



Protocol

A protocol for the gentle purification of virus-like particles produced in plants



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A B S T R A C T

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The purpose of the protocol is to extract and purify virus-like particles (VLPs) that have been produced in plants. More specifically, this method is well suited to the purification of chimaeric and genetically modified VLPs that do not have native surface properties. This will be the case for VLPs used in antigen display experiments. Such particles are often more fragile than their wild-type infectious virus counterparts, and as such can be damaged or lost during procedures that involve pelleting or precipitating the particles. The method presented here is based on ultracentrifugation and density gradients, with no pelleting or precipitation step. It makes virtually no assumptions about the yield of recombinant VLPs or their properties, which means that this protocol is ideally suited to screening new constructs which are expected to lead to the formation of VLPs. This protocol will allow the researcher to determine whether the construct does indeed form VLPs, and if it does, will reduce the likelihood of those particles being lost or damaged during the purification process. Because of its non-specific nature, this protocol may also be suited to the purification of viruses of unknown nature from leaf material where an infection is suspected.

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1. Type of research

i. **Fuscaldo et al. (1971)** described the need for gentle purification methods when dealing with eastern equine encephalitis virus (EEE virus). In particular, they argued that pelleting was liable to damage virus particles. The proposed solution was to use

chromatography followed by a single sucrose cushion, then a sucrose gradient to achieve gentle purification. However, this protocol was based on the assumption that infectious EEE virus was always present in the animal cell cultures that were used to produce the virus. This made the chromatography step reliant on solid information about the properties of the virions being produced.

ii. **Yeh and Iwasaki (1972)** described a purification method for panencephalitis virus nucleocapsids that relied on an initial concentration step over a double sucrose cushion followed by a caesium chloride density gradient.

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- iii. Gugerli (1984) described the purification of many different types of plant viruses thanks to isopycnic centrifugation using Nycodenz. The authors found that Nycodenz was suitable for all plant viruses tested. For some species of viruses, Nycodenz was superior to sucrose or caesium chloride gradients.
- iv. Sathananthan et al. (1997) found that herpes simplex virus type 1 could be purified with a Nycodenz gradient, and that this gave slightly superior results than a Ficoll gradient.
- v. Moon et al. (2014) found that a sucrose gradient was a useful tool in the purification of VLPs of three different plant viruses which were produced in *Nicotiana benthamiana*.

Time required: the complete protocol normally takes 2–3 days.

The breakdown is as follows:

- Leaf harvest and preparation: 30 min
- Leaf disruption and filter: 15 min
- Clarification centrifugation: 20 min
- Syringe filtration (optional): 10 min
- Double sucrose cushion (including preparation and fractionation time): 3.5 h
- Dialysis: 3 h or overnight
- Concentration: 1–4 h
- Nycodenz gradient (including preparation and fractionation time): 3–24 h
- Fraction analysis (SDS-PAGE and/or western blot): 4 h–1 day
- Dialysis: 1–3 days
- Concentration (optional): 1–2 h
- Electron microscopy (including grid preparation): 30 min

2. Materials

2.1. Special equipment

Waring blender (One Cummings Point Road, Stamford CT 06902-7901, U.S.A.) or equivalent, Miracloth (Merck Millipore, Croyley Green Business Park, Watford, Hertfordshire WD18 8YH, UK) or equivalent, syringe filters (such as Minisart syringe filters from Sartorius, Longmead Business Centre, Blenheim Road, Epsom, Surrey, KT19 9QQ, UK), Ultracentrifuge (Thermo Scientific Sorvall WX floor ultracentrifuge, or equivalent), ultracentrifuge swing-out rotor (example: TH641 or Surespin 630/36 from Thermo Scientific, 81 Wyman Street Waltham, MA USA 02451), Ultra-Clear ultracentrifuge tubes (Beckman Coulter, Oakley Court Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK), SpeedVac vacuum concentrator (Thermo Scientific) or equivalent.

2.2. Chemicals and reagents

- Sodium phosphate (Sigma Aldrich, St. Louis, Missouri, United States)
- cOmplete protease inhibitor cocktail tablets (Roche, Grenzacherstrasse 124 CH-4070 Basel, Switzerland) or equivalent
- Sucrose (Sigma Aldrich, St. Louis, Missouri, United States)
- Ammonium bicarbonate (Sigma Aldrich, St. Louis, Missouri, United States)
- Nycodenz (Axis-Shield PoC AS, P.O. Box 6863, Rodelokka, N-0504 Oslo, Norway)

3. Detailed procedure

- i. The agroinfiltrated leaves are harvested and a razor blade or scalpel can be used to remove the areas of the leaves that were not agroinfiltrated. Indeed these non-infiltrated areas will not contain recombinant protein if the expression system used

was non-replicating (such as the pEAQ vector suite) or non-moving (such as a deconstructed potyvirus-based system); or if a moving system was used but given insufficient time for viral movement to take place.

