Newcastle Disease Virus-Like Particles: Preparation, Purification, Quantification, and Incorporation of Foreign Glycoproteins

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ABSTRACT

Virus-like particles (VLPs) are large particles, the size of viruses, composed of repeating structures that mimic those of infectious virus. Since their structures are similar to that of viruses, they have been used to study the mechanisms of virus assembly. They are also in development for delivery of molecules to cells and in studies of the immunogenicity of particle-associated antigens. However, they have been most widely used for development of vaccines and vaccine candidates. VLPs can form upon the expression of the structural proteins of many different viruses. This chapter describes the generation and purification of VLPs formed with the structural proteins, M, NP, F, and HN proteins, of Newcastle disease virus (NDV). Newcastle disease virus-like particles (ND VLPs) have also been developed as a platform for assembly into VLPs of glycoproteins from other viruses. This chapter describes the methods for this use of ND VLPs. *Curr. Protoc. Microbiol.* 30:18.2.1-18.2.21. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

Virus-like particles, VLPs, are formed upon the expression of some or all of the structural proteins of a virus (Jennings and Bachmann, 2008). Expression of structural proteins of both enveloped and nonenveloped viruses can form VLPs. VLP structures, as well as mechanisms of assembly and release from cells, mimic those of cognate infectious virus. However, VLPs do not contain a viral genome.

VLPs have many potential uses (Ludwig and Wagner, 2007). They are powerful tools for the study of mechanisms and requirements for the assembly of specific viruses (e.g., Takimoto et al., 1998, 2001; Schmitt et al., 2002). They are potential means for delivery of molecules to cells (Kaczmarczyk et al., 2012). They are useful in defining immune responses to specific antigens presented in a particulate form (see, e.g., Schmidt et al., 2012). However, their widest use thus far has been as vaccines and vaccine candidates (reviewed in Jennings and Bachmann, 2008; Kang et al., 2009). Indeed, two VLPs are now licensed for use as human vaccines: hepatitis B virus vaccine and papilloma virus vaccine (Harper et al., 2006). Furthermore, there are many preclinical studies and clinical trials of a wide variety of VLPs that are potential vaccines for human use.

Expression of the structural proteins of many different viruses can result in formation of VLPs. Here, we focus on VLP generation and purification of particles formed with the

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structural proteins of Newcastle disease virus, an avian paramyxovirus. The assembly and release of Newcastle disease virus-like particles (ND VLPs) is quite efficient (Pantua et al., 2006). As a result, quantitative amounts of these particles are relatively easy to generate even from transiently transfected cells (McGinnes et al., 2010). They can be purified using protocols adapted from virus purification protocols, and the purified VLPs have minimal cell-protein contamination. Basic Protocol 1 describes the generation of ND VLPs using transient transfection of an avian cell line. Methods for large-scale preparation of quantitative amounts of VLPs are described, as well as methods for smaller-scale preparations (Alternate Protocol 1) useful for more analytical applications. Protocols for preparing radioactively labeled particles are included (see Support Protocol 1). Basic Protocol 2 and Alternate Protocol 2 describe purification for both large and small- scale particle preparations. Basic Protocol 3 and Alternate Protocols 3 and 4 describe several different methods for quantification of the content of VLP preparations. Methods include protein staining, autoradiography, and western blots.

Not all virus systems will produce VLPs, while others produce VLPs at very low efficiency. Because of their efficiency of production, ND VLPs have been adapted for use as a platform for the assembly into particles of antigens from a wide variety of viruses including genetically complex viruses. For example, ND VLPs can assemble with the entire ectodomains of glycoproteins from respiratory syncytial virus (Murawski et al., 2010; McGinnes et al., 2011; Schmidt et al., 2012), influenza viruses, cytomegalovirus, and West Nile virus (T. G. Morrison, unpub. observ.). Basic Protocol 4 describes the general methods for assembly of proteins from other viruses into ND VLPs.

BASIC PROTOCOL 1

LARGE-SCALE GENERATION OF ND VLPs

Newcastle disease VLPs are assembled and released from cells expressing the viral M, NP, HN, and F proteins (Pantua et al., 2006). This protocol describes a method for the large-scale generation of VLPs from cells expressing only these four proteins. This protocol is used when quantitative amounts of VLPs are required. Protein expression is accomplished using transient transfection of tissue culture cells with four plasmids containing the cDNA sequences encoding each of these viral proteins. VLPs, assembled on the plasma membranes of these cells, are released into the cell supernatant. The following protocol steps are based on one 150-cm^2 flask.

Materials

Tissue culture cells (ELL-0 are preferable but COS7, CHO, or 293T cell lines are acceptable; all are available at *http://www.atcc.org*)
Cell culture medium such as DMEM with appropriate supplementation (see recipe) for the cells to be used (see instructions from ATCC)
Plasmids containing cDNAs encoding NDV NP, M, F, and HN proteins (prepared using Qiagen Endo-free kit or the equivalent; use of the pCAGGS vector as the expression vector is highly recommended—this plasmid may be obtained from the Belgian Coordinated Collection of Microorganisms, BCCM/LMBP; *bccm.lmbp@dmbr.UGent.be*)
Lipofectamine (Life Technologies) or similar transfection reagent
Fetal bovine serum (heat inactivated at 56°C for 30 min; see *APPENDIX 2A*)

150-cm^2 (T-150) tissue culture flasks
15-ml conical centrifuge tubes (e.g., BD Falcon)

1. The day before transfection, seed 150-cm^2 flasks with the tissue culture cells of choice so they will be 60% to 65% confluent the next morning.
2. Set up transfection mix as follows. Per 150-cm^2 flask, in a 15-ml tube mix 1.6 ml OptiMEM and 8 μg each DNA (NDV NP, M, F, and HN). In a separate tube, mix

3.2 ml OptiMEM and 80 μl Lipofectamine. Incubate both tubes at room temperature for 15 min. Add the Lipofectamine to the DNA and mix together. Incubate at room temperature for 45 min.

Scale up for number of 150-cm^2 flasks to be used. A good starting point is 24 150-cm^2 flasks for a VLP preparation.

3. Wash cells two times with 15 ml OptiMEM.
4. Add 5.6 ml OptiMEM to DNA/Lipofectamine transfection mix and add the mixture to cells growing in a 150-cm^2 flask.
5. Incubate at 37°C for 5 hr.
6. Remove the DNA/Lipofectamine-containing supernatant and discard. Add 25 ml fresh cell culture medium containing fetal bovine serum. Incubate at 37°C.

Monitor the pH of the medium from the beginning of transfection to the last VLP harvest, replacing medium if it becomes too acid. Supernatants removed after 36 hr should be stored at 4°C.

SMALL-SCALE VLP GENERATION

ALTERNATE PROTOCOL 1

This alternative protocol describes methods for generation of VLPs on a small scale. Small-scale generation of VLPs is used when quantitative amounts of VLPs are not required. For example, this protocol is appropriate to determine the effects on VLP assembly of specific mutations in the expressed proteins or to determine time courses of VLP generation. The following steps are based on one 35-mm plate.

Additional Materials *(also see Basic Protocol 1)*

6-well tissue culture plates (or individual 35-mm plates)

1. The day before transfection, seed 6-well plates so they will be 60% to 65% confluent the next morning.
2. Transfect one 35 mm plate (60% to 65% confluent) with DNAs of interest as follows:
   a. Mix 0.5 μg of each DNA (NP, M, F, HN) with 0.1 ml of OptiMEM.
   b. In a separate tube, mix 6 μl of Lipofectamine with 0.2 ml of OptiMEM.
   c. Incubate at room temperature for 15 min.
   d. Mix DNA and Lipofectamine mixtures together and incubate at room temperature for 45 min.
   e. Wash cells twice, each time with 2 ml OptiMEM, and remove.
   f. Add 0.7 ml OptiMEM to the transfection mix and add to the cell monolayer. Incubate at 37°C for 5 hr. Remove DNA/Lipofectamine and add 2 ml of complete cell culture medium. Incubate at 37°C. Harvest VLPs at 72 hr.

