Chapter 26

Generation of Envelope-Modified Baculoviruses for Gene Delivery into Mammalian Cells

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Abstract

Genetically modified baculoviruses can efficiently deliver and express genes in mammalian cells. The major prerequisite for the expression of a gene transferred by baculovirus is its control by a promoter that is active in mammalian cells. This chapter describes methods for producing second generation baculovirus vectors through modification of their envelope. Envelope modified baculoviruses offer additional new applications of the system, such as their use in in vivo gene delivery, targeting, and vaccination. Methods of generating a recombinant baculovirus vector with a modified envelope and its amplification and purification, including technical scale production, are discussed. A variety of notes give clues regarding specific technical procedures. Finally, methods to analyze the virus and transduction procedures are presented.

Key words Baculovirus, Gene delivery, Targeted vectors, Complement-resistant, Vaccine, Mammalian cell, Envelope modification, Hepatocyte, Vector generation, Gene delivery

1 Introduction

Twenty years ago, we genetically engineered baculoviruses that can efficiently deliver and express genes in mammalian cells [1]. The only prerequisite for the expression of a gene transferred by baculovirus is its control by a promoter that is active in mammalian cells. Such recombinant baculovirus vectors have been used successfully for a variety of applications, including gene transfer studies, generation of monoclonal antibodies, and cell-based assays [2]. The advantages of recombinant baculovirus vectors for mammalian cell expression are their safety due to their "non-mammalian-virus-based" origin. Baculoviruses have the unique property of replicating in insect cells while being incapable of initiating a replication cycle and infectious virus in mammalian cells. The viruses can be readily manipulated and easily generated to high vector titers. They show no cytopathic effect in mammalian cells and have a large capacity for the insertion of foreign DNA. The recent generation of envelope modified baculoviruses offers

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additional potential applications of the system, including in vivo gene delivery, targeting, and vaccination. The methods for the development of recombinant envelope-modified baculoviruses are described.

1.1 Principle of Baculovirus Envelope Modification

The main baculovirus envelope protein, gp64, is responsible for virus uptake by insect cells and hepatocytes [3]. Baculoviruses deleted of their natural single copy gp64 gene cannot be productively propagated in insect cells. Therefore, a strategy to modify the baculovirus envelope is to insert the modified gp64 gene as an additional copy into the baculovirus genome. A site for modification of this additional gp64 gene that successfully leads to display of the modification on the virus surface is the N-terminus in between the gp64 signal sequence and mature gp64 [4]. The modified gp64 can be adequately co-expressed in insect cells, even from the late polyhedrin promoter, during virus amplification and assembles into the viral envelope.

1.2 Selection of the Envelope Sequence Based on Intended Use

1.2.1 Complement-Resistant Vectors

Gene delivery with baculovirus vectors in vivo is hampered by the complement system [5–7]. However, a variety of proteins are described that may protect the virus from complement-mediated inactivation. We selected a functionally active sequence of human decay acceleration factor (DAF) without signal sequence and with the glycosyl-phosphatidylinositol (GPI)-anchor (to insert the resulting protein into the viral membrane) to protect the baculovirus from complement. This sequence was cloned in between an extra copy of the gp64 signal sequence and mature gp64 and inserted into the polyhedrin locus to generate complement-resistant vectors [8, 9].

1.2.2 Targeted Vectors

As described under Subheading 1.1, sequences for specific receptor ligands can also be inserted to generate targeted vectors and to expand the host range of baculovirus vectors. We have generated envelope-modified baculovirus vectors displaying the epidermal growth factor on the virus surface and achieved an enhanced gene delivery into EGF-receptor positive cells in comparison with vectors having the wild-type envelope. In vivo tumor targeting of a systemically administered, tropism-modified baculoviral vector was also shown recently and highlights the potential of baculovirus-mediated targeted therapies [10].

