
22. Wash the NC membrane with PBS-T (0.1 %) with shaking: (a) 1×15 min and (b) 4×5 min.
23. Detect using BM Chemiluminescence Blotting Substrate and analyze using a Kodak Image Station.

3.4.2 Total Protein

The following is a brief description of the standard assay protocol for 5 mL samples from Bio-Rad (*see Note 23*).

1. Add 20 µL of DC reagent S to each 1 mL of DC reagent A. This solution is now called Reagent A.
2. Prepare 3–5 dilutions of a protein standard from 0.2 to 1.5 mg/mL protein. This should be done each time the assay is conducted.
3. Pipet 100 µL of standards and samples into clean and dry tubes.
4. Add 500 µL RC reagent I, vortex, incubate for 1 min at room temperature.

5. Add 500 μL RC reagent II, centrifuge tubes at $15,000 \times g$ for 3–5 min.
6. Discard supernatant.
7. Add 510 μL reagent A to each tube, vortex then incubate at room temperature for 5 min.
8. Add 4 mL of DC reagent B to each tube, vortex then incubate at room temperature for 15 min.
9. Determine the absorbance at 750 nm.

3.4.3 Negative Staining Electron Microscopy

For additional details *see* **Note 24**.

1. Dilute samples with TEN buffer.
2. Place aliquoted diluted samples in 240 μL microtubes with Formvar and carbon coated grids inserted at the bottom of the tube.
3. Centrifuge tubes at $20,000 \times g$ for 5 min.
4. Dry recovered grids and stain with 3 % phosphotungstic acid, pH 6.0.
5. Visualize samples under transmission electron microscope.

4 Notes

1. VLP production can be done using Sf-9 or Tn-5 cells [22]. We believe that a better baculovirus stock is produced in Sf-9 cells; however, the total VLP titers (and viral vectors) have been observed to be comparable [35]. Protein Sciences Corporation has developed a proprietary cell line, expresSF+, with the same large size of Tn-5 cells that also possesses the ability to grow to the same high cell densities as Sf-9 cells (10^8 cells/mL). This cell line has great potential for VLP production.
2. Recombinant baculoviruses can be produced with a number of different commercially available systems. Invitrogen, Clontech and BD Biosciences have easy to use systems based on direct cloning into provided vectors and recombination with baculovirus DNA or bacmid production for recombinant virus generation (Bac-to-Bac, BaculoDirect, BacPAK and BaculoGold). The most widely used promoter is polyhedron (polh), however, other promoters, e.g., p10 can also be used. Another consideration is the use of polycistronic viruses for simultaneous expression of proteins of interest, or individual monocistronic viruses coding for each protein for a higher degree of flexibility for final VLP composition [24]. Additional information about generating recombinant baculoviruses can be found in Chapters 3 and 4.

3. Other serum-free media that have been used for VLP production, e.g., HyClone-SFX [36] and IPL-41 [22]. *See* Chapter 8 for more information regarding serum-free media.
4. Our lab routinely uses disposable plastic Erlenmeyer flasks with a vented top. Glass flasks can be used as well, but it is advisable to consistently use the same flask type when cultivating and maintaining cells.
5. Due to the shear sensitivity of the cells, the bioreactor should be equipped with a low shear impeller to ensure adequate mixing. In our lab, the use of a helical ribbon impeller or pitch blade impeller is common and depends solely on the configuration of the reactor and on the probes that are used with the bioreactor. A dissolved oxygen (DO) probe, pH probe, and temperature probe can be installed in the bioreactor to monitor the DO concentration, pH and temperature, respectively. The resulting information can then be used to control these parameters. In addition, a capacitance probe may be installed in the bioreactor to monitor biomass levels. The DO concentration can be controlled either through the use of mass flowmeters (i.e., by controlling air, oxygen and/or nitrogen flowrates) or by varying the agitation and sparging rates. *See* Chapter 11 for information regarding large scale production.
6. The CEDEX automated cell counter (Innovatis, Germany) using the established Trypan blue exclusion method (*see* Chapter 11) can also be used here. In addition to cell concentration, the CEDEX records viability, average cell diameter and has a useful multi-sampler application.
7. Regardless of whether the VLPs are intracellular or secreted, the first step is to separate the supernatant from the cells directly after harvest. This is traditionally done with centrifugation, although filtration can also be used. In the case of intracellular VLPs, the cells need to be lysed to release VLPs. During primary recovery, VLPs are at the highest risk of aggregating, either with themselves or with cellular debris (e.g., proteins and nucleic acids) left in the culture medium. Chahal et al. [37] investigated different lysing techniques (freeze-thaw, surfactant and two phase extraction), aggregation prevention methods (salt addition, nuclease treatment) and resuspension buffers for AAV vectors. In the case of influenza VLPs, aggregation may also prove to be a problem during exploration of novel bioprocessing steps intended to improve VLP final concentration.
8. After primary recovery, the next step is concentration and/or intermediate purification. This step can utilize ultracentrifugation, ultrafiltration, or diafiltration and should aim to separate VLPs from free proteins. When VLPs are concentrated by ultracentrifugation, different speeds and times have been