   Check cells at 40 to 48 hr. If the medium is getting too acidic, remove 1 ml and keep at 4°C, add 1 ml fresh medium, and continue incubation to 72 hr. Combine all supernatants for VLP purification.

RADIOACTIVE LABELING OF VLPs

SUPPORT PROTOCOL 1

In some instances, it is useful to be able to visualize all components of a VLP made on a small scale. This visualization may be accomplished by autoradiography of radioactively labeled VLP proteins separated on a polyacrylamide gel. This approach may be necessary if no antibody exists for the VLP protein in question or if the amount of a protein incorporated into the VLP relative to the core NP and M proteins is to be assessed. This

protocol describes how protein components of the VLP may be radioactively labeled during their generation.

Additional Materials *(also see Basic Protocol 1 and Alternate Protocol 1)*

DMEM without methionine and cysteine (Life Technologies)
Dialyzed fetal bovine serum (dialyzed FBS; Life Technologies)
[^{35}S]cysteine/methionine (PerkinElmer Express ^{35}S Protein Labeling Mix, or equivalent)

1. Follow steps 1 and 2 in Alternate Protocol 1.
2. At 40 hr post-transfection, wash the plate twice with DMEM without methionine and cysteine and containing dialyzed FBS (this medium is referred to as complete medium without methionine and cysteine).
3. Add 0.5 ml of complete medium without methionine and cysteine.
4. Add [^{35}S]methionine/cysteine (approximately 10 to 20 μCi).
5. Incubate cells for 8 hr at 37°C.
6. Remove medium and replace with complete medium containing methionine and cysteine.
7. Incubate for an additional 24 hr at 37°C.

Follow all safety protocols required for handling radioactive material.

BASIC PROTOCOL 2

LARGE-SCALE PURIFICATION OF VLPs

This protocol describes the purification of a large-scale preparation of ND VLPs. The protocol is designed to purify the VLPs for use as immunogens and, therefore, is designed to minimize contamination of host-cell molecules. The protocol involves multiple differential centrifugations. Cell supernatants containing released VLPs are cleared of cells and large-sized cell debris by low-speed centrifugation. The VLPs in the cleared supernatants are then concentrated by pelleting in an ultracentrifuge. The resuspended VLPs are then purified on sequential sucrose gradients illustrated in Figure 18.2.1. The first gradient, sedimentation through 20% sucrose to a 65% sucrose pad, removes soluble material as well as non-lipid-associated aggregates (illustrated in Fig. 18.2.1, step 1). The second, flotation into a sucrose gradient centrifuged to equilibrium, removes additional non-lipid-associated particulate material, and minimizes non-VLP-associated lipid material (illustrated in Fig. 18.2.1, step 2). The third optional gradient, a more rigorous equilibrium gradient centrifugation, separates authentic VLPs from lighter density lipid vesicles as well as other non-VLP lipid associated material (illustrated in Fig. 18.1.1, step 3).

Materials

Supernatants containing large-scale amounts of VLPs (Basic Protocol 1)
DMEM medium with FBS
TNE buffer (see recipe)
10%, 20%, 25%, 35%, 45%, 50%, 55%, 65%, 80% (w/v) sucrose solutions in TNE buffer (see recipe for buffer)

Adaptors and 250-ml sterile/disposable conical centrifuge bottles (or comparable)
Beckman GS-6R centrifuge (or comparable)
Beckman Type 19 rotor and Type 19 bottles and Deldrin Cap Assemblies (or comparable)

Glass Dounce homogenizer (10 ml) with a tight-fitting pestle, pre-chilled on ice (optional)

Beckman SW28.1, SW41, SW50.1 rotors, buckets, and tubes (30-ml, 17-ml, 11-ml, and 5-ml tubes) or equivalent

Beckman ultracentrifuge (or comparable)

150-cm^2 (T-150) tissue culture flasks

2-ml vials stable at −80°C

Clarify cell supernatants

1. Remove cell supernatants from transfected 150-cm^2 flasks at 48, 72, 96, and 120 hr post-transfection (see Basic Protocol 1), replacing with fresh 25 ml of medium at each collection.

   Maximal release of VLPs begins at 48 hr and proceeds until at least 96 hr.