1.2.3 Vaccine Vectors

The unique possibility of baculovirus vectors to modify the envelope and simultaneously express genes from the viral backbone in mammalian cells may end up in new vaccines [11–14]. Antigenic sequences can be displayed on the viral surface to trigger a humoral immune response and other immunogenic sequences can be expressed to induce a cellular immune response.

2 Materials

2.1 Transfer Plasmid for Envelope Modification and Mammalian Gene Expression

1. Baculovirus transfer plasmid pBACsurf-1 (EMD Biosciences, San Diego, CA).
2. Reagents for PCR, including specific primers with restriction site overlaps for *Sma*I, *Kpn*I, or *Pst*I.
3. Enzymes for manipulation of DNA (restriction endonucleases: *Sma*I, *Kpn*I, or *Pst*I and *Eco*RV, Klenow enzyme, T4 DNA ligase, etc.).
4. Luria–Bertani (LB) medium.
5. Agar plates.
6. Selection agent (ampicillin).
7. Competent *E. coli* cells DH5-alpha (Invitrogen, Carlsbad, CA).
8. Kits and/or solutions for preparation of plasmid DNA from *E. coli* (Qiagen, Inc., Valencia, CA).
9. Kits and/or solutions for gel purification of DNA fragments (Qiagen, Inc., Valencia, CA).
10. Phenol–chloroform–isoamylalcohol solution (25:24:1).

2.2 Insect Cell Culture

1. Sf-9 cells (PharMingen, EMD Bioscience, San Diego, CA).
2. EX-CELL™ 401 medium (JHR Bioscience, Lenexa, KS).
3. Complete EX-CELL™ 401 medium: EX-CELL™ 401 medium, 10 % heat-inactivated fetal calf serum and 100 mg/L streptomycin and 100,000 units/L penicillin.
4. Tissue culture plates and flasks.
5. Cellspin-platform and spinner flasks (Integra Bioscience, Fernwald, Germany; distributed in the USA by Argos Technologies Inc., East Dundee, IL).

2.3 Virus Generation

1. Insect cell culture material, *see* Subheading 2.2.
2. Baculovirus genomic DNA, BaculoGold™ (PharMingen, EMD Biosciences, San Diego, CA).
3. Recombinant transfer plasmid from Subheading 3.1 (pBacsurf_X_mam).
4. Falcon polystyrene tubes.
5. Lipofectamine (Gibco, Carlsbad, CA).
6. Sterile distilled water.

2.4 Titration by Plaque Assay

1. Insect cell culture material, *see* Subheading 2.2.
2. SeaPlaque-Agarose™ (FMC, Rockland, USA).
3. Sterile distilled water.
4. 60 mm plates.
5. Plastic box.

2.5 Amplification of Recombinant Viruses

1. Insect cell culture material, *see* Subheading 2.2.
2. Recombinant virus stock or single plaque virus (**step 15** under Subheading 3.3.2).
3. 1 L spinner flasks (Cellspin, Integra, Fernwald, Germany; distributed in the U.S. by Argos Technologies Inc., East Dundee, IL).

2.6 Virus Concentration and Purification

1. Virus stock to be concentrated and purified.
2. Large capacity ultracentrifuge tubes, e.g., 38 mL polyallomer ultracentrifuge tubes for SW28 rotor, Beckman.
3. Ultracentrifuge tubes with a small diameter, e.g., 17 mL polyallomer ultracentrifuge tubes for SW40 rotor, Beckman.
4. Phosphate-buffered saline (PBS): 1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl, adjust pH to 7.4 through addition of 1 M HCl.
5. Sucrose cushion solution: 25 % sucrose (w/w) in PBS, pH 7.4.
6. Sucrose gradient solutions: 25 % sucrose (w/w) in PBS and 60 % sucrose (w/w) in PBS, pH 7.4.
7. Sucrose-gradient-former (Jule Inc., Milford, CT).
8. Rotors and ultracentrifuge for centrifugation of up to 96,000 × *g*, e.g., SW28 and SW40 rotor, Beckman.

