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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

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.

Contents

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, Croxley 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 potexvirus-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, Croxley 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 × *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 μm 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 × 89 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 × *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 × 89 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 × *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