- ii. The agroinfiltrated leaf material is weighed.
- iii. In a Waring blender (Waring, One Cummings Point Road, Stamford CT 06902-7901, U.S.A.), the leaf tissue is mixed with three volumes of chilled extraction buffer: 0.1 M sodium phosphate, pH 7.2, supplemented with cOmplete protease inhibitor cocktail tablets (Roche, Grenzacherstrasse 124 CH-4070 Basel, Switzerland). For example, 60 g of leaf tissue is mixed with 180 ml of extraction buffer. While this simple buffer with a neutral pH is a good starting point when extraction of a particular virus or VLP has not been optimised, optimisation of the buffer conditions and pH may increase recovery. The leaf tissue is homogenised using the blender in a cold room (maximum speed, 30–60 s).
- iv. The homogenate is filtered through a layer of Miracloth (Merck Millipore, Croyley Green Business Park, Watford, Hertfordshire WD18 8YH, UK). Alternatively, muslin cloth can be used, but this will take longer.
- v. The primary filtrate is centrifuged at $15,000 \times g$ for 20 min at 4°C . The pellet (insoluble fraction) can be kept for insoluble protein fraction analysis if desired. The supernatant (soluble fraction) is recovered.
- vi. Optional: the soluble fraction can be filtered with $0.45 \mu\text{m}$ syringe filters (Merck Millipore, Sartorius, Longmead Business Centre, Blenheim Road, Epsom, Surrey, KT19 9QQ, UK). This will help to provide a cleaner interface fraction in the subsequent sucrose cushion. However, syringe-filtering large volumes may be impractical, as the filters will get clogged with large impurities in the extract. Syringe filters with glass fibre pre-filters are available to partially mitigate this issue. It is also important to note that a small volume will always be lost in each syringe filter that is used.
- vii. Two sucrose solutions, at 25% and 70% (w/v) are prepared in 0.1 M sodium phosphate, pH 7.2. Any spin-out (swinging-bucket) ultracentrifuge rotor can be used depending on the volume of extract to be processed; two example rotors will be given here. With a TH-641 ultracentrifuge spin-out rotor (Thermo Scientific, 81 Wyman Street Waltham, MA USA 02451), the tubes to use are Ultra-Clear 13 ml, $14 \times 89 \text{ mm}$. The double sucrose cushion is prepared by pouring the plant extract in the tube, then carefully underlaying 2 ml of 25% sucrose underneath the extract, then 0.25 ml of 70% sucrose underneath the previous sucrose layer thanks to a long needle. Ultracentrifugation then takes place at 40,000 rpm ($274,000 \times g$) for 2.5 h at 4°C . The TH-641 rotor has six buckets that each hold 13 ml tubes, so the maximum volume of leaf extract that can be processed simultaneously is about 66 ml (which corresponds to 22 g of leaf tissue). With the larger Surespin 630/36 spin-out rotor (Thermo Scientific), the tubes to use are Ultra-Clear 36 ml, $25 \times 89 \text{ mm}$. The double sucrose cushion is prepared by pouring the plant extract in the tube, then carefully underlaying 5 ml of 25% sucrose underneath the extract, then 1 ml of 70% sucrose. Ultracentrifugation then takes place at 30,000 rpm ($167,000 \times g$) for 3 h at 4°C . The Surespin 630/36 rotor has six buckets that each hold 36 ml tubes, so the maximum volume of leaf extract that can be processed simultaneously is about 180 ml (which corresponds to 60 g of leaf tissue).
- viii. After ultracentrifugation, a thick green band will be visible at the interface between the 25% and 70% sucrose layers. VLPs will typically co-sediment slightly below, but will overlap with, this green band. The bottom of each tube is pierced with a

needle and the sucrose is allowed to drip into a collection tube. If the 70% fraction and the interface (green band) fractions are collected, this should include all of the VLPs present in the sample. If only the 70% fraction is collected, the recovered sample will be cleaner, but will not contain all of the particles.