2.7 Virus Analysis

2.7.1 Immunoblotting

1. A4p virus preparation (Subheading 3.3.5).
2. Sample buffer: 62.5 mM Tris–HCl, pH 6.8, 10 % glycerin, 2 % SDS, 300 mM 2-mercaptoethanol, 0.0025 % bromophenol blue.
3. SDS polyacrylamide gels (8 %).
4. 1 mM Dithiothreitol (DTT).
5. Rainbow molecular weight protein marker (Amersham (now part of GE Healthcare), Piscataway, NJ).
6. Western-blot apparatus (Owl Scientific, Portsmouth, NH).
7. Semidry-blotter (Serva Electrophoresis GmbH, Heidelberg, Germany; Crescent Chemicals Corp., Hauppauge, NY).
8. Transfer buffer: 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 5 % (v/v) methanol.
9. Hybond-C extra membranes (Amersham Pharmacia Biotech, Freiburg, Germany; Amersham (now part of GE Healthcare), Piscataway, NJ).
10. Blocking buffer: 5 % (w/v) milk powder, 0.1 % (v/v) Tween 20 in PBS.
11. Primary antibody against AcV5 [15] or directed against your specific protein displayed on the surface.

12. Horseradish peroxidase conjugated antisera (Pierce, St. Augustin, Germany; Rockford, IL).
13. ECL detection system (Amersham Pharmacia Biotech, Freiburg; now a part of GE Healthcare, Piscataway, NJ).

2.7.2 Electron Microscopy

1. A4p virus preparation.
2. Carbon coated grids (Agar Scientific Limited, Stansted Essex; Ted Pella, Inc., Redding, CA).
3. Monoclonal primary antibody.
4. 10 % (w/v) bovine serum albumin (BSA).
5. PBS.
6. 12 nm colloidal Gold-AffiniPure goat anti-mouse IgG (Dianova, Hamburg, Germany).
7. Phosphotungstic acid (PTA).
8. Transmission electron microscope (e.g., 902A, Zeiss, Oberkochen, Germany).

3 Methods

3.1 Transfer Plasmid for Envelope Modification and Mammalian Gene Expression

3.1.1 Basic Vector Elements

The baculovirus transfer plasmid pBACsurf-1 is designed for in-frame insertion of DNA-sequences between the gp64 signal sequence and the mature protein coding sequence (under the control of the polyhedrin promoter). Expressed fusion proteins are incorporated onto the virion surface, anchored by the transmembrane domain of gp64 [4]. Mammalian expression cassettes can be inserted into the *Eco*RV-site (Fig. 1).

3.1.2 Insertion of a Mammalian Expression Cassette

Use standard cloning procedures to generate pBacsurf_mam by inserting an expression cassette that is active in mammalian cells blunt-end into the *Eco*RV-site of pBacsurf-1 (*see* **Note 1**).

3.1.3 PCR-Cloning of New Envelope Sequence

PCR-amplify the sequence to modify the baculovirus envelope and insert it via *Sma*I or *Kpn*I or *Pst*I into pBACsurf_mam to generate pBACsurf_X_mam (*see* **Note 2**).

3.1.4 Baculovirus Genomic DNA

BaculoGold™ is a modified *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) Baculovirus DNA that contains a lethal deletion and does not code for viable virus. Co-transfection of the BaculoGold-DNA with a complementing Baculovirus Transfer plasmid, e.g., pBACsurf_X_mam (Subheading 3.1.3), rescues the lethal deletion after homologous recombination in insect cells (Fig. 1).