used, e.g., 130,000–200,000 $\times g$ for 90 min [22, 33] and 27,000–37,000 $\times g$ with and without a 25 % sucrose cushion [26]. In our lab we use a 25 % sucrose cushion and a centrifugation time of 3 h at 37,000 $\times g$ in a Sorvall Discovery SE 100 ultracentrifuge (Thermo Fisher Scientific, USA). VLPs are then resuspended in another buffer (PBS, Tris-HCl, or HEPES), but studies to determine which buffer is most appropriate for VLP stability still need to be conducted. In the case of diafiltration/ultrafiltration we use a 100 k MW cut off centrifugal filter (PALL Corporation, USA) and follow the guidelines given in the specification booklet for centrifugation speed and time (e.g., for 4 mL filter units, 4000 $\times g$ for 20 min concentrates 10–20 \times influenza VLPs). Using molecular weight cutoff filters are fast and convenient for concentrating smaller volumes (i.e., 4–20 mL filter units).

9. The polishing step to produce clinical grade material can be done using another density gradient ultracentrifugation with sucrose, cesium chloride, or iodixanol, commercially known as Optiprep (Nycomed, Norway). Alternatively, chromatography can be used.
10. Another aspect to take into consideration when producing secreted VLPs is recombinant baculovirus contamination. Both particles can be similar in size and this can complicate downstream processing. See the review by Vicente et al. [1] for more information on downstream processing of VLPs for vaccines.
11. Other methods are currently under intense development for VLP detection and quantification. For example, for influenza VLPs, such methods include kinetic monitoring of secreted VLP production using Fluorescence-Activated Cell Sorting (FACS), and identification of total VLPs and HA by HPLC. Additionally, for influenza VLPs, other methods currently used for whole virus quantification, e.g., the hemagglutination assay or Single Radial Immunodiffusion (SRID) assay, can be used to determine the antigenic activity of the VLPs. While EM and total protein are methods that can be used to verify the presence of VLPs and quantify their total protein content, these methods have drawbacks, including a need for high purity for accurate analysis, thereby making them too laborious for process development. For influenza VLPs, the major drawbacks in using western blots and ELISA are antibody specificity variability and the lack of verified standards. An anti-HA antibody for one strain of influenza may or may not work for other strains, thereby resulting in a wide variety of antibodies to choose from and validate. For AAV quantification and detection, ELISA used to analyze the VLP, while transduction and infection based assays are used to analyze the

activity of the vector form of the particles. *See* Aucoin et al. [38] for a summary of currently available methods.

12. B-mercaptoethanol can also be used.
13. Other Bradford assays could be used, such as the Quick start Bradford, the Bio-Rad protein assay, or the DC protein assay. The Quick start and Bio-Rad assays are compatible with samples containing reducing agent and not with those containing detergents. The DC assay is compatible with samples containing detergents but not reducing agents. The RC DC™ method, however, is compatible with both. This assay has been chosen in our lab out of convenience because it can be used with samples from lysed cells that may contain traces of detergent or with the same samples prepared for SDS electrophoresis. All assays work in the range from 0.2 to 1.5 mg/mL.
14. Cells can be passaged either by inoculating the old cells into fresh medium or by centrifuging cells and resuspending them in fresh medium to achieve the desired concentration of 5×10^5 cells/mL. While completely replacing the medium will ensure that all the toxins are removed, we have found in our experience that inoculation of the old cells into fresh medium is an adequate technique and have not encountered limited cell growth due to existing toxins. However, we do not passage cells at cell densities higher than $5\text{--}6 \times 10^6$ and require a viability of at least 95 %. If cells have been grown to a higher concentration and have a lower viability, then complete medium replacement is generally needed.
15. For shake flasks, loosening the lid and maintaining a low working volume to flask volume ratio (which provides a large liquid–gas interface to volume ratio) results in good oxygen transfer. In a bioreactor the agitation rate is maintained constant for the entire batch to maintain ~constant mass transfer rates. Insect cells do not have a cell wall and are therefore more susceptible to physical damage from hydrodynamic shear stress and bubble formation. Some investigators have used agitation speed as a means to control the DO concentration and have found no adverse effect on the cells until a shear stress of between 1.5 and 4 N/m² is reached, at which point viability decreases. However, it has been shown that cell growth is affected by shear stress before cell death occurs. Therefore, there is an optimal agitation/aeration rate that ensures adequate DO concentration and allows cells to grow and produce optimal amounts of VLPs [18]. By keeping the agitation rate constant, the mass transfer coefficient, $k_{L,a}$, can be assumed to be approximately constant.
16. High MOIs lead to a synchronous infection and faster production, often with the highest yields. This has to be balanced, however, by the volume of virus stock that has to be supplied