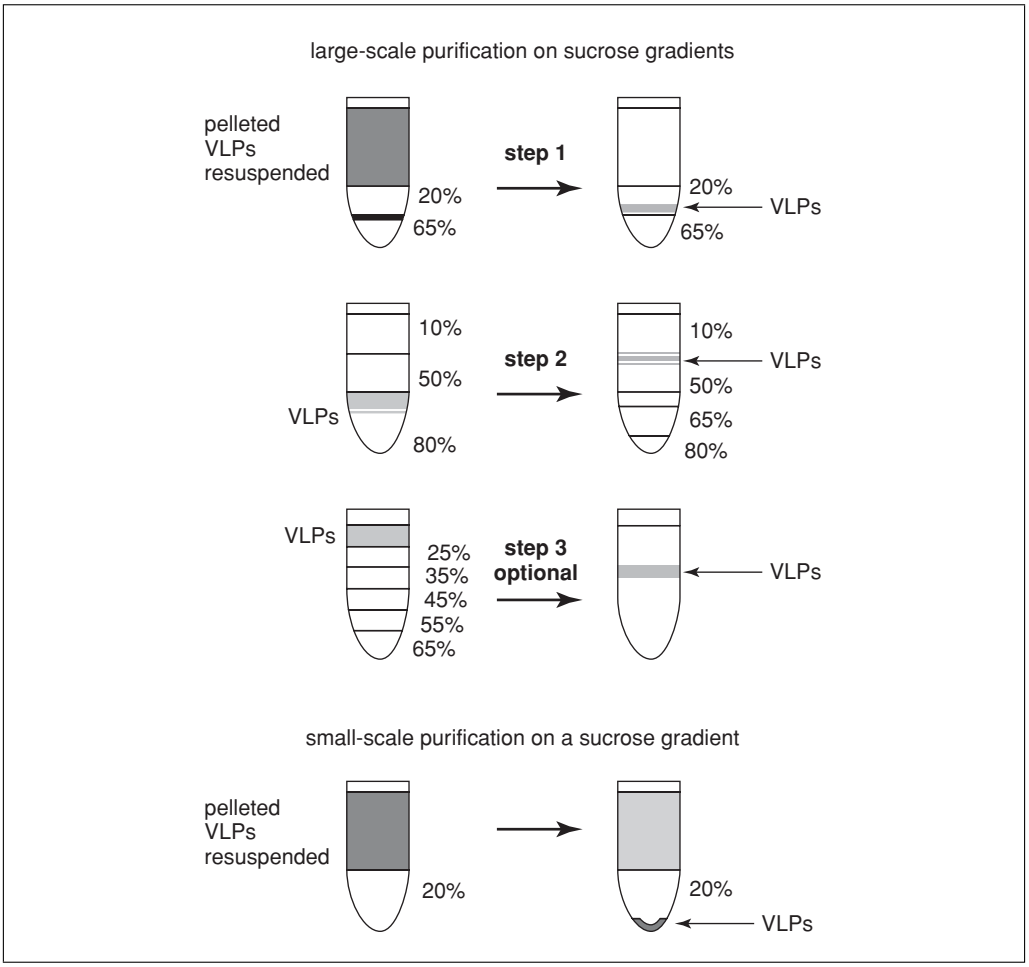

Figure 18.2.1 Diagram of sucrose gradients used to purify VLPs. Large-scale purification of VLPs requires two to three sequential sucrose gradients, which are diagrammed. In step 1, VLPs, pelleted from the cleared cell supernatants, are resuspended in TNE buffer and placed on top of layers of 65% and 20% sucrose, and then subjected to centrifugation for 6 hr at 100,000 × *g* (24,000 rpm). The white fluffy layer between the 65% and 20% layers is harvested. In step 2, the VLPs from step 1 are subjected to flotation into a gradient. The VLPs, made approximately 60% with respect to sucrose, are placed on top of an 80% sucrose pad, and then overlaid with 50% sucrose and then 10% sucrose. The gradients are centrifuged at 242,000 × *g* (38,000 rpm) for at least 18 hr. The VLPs form a white layer in between the 50% and 10% layers. For additional purification, the VLPs can be subjected to sedimentation in a continuous sucrose gradient (step 3). Small-scale purification of VLPs can be accomplished by centrifugation of VLPs through a 20% sucrose layer into a pellet as diagramed at the bottom of the figure. Alternatively, step 1 of the large-scale purification can be done.

2. Place the harvested supernatant fluid directly into chilled 250-ml sterile centrifuge bottles.

   Supernatants can be stored several days in the refrigerator.

3. Centrifuge the pooled supernatants in a Beckman GS-6R centrifuge, precooled (4°C), using adapters, for 15 min at 4000 × *g*, 4°C.

   This spin will remove large-sized cell debris and detached cells.

Concentrate VLPs (pellet VLPs from harvested fluid)

4. Decant the supernatant into sterile, pre-cooled Beckman Type 19 bottles.

5. Add medium (with serum) to completely fill all bottles.

   These bottles have a tendency to collapse if not completely filled. Medium with serum will stabilize VLPs.

6. Add the cap assembly to each bottle.

   Do not autoclave Deldrin cap assemblies. Sterilize with ethanol and UV light.

7. Pellet VLPs for 8 hr (minimum) or overnight in Type 19 rotor in a Beckman ultracentrifuge at 38,000 × *g*, 4°C.

8. Pour off supernatant.

   The supernatant is discarded. The VLPs are in the pellet.

Purify and concentrate VLPs

9. Resuspend all pellets in combined total of 22 ml of TNE buffer.

   The pellet can be sticky. A Dounce homogenizer is useful to resuspend all the particulate material. Homogenize with 10 strokes of the homogenizer.

10. Layer the supernatant (11 ml per gradient) over a discontinuous sucrose gradient formed with 2 ml of 65% sucrose in TNE buffer and 4 ml of 20% sucrose in TNE buffer. Make the gradients in a small (17 ml) (SW28.1) clear ultracentrifuge tube.

    Prepare two gradients for material from 24 to 30 150-ml flasks prepared in Basic Protocol 1. Be sure that the gradients all have the same weight prior to centrifugation, as they will need to be balanced in the centrifuge.

11. Centrifuge the gradients for 6 hr at 100,000 × *g*, 4°C, in a Beckman SW28.1 rotor with small buckets.

12. The white "fluffy" layer between the 65% and 20% sucrose layers is the partially purified VLPs—remove the top 13 to 14 ml of the gradient and discard. Collect the VLP layer into a sterile tube by pipetting the next 2 to 3 ml (containing the "fluffy" material) from the gradient. Discard the tube with the remaining 1 ml or so of 65% sucrose.

13. Add 80% sucrose to bring VLPs, collected from 20%/65% interface (step 12) to approximately 60% with respect to sucrose.

    For use as immunogens, VLPs are further purified by flotation centrifugation to equilibrium in a continuous sucrose gradient, as described in the following steps.

14. In an SW41 clear ultracentrifuge tube, layer VLPs on top of a pad (0.5 ml) of 80% sucrose in a SW41 centrifuge tube. Overlay with 3.5 ml of 50% sucrose and then 2 ml of 10% sucrose in TNE buffer.

15. Centrifuge the gradients overnight (18 hr) at 242,000 × *g*, 4°C, in a Beckman ultracentrifuge using an SW41 rotor.

    The VLPs will band at the 50% to 10% interface and can be seen as a white "fluffy" layer.

16. Collect the top 1 ml of the gradient and discard. Collect the next 1 to 2 ml of VLP material and place in a 15-ml tube.

    After this equilibrium centrifugation, sometimes two bands form. The bottom band (seen at the 60%/50%interface), while it does contain VLPs, is considerably contaminated with cell debris. The majority of the VLPs are in the top band. The purified VLPs can be entirely removed in 1 to 2 ml.

17. *Optional:* For further purification, fractionate concentrated VLPs by more rigorous equilibrium centrifugation. Dilute concentrated VLPs in TNE buffer to 10% to 15% sucrose. Form continuous sucrose gradients, 20% to 65% in TNE with a gradient maker. Alternatively, to make this gradient, slowly add successively 1.5 ml each of 65%, 55%, 45%, 35%, 25% sucrose solutions in TNE buffer to a washed, clear SW41 ultracentrifuge tube (11 ml). Allow the sucrose solutions in the tube to diffuse by incubating the tubes at 4°C overnight. Load 2.5 ml diluted VLPs on top of the gradient and centrifuge 18 hr at 131,000 × *g*, 4°C. Collect VLP band as in step 16.

18. Bring collected VLPs up to 10 ml with TNE buffer (in order to dilute the sucrose to below 25%). Place 5 ml into each of two SW50.1 tubes. Centrifuge the VLPs 6 hr at 146,500 × *g*, 4°C, to pellet.

    This centrifugation serves to concentrate the VLPs.

19. Remove the supernatant from tubes and resuspend the VLPs in 10 to 20 µl of 10% sucrose in TNE buffer per 150-cm^2 flask.

VLP storage

20. Aliquot the VLPs into 2-ml vials and store at −80°C.

    VLPs stored in this way are stable. VLPs are also stable through multiple freeze-thaw cycles.

SMALL-SCALE VLP PURIFICATION

ALTERNATE PROTOCOL 2

This protocol describes methods for purification of small-scale preparations of VLPs. Applications that can use small-scale production of VLPs often do not require rigorous VLP purification, since detection of protein content is usually accomplished by western blotting or immunoprecipitation with specific antibodies. Thus, this protocol is a shortened and modified version of Basic Protocol 2. The following steps are based on the yield from one 35-mm plate.

Additional Materials *(also see Basic Protocol 2)*

Supernatants containing small-scale amounts of VLPs (Alternate Protocol 1)
TNE buffer (see recipe) or gel sample buffer (see recipe)

Beckman GS-6R centrifuge (or comparable)
20% and 65% sucrose solutions dissolved in TNE buffer (weight/volume)
Beckman ultracentrifuge (or comparable)
SW50.1 rotor and buckets or comparable
SW50.1 tubes

1. Collect supernatants from cells transfected as in Alternate Protocol 1 (combine all collected supernatants). Pellet cell debris by subjecting the supernatant to centrifugation 15 min at 4000 × *g*, 4°C.

2. Layer supernatant on top of 2.5 ml of 20% sucrose in an SW50.1 ultracentrifuge tube. Ultracentrifuge gradient 6 hr at 140,000 × *g* (35,000 rpm), 4°C.

   The VLPs will be in the pellet. Alternatively, for increased purity, VLPs can be banded at a 60%/20% sucrose interface as in Basic Protocol 2, steps 10 to 12, adjusting volumes for an SW50.1 centrifuge tube.