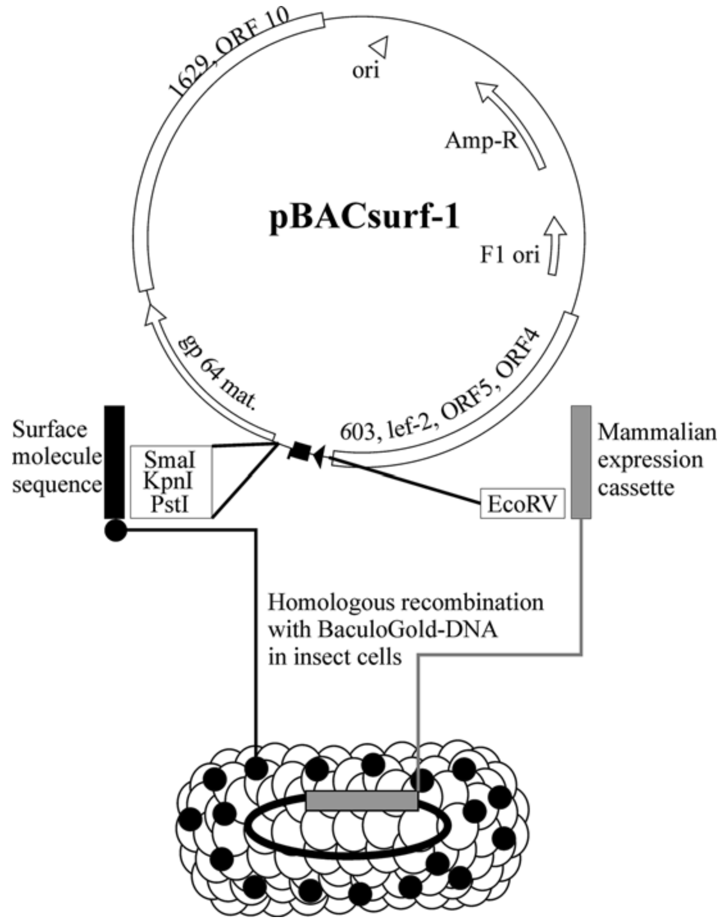

Fig. 1 Map of baculovirus transfer plasmid pBACsurf-1 with insertion sites for envelope modification and expression of genes in mammalian cells. Viruses are generated by homologous recombination with the transfer vector and genomic baculovirus DNA in insect cells. *Black arrow*: polyhedrin promoter; *Black box*: gp 64 signal sequence; gp 64 mat.: gp 64 mature domain; *Open boxes*: 1629, ORF 10 and 603, lef-2, ORF5, ORF4: baculovirus genomic sequences/genes. *Sma*I, *Kpn*I, and *Pst*I are restriction sites to insert surface modification sequences; *Eco*RV can be used to insert a mammalian expression cassette

3.2 Insect Cell Culture, Serum-Free Versus Serum Containing

In general, baculovirus can be produced in serum-free EX-CELL™ 401 medium or complete EX-CELL™ 401 medium. We recommend using complete EX-CELL™ 401 medium at least until large-scale production in spinner flasks (through **step 17** under Subheading 3.3.3). The use of serum-free medium facilitates purification because of the absence of FCS, but results, at least in our hands, in about 1 log less virus output compared to serum-containing medium.

3.3 Virus Generation

3.3.1 Producing the Recombinant Virus by Homologous Recombination in Insect Cells

1. Prepare and label two 25 cm^2 tissue culture flasks with 2×10^6 Sf-9 cells (50–70 % confluent). One flask will be the experimental co-transfection flask, the other a negative control flask.
2. After cell attachment, wash cells gently 3 times with 3 mL EX-CELL™ 401 medium. Finally, add 1 mL EX-CELL™ 401 medium.
3. Experimental co-transfection: Combine 0.5 μg BaculoGold™ DNA and 3 μg recombinant Baculovirus Transfer Vector in a *Polystyrol* tube, add sterile water to a total of 50 μL and mix by pipetting up and down (*see* **Note 3**).
4. Mix 20 μL Lipofectamine and 30 μL sterile water in a separate Polystyrol tube.
5. Add DNA solution (from **step 3**) drop by drop to Lipofectamine–water mixture (from **step 4**).
6. Incubate mixture for 15 min at room temperature (becomes slightly opalescent).
7. Add the mixture from **step 6** drop by drop to the experimental co-transfection flask (from **step 2**). Gently rock the flask back and forth to mix the drops with the medium.
8. Incubate the two flasks at 27 °C for 4 h.
9. After 4 h, remove the medium from the experimental and the negative control flasks. Add 5 mL complete EX-CELL™ 401 medium and incubate the plates at 27 °C for 5 days.
10. After 5 days, cells of the experimental co-transfection flask will have stopped dividing and will often float in the medium. Cells in the control flask will be 100 % confluent.
11. Collect now the virus-containing supernatant of the experimental co-transfection flask and store at 4 °C in the dark.
12. Baculovirus-containing supernatant will be used for isolation of single recombinant viruses by plaque assay.