to achieve the high MOIs. A rule of thumb is to avoid infecting a cell culture with a virus stock volume greater than 10 % of the cell culture volume. Infection at higher cell densities can be done if cells are infected in fresh medium under non-limiting nutrient conditions or under a fed batch mode to increase yields. The advantage of changing medium prior to infection is to ensure that limiting nutrients are replenished and inhibitory compounds are eliminated from the cell culture during the production phase; however, there are significant costs associated with replenishing the entire medium as well as potential damage to the cells through the manipulations involved with removing the medium from the cells. At large scale this step may become difficult without appropriate equipment and the chances of contaminating the culture increase. In some cases, concentration of the virus stocks may be necessary [39]. For more details *see* Chapter 11.

17. The harvest time depends on the downstream processing methodology. If it is desired to keep the VLP associated with the cell, i.e., the VLP is intracellular and loss is to be minimized by avoiding cell lysis, then the harvest should be done by 72 h pi. However, by leaving the culture up to 96 h pi, and treating both the supernatant and the pellet, the process is more robust with respect to the initial MOIs of each baculovirus used.
18. The best method to lyse cells depends upon the scale of operation. Cells are generally lysed after the clarification step. At small scale, freeze-thaw cycles prove to be the best method for product recovery, but are not appropriate at larger scales. Chahal et al. [35] discuss different methods of lysing cells, as well as buffer selection in the case of intracellular VLPs while taking scale into consideration. For secreted influenza VLPs, lysing the cells is not done and particle concentration is usually required prior to conducting additional purification steps. After ultracentrifugation or during ultrafiltration, the VLPs are resuspended in another buffer (PBS, Tris-HCl or other). In theory, this can be advantageous because it removes potentially harmful cell debris that could be left over after clarification; however, to our knowledge no stability studies in different buffers have been completed to verify this assumption. For small volumes (<20 mL), ultrafiltration is the fastest (about 20–45 min to spin down 10×), while the pelleting takes 3 h itself, not including prep time.
19. Due to the amount of host cell proteases, it is advisable to add protease inhibitors during the early steps of downstream processing [1]. Additionally, for secreted VLPs, nucleases can be added before harvest or after clarification to dissociate the VLPs from DNA released by lysed cells. Chelating agents have been shown to have an effect on the stability of SV40 capsid

VLPs [40], i.e., promoting capsid dissociation. In the case of intracellular VLPs, capsid dissociation may be encouraged to remove foreign DNA trapped inside VLPs. In this case the VLPs need to reassemble after DNA removal. All of these agents can be removed in later purification steps. For protease inhibitors and nucleases, this can be accomplished with chromatography or ultracentrifugation, while for chelating agents, such as EDTA, this can be accomplished with a buffer exchange. For protease inhibitors and nuclease detection, Abcam (Cambridge, MA) and Merck KGaA (Darmstadt, Germany) have commercial ELISA kits available.

20. For AAV detection, monoclonal antibodies for Rep 78, 68, 52 and 40 proteins and VP 1, 2 and 3 are used (Maine Biotechnology Services, Portland, ME). For influenza proteins, polyclonal sheep antibodies against HA (detects HA1 and HA0) and NA (NIBSC, Hertfordshire, UK) and a monoclonal mouse antibody for M1 are used (Abcam, Cambridge, MA). For influenza, antibodies were chosen based on their activity with an in-house wild type virus, level of background and cost. Another antibody that was used for HA is a mouse monoclonal anti-HA that detects HA2 and HA0 (Santa Cruz Biotechnology, Santa Cruz, CA), but resulted in a varying signal in our hands (results unpublished). The advantage of monoclonal antibodies over polyclonal antibodies is their specificity, which results in low background noise, while the advantage for polyclonal antibodies is lower cost. When analyzing crude samples, it is important to use an antibody that generates the lowest possible background noise to clearly detect VLP proteins. Background noise can also come from secondary antibodies, so it is advisable to run a control with only the secondary antibody to know what kind of background signals it produces. While laborious, it is best to probe with antibodies for all of the antigens that the VLP is composed of to get an idea of the levels of each in the particles. Another consideration for antibody choice is the strain of virus being used to model VLPs. This is important for influenza VLPs because the virus is classified into three subtypes (A, B, and C) with each containing different strains due to the high mutation rate of the surface proteins (HA and NA). Therefore, one antibody for a strain within subtype A may not be suitable for another in subtype A. There has been some work on developing universal antibodies for influenza focusing on a conserved region of the HA protein that may be of interest in the future to solve this problem of strain-to-strain variability [41].
21. When reducing samples with DTT, we use a mixture of 2 parts sample to 1 part sample buffer with DTT (to give a final DTT concentration of 50 mM).