3. Remove the supernatant and resuspend the pelleted VLPs in 100 µl TNE buffer or gel sample buffer.

4. Store the VLPs at −20°C.

   VLPs may be frozen in aliquots to avoid repeated freeze-thawing. However, the VLPs are quite stable after multiple freeze-thaw cycles.

BASIC PROTOCOL 3

QUANTIFICATION OF PROTEIN CONTENT OF VLPs

For large-scale preparations, particularly, it may be important to quantify the protein content of the VLP preparations. Total protein content can be readily determined using standard protein assays such as the Bradford assay (kits are available for these assays, for example BioRad Bradford Quick Start Assay Kit). However, the concentration of a particular protein may also be important. For example, when VLPs are used as an immunogen, the amount of a particular protein injected into an experimental animal will be important in assessing immune responses to that protein. This protocol describes one method for quantifying the concentration of a particular VLP associated protein in a purified preparation.

Materials

Protein standard (e.g., BSA standards from BioRad Quick Start Protein Assay)
Large-scale purified VLPs (Basic Protocol 2)
Silver stain kit (Thermo Scientific) or Coomassie stain kit (Life Technologies or BioRad or comparable; also see *APPENDIX 3M*)

Rotator (platform shaker)
Gel scanner (Syngene G Box or comparable, *http://www.syngene.com/*)

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*APPENDIX 3M*)

1. Prepare a stock solution of a protein standard, as, for example, bovine serum albumin (1.0 mg/10 ml of water).

2. Separate proteins present in 1, 5, and 10 µl of the large-scale VLP preparation on a polyacrylamide gel (*APPENDIX 3M*). On the same gel, run 2, 5, 10, and 20 µl of the protein standard stock solution to generate a standard curve.

   Because 1 to 10 µl of an undiluted stock of VLPs can overload the polyacrylamide gel, depending upon the gel thickness and sample well size, it is often necessary to prepare a 10-fold dilution of the VLP stock and use 1, 5, and 10 µl of the diluted stock for electrophoresis.

3. Stain the protein bands using a protein-specific stain such as silver stain or Coomassie Brilliant Blue according to the manufacturers' instructions. Agitate gels during staining protocols using a rotator. See Figure 18.2.2 for an illustration of a silver stain of a polyacrylamide gel containing proteins in ND VLPs.

   Different proteins stain with different efficiencies depending upon the protein stain. Thus a rigorous assessment of the concentration of a particular protein will require separation of VLP proteins on several different polyacrylamide gels in order to accomplish different protein staining protocols.

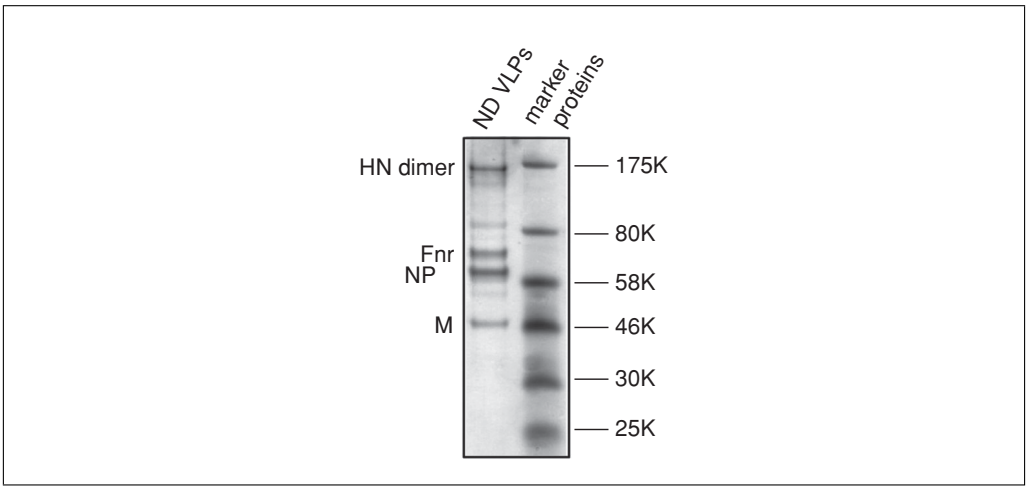

Figure 18.2.2 Silver stain of a preparation of ND VLPs. Proteins in purified VLPs were separated by polyacrylamide gel electrophoresis in a 10% gel in the absence of reducing agent. Molecular-weight protein markers were electrophoresed in an adjacent lane. The resulting gel was stained using a silver-staining kit, and the stained gel was scanned on an Epson printer/scanner. NDV proteins: M, matrix protein; NP, nucleocapsid protein; Fnr, fusion protein not reduced (disulfide linked F_1 and F_2); HN, hemagglutinin-neuraminidase protein, which forms a disulfide linked dimer under nonreducing conditions.

4. Quantify the protein bands in question using appropriate instrumentation that will detect and measure the stain used.

   Gel scanners are available for this application. In the absence of such instrumentation, an approximation of protein content can be made by scanning the stained gel, encased in plastic, on an office printer/scanner (for example, Epson or Hewlett Packard) and then quantifying the intensity of the bands using Photoshop.

5. Using the standard curve generated with a protein standard, estimate the concentration of VLP associated protein for each volume of VLP in order to calculate the concentration of the protein in the undiluted VLP stock.

USE OF WESTERN BLOTTING FOR CHARACTERIZATION OF VLP PROTEIN CONTENT

ALTERNATE PROTOCOL 3

If a purified protein version of a particular VLP protein is available or if a source of this protein with a known concentration is available, the concentration of a particular VLP associated protein can be determined using western blotting. This protocol also requires an antibody specific for the protein in question.

In the absence of a source of purified protein, this protocol can also be used to assess the relative incorporation of different versions of the same protein into VLPs.

Additional Materials *(also see Basic Protocol 3)*

Specific protein standard (same as protein of interest)
Large-scale purified VLPs (Basic Protocol 2) or small-scale purified VLPs (Alternate Protocol 2)
Western blot transfer system (iBlot gel transfer system from Invitrogen, or comparable)
Western blot chemiluminescence detection reagents (ECL from GE Health Care, or comparable)
PBS-Tween: phosphate-buffered saline (PBS; see recipe) containing 0.5% (v/v) Tween 20

Nonfat powdered milk
Protein-specific antibodies

Rotator
X-ray film (Thermo Scientific CL-XPosure Film) or comparable or instrument designed to detect western blots as, for example, Syngene G box)

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*APPENDIX 3M*) and western blotting (immunoblotting; see *UNIT 14.2*, Basic Protocol 1 and Gallagher et al., 2008)

1. Follow steps 1 and 2 of Basic Protocol 3, except use the specific purified protein in question as standard.

2. Transfer separated proteins from gel, using iBlot system or comparable, onto a PVDF membrane (included in the iBlot western blot system) according to manufacturer's directions.

   The iBlot Western Detection System is a dry transfer system that works quite well. Other types of western blot systems, including semi-dry transfer or wet transfer, can be used. See Basic Protocol 4 in UNIT 14A.2 or Gallagher et al. (2008).