3.3.2 Isolation of Single Recombinant Viruses, Titration, and Storage

1. Seed Sf-9 cells on 60 mm plates (2×10^6 cells per plate using a sufficient volume of medium to totally cover the surface). Seed up to 7 plates (for the virus dilutions 10^{-2} to 10^{-8}—*see* **step 2**). Allow the cells to attach for at least 10 min. It is important that this is done on a level surface to allow the cells to spread evenly over the bottom of the plate.
2. Prepare serial dilutions (10^{-2} to 10^{-8}) of virus-containing supernatant (e.g., from **step 12** under Subheading 3.3.1) in 1 mL complete EX-CELL™ 401 medium.
3. Aspirate medium from **step 1** and immediately add respective dilution from **step 2**.

4. Incubate the plates at 27 °C for 1 h to allow virus particles to infect the cells.
5. While the cells are incubating, prepare a 0.8 % SeaPlaque-Agarose in complete EX-CELL™ 401 medium. Make up 4 mL for each 60 mm tissue-culture plate to be overlaid: Prepare a solution of 4 % SeaPlaque-agarose in sterile distilled water and autoclave. Alternatively, melt in advance prepared by microwaving. Heat complete EX-CELL™ 401-medium to 60 °C in a water bath. Cool the melted SeaPlaque-Agarose to 60 °C in a water bath. Dilute the SeaPlaque-agarose to 0.8 % (w/v) with heated complete EX-CELL™ 401-medium and cool the 0.8 % agarose–medium mixture to 40 °C.
6. Remove the virus inoculum from cells and take care that the monolyer does not dry out.
7. Overlay cells with 4 mL of the 0.8 % agarose/complete EX-CELL™ medium mixture.
8. Allow plates to sit undisturbed on a level surface until agarose hardens (about 20 min).
9. Plates should be kept inverted in a humid atmosphere (e.g., a plastic box with wet paper) at 27 °C.
10. Plaques develop after about 5–10 days and can be visualized and counted by inverting the plates on a dark background.
11. Determine the titer by recalculating the number of counted plaques with the respective dilution.
12. Isolate three single plaque recombinant viruses from a "high" dilution plate by picking with a pipette into a plaque and transferring virus in agarose into an Eppendorf tube prefilled with 1 mL complete EXCELL™-medium.
13. Allow virus to diffuse out of the agarose for 1 h at room temperature, mix and divide into two aliquots. Label the tubes as A0 (amplification zero, any additional amplification will produce higher stock numbers, e.g., A1, A2, A3).
14. Freeze one (back-up) aliquot (500 μL) at −80 °C (*see* **Note 4** and Chapter 9).
15. The other aliquot can be stored at 4 °C in the dark until amplification.

3.3.3 Amplification of Recombinant Viruses

1. For each virus from **step 13** under Subheading 3.3.1 seed 2×10^6 Sf-9 cells (50–70 % confluent) into a 25 cm^2 tissue culture flask. Also seed a control flask that will not be infected with virus. Use a sufficient volume to totally cover the flask surface. Allow the cells to attach for 15 min, remove the medium, and add 4.5 mL fresh complete EX-CELL™ 401 medium.
2. Add the 500 μL of the low titer A0 recombinant stock (**step 13** under Subheading 3.3.1) to a flask.