22. Coomassie blue is able to detect as low as 50 ng of protein; however, silver stain is 50× more sensitive. Therefore, silver stain will not only allow the visualization of the protein of interest, but can also identify contaminant proteins that may be present at low levels.
23. The total protein assay is used as a method to determine the amount of loading material for gels and also to analyze protein content during all stages of the process. Bradford protein assays are robust and relatively cheap ways to gain insight into variations of the total protein content during downstream processing steps. This is important for influenza VLPs because as of date there is no proven standardized method to process and quantify. With all these unknown variables coming into play at once, using a universally known and robust method to quantify total protein at all stages of process, and method development, is a valuable tool that can take one unknown variable out of the equation.
24. The method described here has been used in our lab for previous virus samples and could also be used for VLPs. It has been adapted from Alain et al. [42]. NSEM provides a method to physically quantify total particles and is usually done after the second purification step, i.e., density gradient ultracentrifugation, on fractions containing VLPs identified from Western blotting. It can be done earlier, but VLPs may be harder to visualize with remaining contaminants. A concentration of 10^5 – 10^6 virus particles per mL is typically needed for visualization [43].

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References

1. Vicente T, Roldão A, Peixoto C et al (2011) Large-scale production and purification of VLP-based vaccines. *J Invertebr Pathol* 107: S42–S48
2. Roldão A, Mellado M, Castilho L et al (2010) Virus-like particles in vaccine development. *Expert Rev Vaccines* 9:1149–1176
3. Mena J, Kamen A (2011) Insect cell technology as a versatile and robust vaccine manufacturing platform. *Expert Rev Vaccines* 10:1063–1081
4. Zheng Y, Greenfield P, Reid S (1999) Optimized production of recombinant blue-tongue core-like particles produced by the baculovirus expression system. *Biotechnol Bioeng* 65:600–604
5. Maranga L, Cruz P, Aunins J et al (2002) Production of core and virus-like particles with baculovirus infected cells. *Adv Biochem Eng Biotechnol* 74:183–206
6. Palomares L, Ramírez O (2009) Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles. *Biochem Eng J* 45:158–167
7. Roldão A, Vieira H, Charpilienne A et al (2007) Modeling rotavirus-like particles production in a baculovirus expression vector