3. Detect the protein in question on the membrane, using antibody specific for that protein, according to the following steps (also see *UNIT 14.2*, Basic Protocol 1 and Gallagher et al., 2008):

   The PVDF membrane should be continuously agitated during each step by placing it in a plastic box or a sealed plastic bag on a rotator.

   a. Incubate PVDF membrane (blot) containing proteins in PBS-Tween with 10% (w/v) powdered milk for 1 to 4 hr at room temperature or 4°C overnight.
   b. Incubate blot in PBS-Tween containing diluted primary antibody (1/1000 to 1/5000 dilution) for 1 hr at room temperature or overnight at 4°C.
   c. Wash blot extensively with PBS- Tween.
   d. Incubate blot for 1 to 2 hr at room temperature with diluted secondary antibody (anti-mouse or anti-rabbit antibody depending upon the primary antibody used) coupled to HRP at the dilution recommended by the supplier (often ∼1/40,000 dilution).
   e. Wash blot extensively in PBS-Tween.
   f. Incubate blot with chemiluminescence detection reagents as recommended by manufacturer.

4. Detect and quantify the signals generated from the western blot using X-ray film or instrumentation designed to detect western blot signals.

   Gel scanners are available for this application. In the absence of such instrumentation, an approximation of band intensity can be made by scanning the X-ray film used to detect signal on an office printer/scanner (for example, Epson or Hewlett Packard) and then quantifying the intensity of the bands using Photoshop. In detection of signal, insure that the signal obtained is in the linear range of the film or the instrumentation.

5. Follow step 5 in Basic Protocol 3 for quantification of the protein in question if a protein standard is available.

USE OF AUTORADIOGRAPHY FOR ASSESSMENT OF PROTEIN INCORPORATION INTO VLPs

ALTERNATE PROTOCOL 4

In the absence of an antibody specific for a VLP-associated protein, assessment of the incorporation of a protein into VLPs, relative to other versions of that protein or relative to the NP and/or M protein core components of the VLPs, can be accomplished by detecting radioactively labeled proteins in purified VLP proteins. Support Protocol 1 describes the generation of radioactively labeled VLPs. This protocol describes the detection and quantification of the radioactively labeled VLP proteins.

Materials

Radioactively labeled VLPs prepared in Support Protocol 1 and purified by Alternate Protocol 2

Gel dryer (BioRad or comparable)

X-ray film for detection of radioactively labeled proteins (Kodak BioMax XAR Film from Carestream Health, *http://www.carestream.com/*, 5 × 7 in.; cat. no. 05-728-36, or comparable)

X-ray film processor

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*APPENDIX 3M*)

1. Separate proteins in 1, 5, and 10 μl of a radioactively labeled VLP preparation on a polyacrylamide gel (*APPENDIX 3M*).

   The proteins in radioactively labeled VLPs can be directly separated on polyacrylamide gels or, alternatively, the VLPs can be solubilized in, for example, 1% Triton X-100 and the proteins then immunoprecipitated with protein specific antibody prior to separation on polyacrylamide gels, as described in Pantua et al. (2006).

2. Dry the gel.

3. Detect radioactively labeled proteins by autoradiography by placing, in a light-tight room, the dried gel onto X-ray film and then placing the film/gel in a light-tight envelope.

   The film should be firmly held against the completely dry gel by placing the light-tight envelope between two rigid boards, and the boards should be held together with clamps. Signals are enhanced by storing the film and gel together at −80°C. Exposure times can vary depending upon the levels of radioactively in the VLPs. Typically exposure times are from 12 to 72 hr. The film is developed in an X-ray film processor. Alternatively, radioactively labeled proteins can be detected using appropriate instrumentation.

4. Detect and quantify the signals generated using X-ray film or instrumentation designed to detect ^{35}S.

   Gel scanners are available for this application. In the absence of such instrumentation, an approximation of band intensity can be made by scanning the X-ray film used to detect signal on an office printer/scanner (for example, Epson or Hewlett Packard) and then quantifying the intensity of the bands using Photoshop. In detection of signal, insure that the signal obtained is in the linear range of the film or the instrumentation.

   Relative incorporation of different proteins into VLPs can be directly compared by this method. Calculations must take into consideration the methionine and cysteine contents of proteins being compared.

INCORPORATION OF FOREIGN GLYCOPROTEINS INTO ND VLPs

BASIC PROTOCOL 4

Full-length, foreign glycoproteins expressed with the NDV proteins are minimally incorporated into ND VLPs (unpub. observ.). Specific, directed incorporation of a foreign glycoprotein ectodomain into ND VLPs can be achieved by constructing a chimera

protein gene composed of sequences encoding the foreign protein sequences fused to those encoding the TM and CT domains of the appropriate NDV glycoprotein (Morrison, 2010; Murawski et al., 2010; McGinnes et al., 2011). Both type 1 and type 2 glycoproteins can be assembled into these particles. The NDV F protein is a type 1 glycoprotein; thus, foreign type 1 glycoprotein ectodomains can be fused to the transmembrane (TM) and cytoplasmic tail (CT) domains of the F protein. The NDV HN protein is a type 2 glycoprotein and type 2 glycoprotein ectodomains can be fused to the CT and TM domains of the HN protein (see Fig. 18.2.3).