- ix. The recovered VLP sample is dialysed against 20 mM ammonium bicarbonate, pH 8.5 (Sigma Aldrich, St. Louis, Missouri, United States). Note that the alkaline pH may be unsuitable for some viruses or VLPs and may need to be optimised. Also note that due to the high osmotic pressure of the sucrose, the volume of the dialysate can increase 2–3-fold.
- x. If the interface fraction from the sucrose cushion in step viii was included, the dialysate can be clarified by centrifugation at $15,000 \times g$ for 20 min at 4 °C. Alternatively, or in addition, the dialysate can be filtered through a 0.2 μm syringe filter. Note that a small volume is always lost in the syringe filter.
- xi. The dialysate is then concentrated in a SpeedVac vacuum concentrator (Thermo Scientific). Note: the ammonium bicarbonate will decompose to volatile compounds as the water evaporates. The sample will spontaneously remain cold while evaporation is taking place, but will heat up rapidly after the end of concentration due to the ambient heat in the vacuum concentrator. To avoid heat shock, the sample should be placed on ice immediately after centrifugation/concentration has finished. Because impurities will concentrate along with the sample, short (10 min) centrifugation in a microcentrifuge is recommended halfway through concentration in order to pellet some of the impurities, particularly if the sample is being concentrated more than 5-fold. The sample should not be concentrated to dryness. The desired final volume depends on the downstream application. If further purification is required, a final volume of 2 ml is appropriate for the Nycodenz gradient step described in step xii. Alternatively, the sample can be concentrated further for SDS-PAGE or transmission electron microscope (TEM) analysis.
- xii. Further purification can be achieved with a Nycodenz gradient. If using a TH641 ultracentrifuge rotor, an UltraClear 14×89 mm ultracentrifuge tube should be used. Solutions of Nycodenz (Axis-Shield PoC AS, P.O. Box 6863, Rodelokka, N-0504 Oslo, Norway) at 20, 30, 40, 50, and 60% should be prepared and the gradient is set up by pouring the plant extract in the tube, then using a long needle to carefully underlay 2 ml of each of the Nycodenz solutions underneath the concentrated extract. Ultracentrifugation then takes place at 40,000 rpm ($274,000 \times g$) for 3 h at 4 °C. Alternatively, the extract can be mixed with a solution of 40% Nycodenz and ultracentrifuged for 16–24 h: the gradient will form spontaneously.
- xiii. After ultracentrifugation, the green contaminants will have sedimented towards the top of the gradient, while VLPs will typically sediment elsewhere in the gradient, usually below the green impurities. The VLPs might appear as a distinct iridescent band in the Nycodenz, or could instead appear as a more diffuse brown-grey band. The gradient should be fractionated by piercing the bottom of the tube with a needle and collecting 1 ml fractions. Alternatively, visible bands in the Nycodenz can be collected by piercing the side of the tube just below the band with a needle, then aspirating the band with a syringe. The collected fractions can be analysed by SDS-PAGE or western blotting.
- xiv. The Nycodenz fractions containing recombinant protein can then be checked for presence of VLPs by transmission electron microscopy (TEM) and the protein content quantified using a Modified Lowry Assay. Note that Nycodenz will interfere with UV/Vis spectroscopy measurements.

4. Results

Blended leaf material forms a thick slurry which is significantly more fluid after filtration over Miracloth. The clarification centrifugation step yields a thick green pellet and a brownish supernatant. The double sucrose cushion will always result in a green band of dense impurities forming at the interface between the 25% and 70% sucrose layers, but the exact nature of the extraction buffer (in particular the addition of detergent) can affect how thick and diffuse this green band is. To illustrate, Fig. 1a shows ultracentrifuge tubes after a double sucrose gradient which concentrates and partially purifies fluorescently labelled VLPs. In this case, the VLPs are “anti-GFP tandibodies”: hepatitis B core (HBcAg)-based VLPs with an anti-GFP camelid nanobody genetically fused to a surface-exposed loop, and with GFP bound to those surface-exposed nanobodies via antibody–antigen interaction (Peyret et al., 2015). These VLPs have been expressed in *Nicotiana benthamiana* using the pEAQ-HT expression system (Sainsbury et al., 2009; Peyret and Lomonosoff, 2013). If VLPs are present in the sample, they should be detectable in the 70% sucrose fraction as well as in the interface, although the 70% fraction will normally look cleaner (Fig. 1b). Dialysis of sucrose fractions will generally lead to a significant increase in sample volume unless care is taken to prevent this. This is overcome by concentration of the sample on the vacuum concentrator. Other methods such as centrifugal filter concentrators may be used but often require pure starting material in order to avoid clogging the filter unit during centrifugation. Moreover, some VLPs may adsorb onto the filters, thereby reducing recovery.