3. Incubate the cells at 27 °C for 5 days. Check for signs of infection 3 days post-infection (pi) (floating cells in comparison to a confluent monolayer in the control flask).
4. Harvest the supernatant from the flask and centrifuge at $500 \times g$ for 15 min to remove cellular debris.
5. Store the virus supernatant (A1) in a sterile tube at 4 °C in the dark and freeze 500 μL at −80 °C as A1-back-up (*see* **Note 4**).
6. At this stage, rather than determining the virus titer, which is normally around 10^6–10^7 pfu/mL, try to amplify your specific inserts by PCR using 1 μL (heated for 1 min at 95 °C) of your virus-containing insect cell supernatant as template for each reaction.
7. For further amplification, seed 6×10^6 Sf-9 cells (50–70 % confluent) into 75 cm^2 tissue culture flasks. Allow them to attach for 15 min and change to 3 mL fresh complete EX-CELL™ 401 medium. Add 1 mL of your A1 stock (PCR-positive for the inserts) and incubate for 1 h at on a rocking plate.
8. Add 11 mL fresh complete EX-CELL™ 401 medium and incubate at 27 °C for 3–5 days until nearly all cells are floating (in comparison to the control flask).
9. Harvest the supernatant from the flask and centrifuge to remove cellular debris ($500 \times g$, 15 min).
10. Store the virus supernatant (A2) in a sterile tube at 4 °C in the dark and freeze 500 μL at −80 °C as A2-back-up (*see* **Note 4**).
11. Proceed with amplification (**steps 7–10**) by using three 225 cm^2 tissue culture flasks (i.e., scale-up cells and virus by a factor 3) to end up with 135 mL virus stock A3.
12. Titrate the A3 stock by plaque assay (**steps 1–11** under Subheading 3.3.2). The titer should reach about $1–2 \times 10^8$/mL at this stage.
13. Amplification in spinner flasks: Seed approximately 5×10^5 Sf-9 cells/mL in a total of 250 mL medium in a 1 L spinner flask. The cells should be healthy (95–100 % viable) and have a population doubling time of ~24 h (*see* Chapter 1).
14. Cultivate cells (*see* Subheading 3.2) for about 2–3 days until a cell density of $2–3 \times 10^6$/mL is reached (exponential growth phase; *see* Chapter 1) and then add A3 virus stock at an MOI of 1. Repetitive infections with an MOI of substantially higher than 1.0 will select for deletion mutants that may no longer contain your inserts.
15. Cultivate cells for an additional 1.5–2.5 (absolute maximum) days. Check the progress of infection by examining aliquots of the culture under the microscope. Aliquots should also be used to monitor virus production (plaque assay) over time (*see* **Note 5**).

16. To harvest the virus, first pellet cells by centrifugation at 500 × *g* for 15 min and then centrifuge the resulting supernatant at 5000 × *g* for 15 min. Virus containing insect cell medium should be clear (*see* **Note 5**).
17. Titrate the A4 by plaque assay (**steps 1–11** under Subheading 3.3.2) and store at 4 °C in the dark until purification.

3.3.4 Virus Concentration

1. Load virus stock (A4) to high capacity polyallomer ultracentrifuge tubes.
2. Underlay with the sucrose cushion solution (10 % of the total volume of the virus stock).
3. Centrifuge at 80,000 × *g* for 75 min at 4 °C.
4. Decant the supernatant and carefully remove all liquid. The virus pellet should be translucent white.
5. Resuspend the pellets in a total of 2 mL PBS, retain an aliquot for titration to monitor a potential virus loss and purify as described under Subheading 3.3.5.