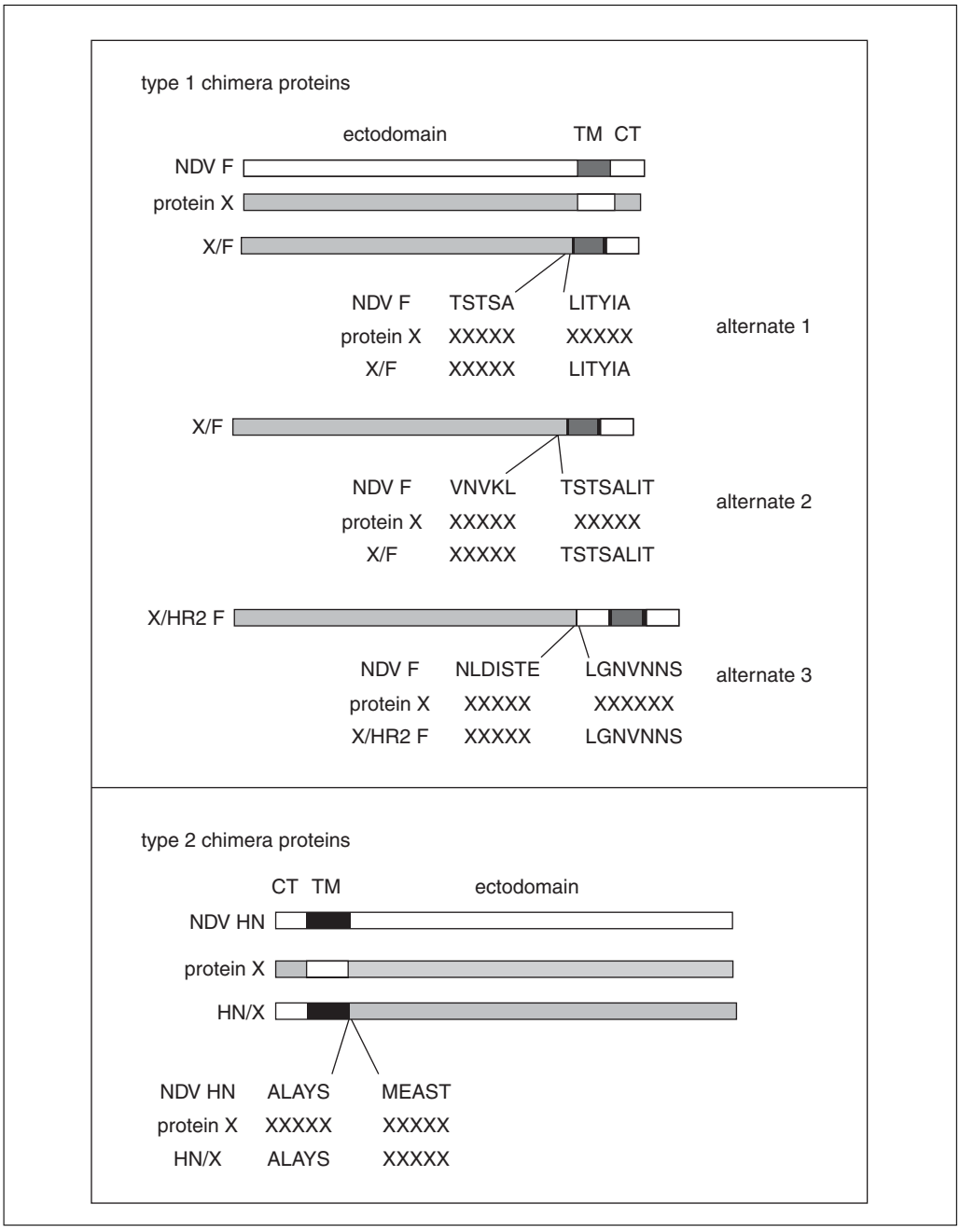

Figure 18.2.3 Protein junctions in chimera proteins. The top panel shows linear diagrams of type 1 glycoproteins with the ectodomain of NDV F protein replaced with that of protein X. The panel shows several alternative NDV F protein sequences at the chimera junction. The bottom panel shows linear diagrams of type 2 glycoproteins with the ectodomain of the NDV HN protein replaced with that of protein X. The panel shows the HN protein sequence at the junction of the chimera protein.

This protocol describes the logic for the construction of the chimera glycoproteins. Any candidate chimera proteins resulting from this construction must be characterized for cell-surface expression, since incorporation of the protein into the ND VLP platform requires that the chimera be expressed efficiently on cell surfaces. This protocol describes a method for analysis of the efficiency of that surface expression. Once candidate chimera proteins are identified, incorporation of chimera glycoproteins into VLPs is accomplished as described in Alternate Protocols 1 and 2. Efficiency of incorporation is assessed as described in Alternate Protocols 3 or 4. Once chimera proteins with optimal efficiency of incorporation into VLPs are defined, quantitative amounts of VLPs containing chimera proteins are generated, purified, and quantified as described in Basic Protocols 1, 2, and 3, respectively.

Additional Materials *(also see Alternate Protocols 1 and 2)*

Restriction enzymes
Tissue culture cells (ELL-0 are preferable but COS7, CHO, or 293T cell lines are acceptable; all are available at *http://www.atcc.org*)
PBS-CM (see recipe)
Sulfo-NHS-SS-biotin (Pierce)
Complete cell culture medium containing serum
Cell lysis buffer (see recipe)
Neutravidin agarose resin (Pierce)
Tween 20
10% sodium dodecyl sulfate (SDS)
Gel sample buffer (see recipe)

DNA synthesis facility (e.g., GeneWiz, Gene Art, Celtek Genes)
35-mm tissue culture plates
Rotator

Additional reagents and equipment for basic molecular biological techniques (primer design, PCR, restriction digestion, ligation; Ausubel et al., 2013), small-scale VLP generation (Alternate Protocol 1), polyacrylamide gel electrophoresis (*APPENDIX 3M*), and western blotting (immunoblotting; see Alternate Protocol 3; *UNIT 14.2*, Basic Protocol 1; and Gallagher et al., 2008), generation of VLPs containing chimera proteins (Alternate Protocol 2), and assessment of efficiency of incorporation of chimera protein into ND VLPs (Alternate Protocol 3 or 4)

Construction of chimera proteins

1. For incorporation of a type 1 glycoprotein into ND VLPs, design chimera protein junctions using NDV F protein TM domain sequences and the sequences at the carboxyl terminus of the ectodomain of the foreign glycoprotein.

   See Figure 18.2.3 for the suggested fusion junctions. Three alternatives that have been successful with specific foreign glycoproteins are illustrated. Alternatively, changing the specific NDV sequences (such as the TSTSA sequence at the junction of the NDV F ectodomain and TM domain) at the junction sites have also been successful. The carboxyl terminus of the ectodomain of the foreign protein at the fusion junction may also be varied.

2. For incorporation of a type 2 glycoprotein into ND VLPs, design of chimera protein junctions using NDV HN protein TM domain sequences and the amino terminal sequences of the ectodomain of the foreign glycoprotein.

   See Figure 18.2.3 for the suggested fusion junction. This junction has been successful for a number of different type 2 glycoproteins.

3. Generate chimera protein genes. See Ausubel et al. (2013) for the described techniques.

   This can most easily be accomplished by ordering the codon-optimized gene encoding the chimera protein from one of numerous companies offering such services (for example, GeneWiz, Gene Art, Celtek Genes). Request that the DNA be inserted into the expression plasmid of choice, for example, the pCAGGs vector.

   Alternatively, the chimera gene can be made using standard PCR protocols as documented in McGinnes et al. (2011) and Murawski et al. (2010). For type 2 glycoproteins, the HN protein CT and TM domain sequences should be generated by PCR using primers that will encode restriction enzyme sites XhoI at the 5′ end and NotI at the 3′ end. The ectodomain of the type 2 glycoprotein of interest should be generated by PCR with primers containing restriction sites NotI at the 5′ end and MscI (or any restriction site generating blunt ends upon digestion) at the 3′ end.

   Care should be taken to insure that ligation of the NotI sites at the ends of the HN TM domain and the glycoprotein ectodomain will result in a protein sequence in frame without addition of extra amino acids. Computer programs to identify restriction sites to insert or mutate without changing translation are useful (for example, EMBOSS 6.3.1:silent).

   For type 1 glycoproteins, the F protein TM and CT domain sequences should be generated by PCR using primers with restriction enzyme sites SnaB1 at the 5′ end and MscI at the 3′ end (as in Fig. 18.2.3, "alternate 1"). The sequences of the ectodomain of a glycoprotein of interest should be generated by PCR using primers containing XhoI at the 5′ end and SnaB1 at the 3′ end.

   Care should be taken to ensure that ligation of the SnaBI sites at the ends of the F protein TM domain and the glycoprotein ectodomain will result in a protein sequence in frame, without addition of new codons (and, therefore, additional amino acids at the chimera junction).

4. Generate plasmid DNA encoding candidate chimera type 2 protein by ligating pCAGGs vector cut with *Xho*I and *Msc*I with the HN CT and TM domain sequences (flanked by *Xho*I and *Not*I sites and the sequences encoding the ectodomain of the type two protein of interest (flanked by *Not*I and *Msc*I sites). Transform competent *E. coli* with ligated molecules and select ampicillin-resistant colonies.

5. Generate plasmid DNA encoding candidate chimera type 1 protein by ligating pCAGGs vector cut with restriction enzymes *Xho*I and *Msc*I with DNA sequences encoding the ectodomain of the type 1 glycoprotein of interest, flanked by *Xho*I and *Sna*B1 sites, and DNA sequences encoding the TM and CT domains of the NDV F protein, flanked by *Sna*B1 and *Msc*I sites.

6. Prepare plasmid DNA stocks from *E. coli* transformants and verify candidate chimera genes by sequencing the DNA inserted into pCAGGs vector.

7. Generate large-scale preparations of plasmid DNA encoding candidate chimera genes.

Assessment of surface expression of candidate chimera proteins

Transfection of cells

8. The day before transfection, seed 35-mm plates with the tissue culture cells so cells will be 60% to 65% confluent the next morning.

9. Transfect cells growing in 35-mm plates with plasmids encoding chimera candidate proteins using Alternate Protocol 1.

   Include transfection of cells with the wild-type version of the foreign protein as a positive control for surface expression.