If the interface fraction from the sucrose cushion is recovered along with the 70% fraction, the green impurities will be concentrated during vacuum evaporation. To address this, the concentrate can be centrifuged to remove some of these impurities, but the Nycodenz gradient will separate them from the VLPs much more efficiently. The green impurities will typically form a band of lower density than the VLPs (depending on the exact density of the VLPs in question), so fractionation of the Nycodenz gradient can easily be done in such a way as to separate VLPs from impurities (Fig. 2a). The fractions containing VLPs may appear as a single iridescent band, multiple bands (if there are sub-populations of material, as in Fig. 2b) or a diffuse band (if the population is very heterogeneous). TEM analysis of Nycodenz gradient fractions containing VLPs will appear cleaner than fractions obtained from the sucrose cushion (Fig. 2b). In a typical purification (using a Surespin 630/36 ultracentrifuge rotor), 60 g of fresh-weight infiltrated leaf material will form 180 ml of extract, which will be concentrated to 12 ml after the sucrose cushion (15-fold concentration, assuming both the 70% and interface fractions are collected), and 2 ml after the Nycodenz gradient (90-fold concentration, assuming the recombinant protein of interest is spread over two recovered fractions). After Nycodenz purification, the sample can be concentrated even further by dialysing against ammonium bicarbonate and concentrating by vacuum evaporation.

5. Discussion

The protocol described here is suitable for determining whether VLPs are formed after recombinant protein expression in plants. Because the protocol avoids harsh chemical treatment, chromatography, precipitation, and pelleting, it is suitable for fragile VLPs. Moreover, this protocol allows for significant concentration of the sample, which is very useful for detecting VLPs produced in low yield. In essence, the only assumption that is made with this protocol about the properties of the recombinant protein is that the protein forms particulate material which will migrate through a sucrose cushion and Nycodenz gradient. Every correctly assembled

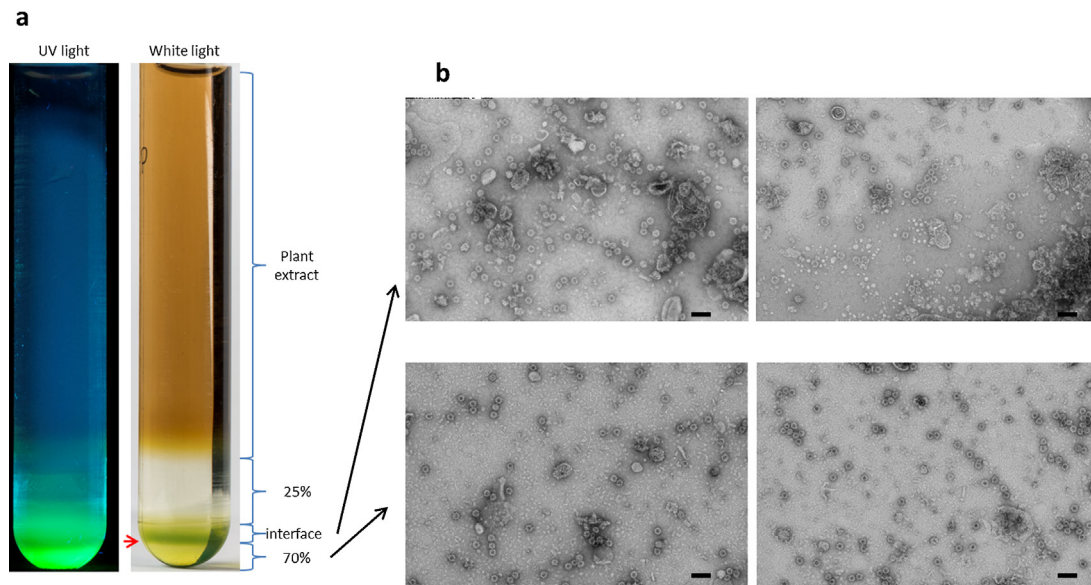


Fig. 1. Using a double sucrose cushion to concentrate and partially purify VLPs. (a) UV light (left) and white light (right) photographs of a 14×89 mm ultracentrifuge tube after ultracentrifugation. A visible band of green impurities (red arrow) will sediment at the interface between the 25% and 70% sucrose layers. VLPs will sediment within that interface layer and in the 70% sucrose layer below. For clarity, the VLPs used here are fluorescently labelled and can be visualised under UV light. (b) Comparison of the interface (top two micrographs) and 70% sucrose fractions (bottom two micrographs). While VLPs are found in both, the 70% sucrose fraction is noticeably cleaner. All scale bars are 100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