3.3.5 Virus Purification (See **Note 6**)

1. For 2 mL concentrated virus (**step 5** under Subheading 3.3.4), pour two 14 mL 25–60 % linear sucrose gradient into a 17 mL polyallomer ultracentrifuge tube.
2. Carefully load the concentrated virus (1 mL/tube) (**step 5** under Subheading 3.3.4) onto the gradient.
3. Centrifuge at 96,000 × *g* for 3 h at 4 °C.
4. Collect the white band (budded virus) in a minimum volume (at around 47–49 % sucrose).
5. Dilute the virus-bands about tenfold.
6. Centrifuge at 80,000 × *g* for 75 min at 4 °C.
7. Decant the supernatant and remove any liquid.
8. Resuspend the pellet in a small volume (1–2 mL) of PBS, aliquot (A4p, "A4purified") and freeze at −80 °C before titration.

3.4 Virus Analysis

Virus analysis is an important quality aspect to compare, e.g., newly acquired features of envelope-modified viruses to vectors with wild-type envelope. Besides the three analytical methods described in this section, a Southern-blot analysis should be performed with every large scale preparation of purified baculoviruses to confirm insert integrity. In addition, a Southern-blot analysis allows for calculating the total number of baculovirus genomes in a given sample that includes both infectious and noninfectious viruses. Dividing this number by the number of infectious viruses determined from plaque assay experiments yields the particle to infectious virus ratio. The smaller this ratio, the better is your preparation.

3.4.1 Functional Assays

Functional assays to determine envelope-modified virus integrity depend on the specific construct (*see* Subheading 1.2) and should be performed with A4p preparations (**step 8** under Subheading 3.3.5). For example, we generated complement-resistant vectors by incorporating decay acceleration factor (DAF) into the viral envelope. In addition, the vectors were able to mediate ß-galactosidase expression in insect and mammalian cells. The functionality was therefore tested in an Sf-9 insect cell-based and an hepatocyte-based assay [8] by incubating the viruses with active complement. Targeted vectors can be assessed by their eventually acquired new function to bind to cells or to mediate gene delivery in previously non-susceptible cell lines or respective animal models [10]. Vaccine vectors can be tested based on their ability to mediate a humoral and/or cellular immunity against displayed or expressed antigens after injection into animals [11–14].

3.4.2 Immunoblotting

In order to determine the composition of envelope modification, vectors from A4p preparations (**step 8** under Subheading 3.3.5) with wild-type envelope and modified envelope should be analyzed by immunoblotting. If analysis is done under non-reducing and reducing conditions and development is with a specific antibody against the displayed protein and gp64, then 4 identical gels/blots are required. Non-reducing conditions allow for detection of multimeric forms of the displayed protein, while reducing conditions offer the possibility to assess the portion of proteinX-gp64-fusion-protein in comparison to native gp64 (can be 50 %).

1. Denature 2×10^6 pfu A4p virus preparation (Subheading 3.3.5) per slot with sample buffer for 5 min at 95 °C. If you want to quantitatively compare vectors, then make certain that equal amounts of virus protein are loaded onto the gel.
2. Load onto SDS polyacrylamide gels (8 %) under non-reducing or reducing conditions (sample buffer + 1 mM DTT) and separate at 150 V.
3. Wash Gel in transfer buffer for 5 min.
4. Transfer onto Hybond-C extra membranes at 0.85 mA/cm^2.
5. Block membrane for 90 min with blocking buffer.
6. Incubate membrane with primary antibody, e.g., *Ac*V5 against gp64 in a 1:1000 dilution and/or your displayed protein in blocking buffer overnight.
7. Wash membrane with blocking buffer and detect primary antibodies with respective horseradish peroxidase conjugated antisera (dilution 1:1000, for 30 min).
8. Carefully wash membrane 5 times with PBS and visualize bands by the ECL detection system.

3.4.3 Electron Microscopy

1. Prepare serial dilutions of the A4p virus preparation.
2. Float virus dilutions from **step 1** on carbon coated grids. This is done by simply adding a drop of the virus solution on the grid for at least 1 min and then excess virus solution is drained off by touching the edge of the grid to a piece of clean filter paper. The same procedure is performed with antibodies.
3. Expose with monoclonal primary antibody in 10 % (w/v) BSA. Use concentration recommended for immunofluorescence studies.
4. Wash grids 3 times with PBS and incubate with a 1:30 solution of 12 nm colloidal Gold-AffiniPure goat anti-mouse IgG in 10 %w/v BSA.
5. Wash grids 5 times with PBS.
6. Stain preparations with PTA and analyze with an electron microscope (*see* **Note** 7).