10. Incubate transfected cells at 37°C for 40 to 48 hr.

Biotinylation of cell surfaces

11. Wash cell monolayer three times, each time with 2 ml PBS-CM (cold).

12. Add 1 ml of PBS-CM and incubate at room temperature for 10 min, then remove PBS-CM.

13. Add 1 ml PBS-CM containing 0.5 mg/ml Sulfo-NHS-SS-biotin. Incubate at room temperature for 30 min.

    Sulfo-NHS-SS-biotin will covalently link the biotin to primary amines such as the side chain of lysines in proteins. By incubating the reagent (which does not cross cell membranes) with intact cells, only cell-surface proteins are modified by biotin, and the biotin thus serves as a marker for cell surface expression.

14. Remove buffer with Sulfo-NHS-SS-biotin, add 2 ml complete cell culture medium, and incubate 5 min at room temperature, remove and wash cells three times with PBS-CM.

15. Lyse cells with 400 µl of cell lysis buffer (without reducing agent).

    The presence of reducing agent will cleave the biotin from proteins.

Immunoprecipitate cell-surface proteins containing covalently linked biotin

16. Add 100 µl of cell extract to a fresh microcentrifuge tube and add 50 µl of washed neutravidin agarose resin.

    To wash neutravidin-agarose resin, mix bottle well and remove what is needed (e.g., 50 µl multiplied by the number of samples). Pellet resin for 30 sec in a microcentrifuge at 10,000 rpm. Wash twice with PBS containing 0.5% (v/v) Tween 20. Resuspend to original volume in PBS containing 0.5% Tween 20. Streptavidin resin can also be used in place of neutravidin-agarose.

17. Mix extract-resin mixture at 1 hr at room temperature (or overnight at 4°C).

    Mixing can be accomplished automatically by using a rotator. Biotinylated surface proteins are bound to the neutravidin coupled to resin.

18. Pellet resin–biotinylated protein complexes 30 sec at 10,000 rpm in a microcentrifuge, remove supernatant, and wash resin-protein complexes three times with 1 ml of PBS containing 0.5% Tween 20 plus 0.2% SDS. After the final wash, pellet the resin-protein complexes by microcentrifuging 2 min at 10,000 rpm, 4°C, and remove all traces of supernatant.

19. Resuspend the pellet in 30 µl gel sample buffer (without reducing agent if the protein is to be detected using the biotin label), heat 5 min in boiling water bath, and pellet resin by microcentrifuging 2 min at 10,000 rpm, 4°C.

    The biotinylated cell-surface proteins are in the supernatant. Do not use reducing agent in the gel sample buffer if the protein is to be detected using the biotin label, since the biotin will be removed. If the protein is to be detected by western blots using protein specific antibody or by autoradiography of radioactively labeled proteins, then reducing agent in the sample buffer will not affect protein detection.

20. Load 15 to 30 µl of the supernatant onto a polyacrylamide gel (*APPENDIX 3M*). Load aliquots of total cell extract in parallel lanes. After electrophoretic separation of proteins, transfer separated proteins to a PVDF membrane. Perform a standard western blot to detect proteins of interest using appropriate antibody as described in Alternate Protocol 3.

    Alternatively, all cell-surface biotinylated proteins can be detected by western blots using HRP-coupled neutravidin for detection, or by autoradiography of radioactively labeled proteins.

Quantitative analyses of amounts of surface protein (biotinylated molecules precipitated with neutravidin-agarose) and total cell protein (in total cell extracts) allow determination of the efficiency of surface expression of the chimera protein. Inclusion of control monolayers transfected with the cDNAs encoding the wild-type foreign protein will serve as a positive control for efficient surface expression. Chimera proteins efficiently expressed on cell surfaces (comparable to wild-type protein) are candidates for inclusion into ND VLPs.

Assessment of the efficiency of incorporation of a chimera protein into VLPs

21. Generate VLPs containing chimera proteins using Alternate Protocol 1.
22. Purify VLPs containing chimera proteins using Alternate Protocol 2.
23. Assess the efficiency of incorporation of a chimera protein into ND VLPs using Alternate Protocols 3 or 4.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cell lysis buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
10 mM NaCl
1.5 mM $MgCl_2$
1% (v/v) Triton X-100 (1 ml of a 10% stock solution per 10 ml buffer)
0.5% (w/v) sodium deoxycholate (0.5 ml of a 10% stock solution per 10 ml buffer
25 mg/ml *N*-ethylmaleimide (25 mg ethylmaleimide per 1.0 ml buffer)
Store up to 1 hr at 4°C

DMEM, supplemented

Dulbecco's modified Eagle medium (DMEM; e.g., Life Technologies) supplemented with:
10% fetal bovine serum (FBS; numerous sources including Life Technologies) *or* dialyzed fetal bovine serum
100 U/ml penicillin (add from concentrated pen/step, 10,000 U penicillin/10,000 μg/ml streptomycin; e.g., Life Technologies)
100 μg/ml streptomycin (add from concentrated pen/step, 10,000 U penicillin/10,000 μg/ml streptomycin; e.g., Life Technologies)
1× vitamins (obtained as a 100× stock solution from Life Technologies)
1× glutamine (obtained as a 100× stock solution from Life Technologies)
Store up to 3 months at 4°C

Other additions to the medium will depend upon the requirements of the cells used. For example, some cells require added amino acids (nonessential amino acids).

Gel sample buffer, 2×

20% (v/v) glycerol
0.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
4% (v/v) bromphenol blue (0.4 ml of a 10% solution per 10 ml buffer)
10% (w/v) sodium dodecyl sulfate
0.1 M 2-mercaptoethanol (optional)
Store up to 1 month at room temperature

PBS-CM

Prepare phosphate-buffered saline (PBS; see recipe) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Store up to 6 months at room temperature.

Phosphate-buffered saline (PBS)

145 mM NaCl
7.6 mM K_2HPO_4
2.4 mM KH_2PO_4
Adjust pH to 7.2
Store up to 1 year at room temperature

TNE buffer

0.150 M NaCl
0.025 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.005 M EDTA
Store up to 1 year at room temperature

COMMENTARY

Background Information

VLPs are particles with sizes similar to authentic virus. They contain repeating protein complexes in ordered arrays on their surfaces and in their cores similar to those of infectious viruses (reviewed in Noad and Roy, 2003; Jennings and Bachmann, 2008). VLPs derived from nonenveloped virus proteins are empty capsids structurally similar to those of infectious virus. VLPs derived from enveloped virus proteins may contain surface glycoproteins that are properly folded and inserted into membranes in repeating arrangements typical of the enveloped virus. Internal core or capsid proteins in enveloped VLPs are also likely folded and assembled in ways that are typical of a virus. The protocols described here were developed for the production and purification of VLPs formed with structural proteins of Newcastle disease virus, an enveloped virus.

Newcastle disease virus (NDV) is a paramyxovirus. Paramyxoviruses are enveloped, negative-stranded RNA viruses (Collins and Crowe, 2007; Karron and Collins, 2007; Lamb and Parks, 2007). All paramyxovirus virions contain three membrane proteins. Two of these proteins are glycoproteins, an attachment protein, termed HN protein for Newcastle disease virus, and a fusion (F) protein. The third membrane protein is a nonglycosylated matrix protein (M protein), which lines the inner surface of the membrane. The virus also contains three core proteins, the nucleocapsid protein (NP), which binds to the RNA genome, as well as a phosphoprotein (P) and the viral polymerase, the L protein. It has been reported that paramyxovirus VLPs can be produced upon expression of the M protein or M protein and various combinations the glycoproteins and NP (Coronel et al., 1999; Takimoto et al., 2001; Schmitt et al., 2002; Sugahara et al., 2004; Ciancanelli and Basler, 2006; Patch et al., 2007; Li et al., 2009). Indeed, cells expressing the NDV HN, F, NP, and M proteins release particles that both structurally and functionally resemble virus particles (Pantua et al., 2006; McGinnes et al., 2010). What distinguishes ND VLPs from other paramyxovirus VLPs, and, indeed, from many other types of VLPs, is their efficiency of release (Pantua et al., 2006). As a result, quantitative amounts of these particles are relatively easy to prepare even from transiently transfected cells (McGinnes et al., 2010; McGinnes et al., 2011). They can be purified using protocols modified from those used for virus purification; the purified VLPs show minimal cell protein contamination. Furthermore, the ratios of viral proteins are similar to those in virus particles.