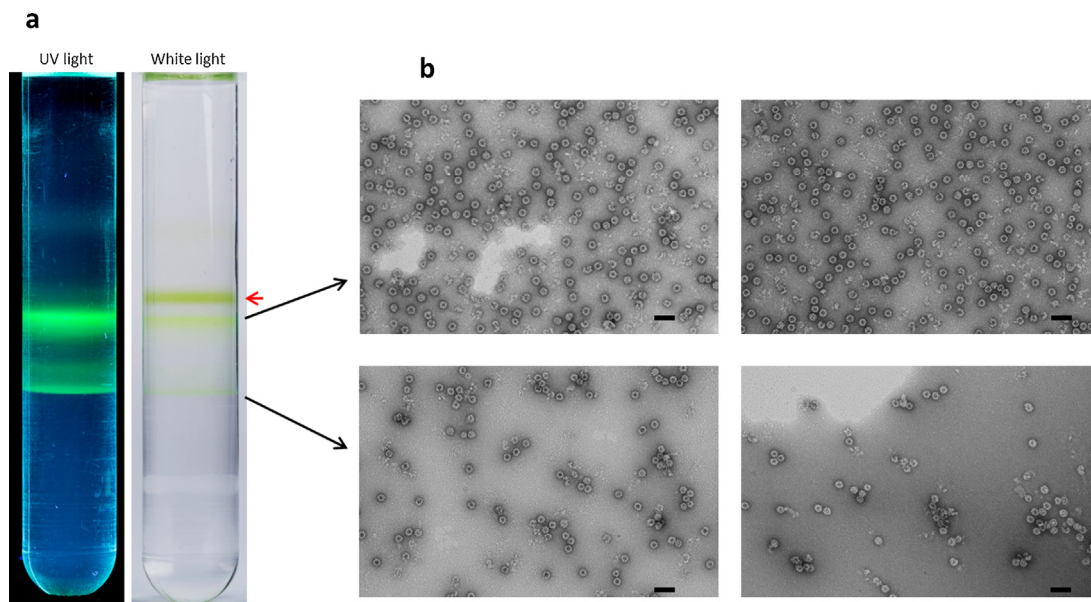


Fig. 2. Using a Nycodenz gradient to purify VLPs. (a) UV light (left) and white light (right) photographs of a 14×89 mm ultracentrifuge tube after ultracentrifugation. The impurities will form a green band (red arrow), which will be separate from the VLPs (fluorescent bands), allowing greater purification than the sucrose cushion alone. Because this method of purification is based on density, the Nycodenz gradient can also separate subpopulations of VLPs present in a sample, as seen here with two distinct fluorescent bands. (b) TEM analysis of these bands reveals that both contain VLPs. The difference between the two sub-populations is due to nucleic acid content. Note that TEM analysis of the Nycodenz-purified VLPs indicates that they are cleaner than after the double sucrose cushion alone (see Fig. 1). All scale bars are 100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

virion or VLP (and even some incorrectly assembled ones) should fulfil this assumption. This protocol is therefore a powerful tool for testing whether a gene construct directs the formation of VLPs in plants, and information gained from completing this protocol can be used as a starting point to optimise extraction and purification conditions for the specific VLP that is being produced. Because of its non-specific nature, this protocol can also be used to purify plant viruses for which an optimised protocol has not been developed. It should be noted that the use of centrifugation imposes a limit on the volume of plant extract that can be handled at any one time, which makes this protocol unsuitable for significant scale-up.

5.1. Trouble-shooting

i. TEM analysis of the sucrose fraction does not reveal VLPs.

It is useful to collect four different fractions from the sucrose cushion: the 70% sucrose fraction, the interface fraction, the 25% sucrose fraction, and the supernatant. These different fractions can be analysed by SDS-PAGE and/or western blot. If the recombinant protein is found in the supernatant or the 25% sucrose fraction, but not in the interface or the 70% sucrose fraction, it is unlikely that VLPs have formed.