- system: infection kinetics, baculovirus DNA replication, mRNA synthesis and protein production. *J Biotechnol* 128:875–894
8. Palomares L, Lopez S, Ramirez O (2002) Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins during simultaneous production by insect cells. *Biotechnol Bioeng* 78:635–644
 9. Jiang B, Barniak V, Smith R et al (1998) Synthesis of rotavirus-like particles in insect cells: comparative and quantitative analysis. *Biotechnol Bioeng* 60:369–374
 10. Park J, Kim H, Hwang H et al (2004) Large-scale production of rotavirus VLP as vaccine candidate using Baculovirus Expression Vector System (BEVS). *J Microbiol Biotechnol* 14:35–40
 11. Shelly D, Cleave V (2009) Parvovirus B19 VLP vaccine manufacturing. *Genet Eng Biotechnol News* 29:1–4
 12. Tsao E, Mason M, Cacciuttolo M et al (1996) Production of parvovirus B19 vaccine in insect cells co-infected with double baculoviruses. *Biotechnol Bioeng* 49:130–138
 13. Maranga L, Brazao T, Carrondo M (2003) Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. *Biotechnol Bioeng* 84:245–253
 14. Maranga L, Cunha A, Clemente J et al (2004) Scale-up of virus-like particles production: effects of sparging, agitation and bioreactor scale on cell growth, infection kinetics and productivity. *J Biotechnol* 107:55–64
 15. Maranga L, Rueda P, Antonis A et al (2002) Large scale production and downstream processing of a recombinant porcine parvovirus vaccine. *Appl Microbiol Biotechnol* 59:45–50
 16. Cruz P, Maranga L, Carrondo M (2002) Integrated process optimization: lessons from retrovirus and virus-like particle production. *J Biotechnol* 99:199–214
 17. Pillay S, Meyers A (2009) Optimization of chimeric HIV-1 virus-like-particle production in a baculovirus-insect cell expression system. *Biotechnol Prog* 25:1153–1160
 18. Cruz P, Cunha A, Peixoto C et al (1998) Optimization of the production of virus-like particles in insect cells. *Biotechnol Bioeng* 60:408–418
 19. Hu Y, Bentley W (2009) Enhancing yield of infectious bursal disease virus structural proteins in baculovirus expression systems: focus on media, protease inhibitors, and dissolved oxygen. *Biotechnol Prog* 15:1065–1071
 20. Hu Y, Bentley W (2000) A kinetic and statistical-thermodynamic model for baculovirus infection and virus-like particle assembly in suspended insect cells. *Chem Eng Sci* 55:3991–4008
 21. Haynes J (2009) Influenza virus-like particle vaccines. *Expert Rev Vaccines* 8:435–445
 22. Krammer F, Schinko T, Palmberger D et al (2010) *Trichoplusia ni* cells (High Five) are highly efficient for the production of influenza A virus-like particles: a comparison of two insect cell lines as production platforms for influenza vaccines. *Mol Biotechnol* 45:226–234
 23. Sun Y, Carrion R Jr, Ye L et al (2009) Protection against lethal challenge by Ebola virus-like particles produced in insect cells. *Virology* 383:12–21
 24. Sokolenko S, George S, Wagner A et al (2012) Co-expression vs. co-infection using baculovirus expression vectors in insect cell culture: benefits and drawbacks. *Biotechnol Adv* 30:766–781
 25. Haynes J, Dokken L, Wiley J et al (2009) Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. *Vaccine* 27:530–541
 26. Pushko P, Tumpey T, Bu F et al (2005) Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* 23:5751–5759
 27. Ghosh S, Parvez K, Banerjee K et al (2000) Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. *Mol Ther* 6:5–11
 28. Urabe M, Ding C, Kotin R (2002) Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* 13:1935–1943
 29. Meghrou J, Aucoin M, Jacob D et al (2005) Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: from shake flasks to a 20-L bioreactor. *Biotechnol Prog* 21:154–160
 30. Aucoin MG, Perrier M, Kamen AA (2006) Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. *Biotechnol Bioeng* 95:1081–1092
 31. Aucoin MG, Perrier M, Kamen AA (2007) Improving AAV vector yield in insect cells by modulating the temperature after infection. *Biotechnol Bioeng* 97:1501–1509
 32. Kamen AA, Bédard C, Tom R et al (1996) On-line monitoring of respiration in recombinant-baculovirus-infected and uninfected insect cell bioreactor cultures. *Biotechnol Bioeng* 50:36–48

33. Latham T, Galarza JM (2001) Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. *Society* 75: 6154–6165
34. Peixoto CC, Sousa MFQ, Silva AC et al (2007) Downstream processing of triple layered rotavirus like particles. *J Biotechnol* 127:452–461
35. Aucoin MG, Mena JA, Kamen AA (2010) Bioprocessing of baculovirus vectors: a review. *Curr Gene Ther* 10:174–186
36. Pushko P, Pearce MB, Ahmad A et al (2011) Influenza virus-like particle can accommodate multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. *Vaccine* 29:5911–5918
37. Chahal PS, Aucoin MG, Kamen AA (2007) Primary recovery and chromatographic purification of adeno-associated virus type 2 produced by baculovirus/insect cell system. *J Virol Methods* 139:61–70
38. Aucoin MG, Perrier M, Kamen AA (2008) Critical assessment of current adeno-associated viral vector production and quantification methods. *Biotechnol Adv* 26:73–88
39. Mena JA, Aucoin MG, Montes J et al (2010) Improving adeno-associated vector yield in high density insect cell cultures. *J Gene Med* 12:157–167
40. Ishizu K, Watanabe H, Han S et al (2001) Roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein. *J Virol* 75:61–72
41. Chun S, Li C, Van Domselaar G et al (2008) Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza a viral hemagglutinins. *Vaccine* 26:6068–6076
42. Alain R, Nadon F, Séguin C et al (1987) Rapid virus subunit visualization by direct sedimentation of samples on electron microscope grids. *J Virol Methods* 16:209–216
43. Goldsmith CS, Miller SE (2009) Modern uses of electron microscopy for detection of viruses. *Clin Microbial Rev* 22:552–563