ND VLPs contain biologically active glycoproteins, indicating that they have folded into an authentic conformation during VLP assembly. Similar to virion-associated HN protein, the VLP-associated HN protein mediates cell binding and possesses neuraminidase activity (McGinnes et al., 2010). ND VLPs have hemagglutination titers comparable to equivalent amounts of virus (McGinnes et al., 2010). Typical of virion-associated F protein, the VLP-associated F protein can direct the fusion of the VLP membrane with red blood cell membranes (McGinnes et al., 2010).

In a murine model, ND VLPs are effective immunogens. Levels of soluble antibodies, characterized by ELISA and by neutralizing antibody titers, resulting from ND VLP immunization are as high or higher than those resulting from immunization with inactivated virus (McGinnes et al., 2010). Furthermore, ND VLPs stimulate T cell responses at levels slightly higher than those stimulated by the vaccine virus (McGinnes et al., 2010).

The protocols described here can be readily adapted for VLPs produced by proteins from other enveloped viruses. Indeed, we have used these protocols for the generation of quantitative amounts of VLPs formed by expression of West Nile Virus structural proteins (unpub. observ.).

The protocols described here are not the only ones suitable for generation and purification of VLPs. The generation of VLPs requires significant levels of expression of the viral structural proteins in cells that will efficiently assemble and release these particles. The protocols described here utilize transient transfection of avian cells. While avian cells most efficiently release these particles, other cell types, such as COS7 cells or 293T cells, can be used with only a slight decrease in yields (Murawski et al., 2010; McGinnes et al., 2011). Another widespread method for the production of VLPs is the use of baculovirus-insect cell expression systems (for example, (Ye et al., 2006; Kang et al., 2009; Quan et al., 2011). Baculoviruses can be engineered to encode VLP proteins. Infection of insect cells with these viruses often results in very efficient release of VLPs. This is an excellent method for cost effective, large-scale production of VLPs. Indeed, this is a preferred method if the particular proteins are efficiently released as VLPs from insect cells. Use of baculovirus-insect cell systems for VLP production, however, must take into consideration that VLP preparations can be contaminated with infectious baculoviruses. In addition, the glycosylation of glycoproteins in insect cells is somewhat different than that in mammalian or avian cells. Furthermore, there are VLPs that are not efficiently released from insect cells infected with recombinant baculoviruses, including ND VLPs. Thus alternative methods are required for production of these VLPs, one of which is described here.

The protocol for purification of VLPs described here utilizes differential centrifugation. However, other protocols utilizing column chromatography and size-exclusion chromatography are possible.

Not all virus systems can be adapted to produce virus-like particles. Some systems do not yield VLPs at levels sufficient for their use for various applications, notably as vaccine candidates (see, e.g., McGinnes et al., 2011). To overcome this problem, established, well characterized VLPs have been adapted for assembly of foreign glycoproteins and peptides into particles. Several different approaches have been reported. One approach, described here, uses directed incorporation of a foreign protein into ND VLPs by creating chimera proteins with NDV domains. Alternatively, the passive incorporation of wild-type, intact proteins into VLPs has been reported. For example, influenza HA glycoprotein has been incorporated into a VLP formed with lentivirus gag (Haynes et al., 2009). The RSV G and F proteins have been passively incorporated into influenza VLPs (Quan et al., 2011). The efficiency of this passive incorporation is not well characterized. Passive incorporation of wild-type foreign glycoproteins into ND VLPs is extremely inefficient, requiring the use of chimera proteins described here.

In another approach, immunodominant molecules can be chemically cross-linked to the surfaces of VLPs (reviewed in Jennings and Bachmann, 2008). Alternatively, key epitopes of a target virus have been genetically fused to a structural protein of a well characterized VLP. If a sequence of a protein have been identified as a domain that stimulates neutralizing antibody responses, incorporation of this domain into a VLP platform could stimulate these antibodies. Incorporation of T cell epitopes could enhance the ability of the VLPs to stimulate cell-mediated immune responses to specific pathogens. There are many variations on this approach (for example, Sadeyen et al., 2003; Saini and Vrati, 2003; Deml et al., 2005; Grgacic and Anderson, 2006; Halsey et al., 2008; Jennings and Bachmann, 2008; Fu et al., 2009; Li et al., 2009), but most of these chimera VLPs have been reported for lentivirus gag, the HBcAg, or the HBsAg VLPs.

Critical Parameters and Troubleshooting

The most important parameters are the condition of the cells and the efficiency of expression of the VLP proteins.

Table 18.2.1 lists possible problems that may arise with the protocols in this unit, along with their possible causes and solutions.

Anticipated Results

For preparation of ND VLPs, transfections of 60 to 75 150-cm^2 plates will yield ~1.5 ml of 2-4 mg/ml of total protein of purified VLPs.

Time Considerations

Large-scale transfections (Basic Protocol 1): Seeding of cells for transfection and transfection will take 2 days. Harvesting of cell supernatants containing VLPs proceeds for 96 to 110 hr.

Table 18.2.1 Troubleshooting Guide for Working with VLPs

Problem	Possible cause(s)	Possible solution(s)
Inefficient VLP release	Cell type used	Use a different cell type (preferably ELL-0 cells)
	Condition of cells	Cells need to be in log phase growth at the time of transfection
	Altered cell type due to multiple passages	Use a new clone of cells
	Inefficient transfection	Use a different transfection reagent Clean up plasmid preparations
	VLPs are rebinding to cells	Inhibit VLP binding by including, for example, neuraminidase or heparin
	Cell pH	Replace medium when cell supernatants become acid
Poor incorporation of a glycoprotein into VLPs	Detection of the protein	Use another antibody for detection Use another protein stain
	Poor surface expression of chimera protein	Adjust chimera construction by changing the junction sequences
Host cell contamination of large scale VLP preparations	Condition of the cells	Monitor pH of medium
		Eliminate last VLP collection

Small-scale transfections (Alternate Protocol 1): Times are the same as for large-scale transfections, although harvesting of cell supernatants can be terminated after 72 hr.

Large-scale VLP purification (Basic Protocol 2): The time required for the multiple centrifugations is between 40 and 50 hr.

Small-scale VLP purification (Alternate Protocol 2): This more modest purification requires approximately 7 hr.

Quantification of protein content of VLPs (Basic Protocol 3): This protocol requires polyacrylamide gel electrophoretic separation of proteins as well as protein using silver staining or Coomassie Brilliant Blue staining of gels. Either staining procedure can be accomplished in 8 hr.

Quantification of protein content by western blotting (Alternate Protocol 3): The protocols involved will require ~2 days.

Construction of chimera proteins (Basic Protocol 4, steps 1 to 2): Depending upon the complexity of the construction, this protocol can be accomplished in 1 to 2 weeks.

Assessment of surface expression (Basic Protocol 4, steps 8 to 20): This protocol requires 48 hr for transfection and incubation of cells. Biotinylation, immunoprecipitation, and western blots will require a minimum of 2 days.

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