- ii. **Western blot analysis does not reveal the presence of recombinant protein in any of the fractions from the sucrose cushion.** This suggests that the recombinant protein could be insoluble: the pellet from step v (clarification of the crude filtrate) should be resuspended in a small volume of buffer and analysed by western blotting. If the recombinant protein is not detected in the pellet or the supernatant of the clarification spin, then the protein was probably not expressed in the plants (or at least not accumulated).
 - iii. **The majority of the recombinant protein is insoluble.** Because this protocol allows for the sample to be concentrated, VLPs can still be detected even if only a small percentage of the recombinant protein is present in the soluble extract (so long as this soluble protein forms VLPs).
 - iv. **VLPs are detected in the sucrose cushion fractions but not in the Nycodenz gradient fractions.** Western blot analysis should be carried out on the sucrose fractions before and after dialysis, after vacuum evaporation, and on all of the Nycodenz gradient fractions. The VLPs could have been lost during dialysis if the equipment malfunctioned, or during vacuum evaporation if the samples were not placed on ice immediately after concentration (as this can cause the sample to heat up very quickly, which might denature the VLPs).
 - v. **Recombinant protein is detected in the 70% sucrose fraction but VLPs are not seen after dialysis in ammonium bicarbonate.** It is possible that the recombinant protein forms large aggregate structures that can migrate into the sucrose cushion, but are not recognisable as VLPs by electron microscopy. It is also possible that ammonium bicarbonate (which is alkaline) causes disassembly of some VLPs. The sucrose fractions could be dialysed against deionised water for concentration by vacuum evaporation, or against another buffer such as phosphate-buffered saline (PBS) for immediate purification with Nycodenz. Whatever the buffer used, VLPs should not be spun to dryness, and tubes should be placed on ice immediately after vacuum evaporation to avoid heating the samples.
 - vi. **VLPs are present before the Nycodenz gradient but are not visible in the fractions after the gradient.** While unlikely, it is possible that Nycodenz has a deleterious effect on the VLPs. Other gradient-forming agents such as Ficoll, sucrose, or caesium chloride could be used.
- ## 6. Quick Procedure
- i. Harvest protein-producing leaf tissue.
 - ii. Weigh the leaf tissue.
 - iii. In a cold room, homogenise the leaf tissue in three volumes of extraction buffer (0.1 M sodium phosphate, pH 7.2, supplemented with cOmplete protease inhibitor cocktail tablets) in a Waring blender.
 - iv. Filter the homogenate through a layer of Miracloth.
 - v. Centrifuge the primary filtrate at 15,000 × g for 20 min at 4 °C and recover the supernatant.
 - vi. Optional: Filter the clarified lysate with 0.45 μm syringe filters.
 - vii. In an ultracentrifuge tube, prepare a double sucrose cushion by carefully underlying a thick layer of 25% sucrose underneath the clarified lysate and a thin layer of 70% sucrose underneath the 25% sucrose layer. Centrifuge the samples at maximum speed (160,000–270,000 × g depending on the ultracentrifuge rotor used) for 2.5–3 h (depending on the rotor used) at 4 °C.
 - viii. Pierce the bottom of the ultracentrifuge tube with a needle and recover the bottom (70% sucrose) fraction and the interface fraction between the 25% and 70% sucrose layers.
 - ix. Dialyse the recovered VLPs against 20 mM ammonium bicarbonate, pH 8.5.
 - x. Syringe-filter the dialysate with a 0.2 μm syringe filter and/or centrifuge at 15,000 × g for 20 min at 4 °C and recover the supernatant.
 - xi. Concentrate the sample by vacuum evaporation using a Speed-Vac until a final volume of 2 ml is reached.
 - xii. Load the concentrated sample onto a 20–60% Nycodenz gradient in an ultracentrifuge tube. If using the recommended TH641 rotor, centrifuge at maximum speed (274,000 × g) for at least 3 h at 4 °C.
 - xiii. Fractionate the gradient by piercing the bottom of the ultracentrifuge tube and collecting 1 ml fractions as they drip out. Alternatively if iridescent bands are clearly visible in the Nycodenz gradient these can be aspirated with a syringe and needle inserted just below the band of interest.
 - xiv. The Nycodenz fractions containing recombinant protein can then be checked for presence of VLPs by TEM and the protein content quantified (after removal of the Nycodenz by dialysis) using a Modified Lowry Assay.

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