3.5 Gene Delivery into Mammalian Cells

Baculovirus-mediated gene transfer was initially described to be highly efficient into hepatocytes [1, 16]. Gene delivery and expression in mammalian cells depends on the promoter used, the not-yet known receptor on the surface and differences [17] in the ability of cells to differentially repress transgene expression.

3.5.1 Transduction Procedure

1. Seed mammalian cells at a density of 3×10^4 cells/cm^2 (can be done from 96-well plate to big T-flask) so that they are 50–70 % confluent the next day. Untreated FCS or sera from other sources contains active complement, which inactivates the virus. It must be heat-inactivated for 30 min at 56 °C before adding to the cell culture medium.
2. The next day, remove medium and replace with fresh medium including the A4p virus preparation (Subheading 3.3.5) at a volume of 100 μL/cm^2. Incubate at 37 °C for 1 h. An MOI of 100 is a good starting concentration if no further information on the cell line is available. This MOI generates a 50 % transduction efficiency in the hepatocarcinoma cell line HuH7, which can be used as a control (*see* **Note 8**).
3. Remove virus inoculum from cells after 1 h and add fresh medium.
4. Analyze efficiency of gene delivery after 36 h (peak with a recombinant luciferase expression baculovirus in Huh7 cells [1]).

3.5.2 Analysis of Gene Delivery

Analysis of gene delivery is dependent on the inserted mammalian expression cassette and based on numerous options. This issue is not discussed with a specific example. In order to experience this system, reporter gene expression cassettes, e.g., ß-galactosidase, GFP,

or luciferase, under control of a mammalian cell active promoter should be inserted into the baculoviral genome. Standard analytical methods are available in most labs (e.g., *see* Chapter 22).

4 Notes

1. This plasmid (pBacsurf_mam) is the basis of the reference baculovirus vector with wild-type envelope. Always generate this virus according to the procedure given under Subheading 3.3.1 for comparative studies with your envelope-modified variant.
2. Inserts must lack an internal stop codon and maintain the appropriate open reading frame. Select a unique cloning site based on the sites of pBACsurf_mam. If all three sites, i.e., *Sma*I, *Kpn*I, and *Pst*I, are within your mammalian expression cassette, then develop a strategy to first insert the PCR-fragment for envelope modification and then the mammalian expression cassette via *Eco*RV.
3. This procedure can be scaled down by at least a factor of 5 to save material and money. If you decide to scale down transfection, then use 24-well plates for cell culture and 96-well Polystyrol plates with round bottoms for the Plasmid/BaculoGold–Lipofectamine mixture.
4. At these steps, we recommend to prepare backups, which can be thawed and used in case a later procedure fails. It also helps retrospectively to define the step that led to an eventual discrepancy between your plasmid construct and virus preparation in the course of virus analysis (Subheading 3.4).
5. Virus titer peaks normally at about 24–36 h. Longer cultivation ends up in a lot of cell debris that is more difficult to purify later.
6. Although the use of unpurified viruses is described in the literature for gene delivery into mammalian cells, this step avoids potential pseudotransduction.
7. A variety of publications describe that gp64 and gp64-fusion proteins are localized at the poles of the virions. By electron microscopy, we mainly detected viruses that displayed either gp64 or fusion-proteins completely surrounding their loose-fitting envelope.
8. Addition of butyrate at a concentration of 1–10 mM or trichostatin at a concentration of 1 μM to the cells prior to the transduction procedure de-represses transgene expression and leads in most cells to increased infection efficiencies—the compounds are, however, to a certain extent toxic to the cells.

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