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Recombinant Expression of Tandem-HBc Virus-like Particles (VLPs)

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Summary

The core protein of hepatitis B virus (HBc) has formed the building block for virus-like particle (VLP) production for more than thirty years. The ease of production of the protein, the robust ability of the core monomers to dimerise and assemble into intact core particles, and the strong immune responses they elicit when presenting antigenic epitopes all demonstrate its promise for vaccine development (reviewed in (1)). HBc has been modified in a number of ways in attempts to expand its potential as a novel vaccine platform. The HBc protein is predominantly α-helical in structure and folds to form an L-shaped molecule. The structural subunit of the HBc particle is a dimer of monomeric HBc proteins which together form an inverted T-shaped structure. In the assembled HBc particle the four-helix bundle formed at each dimer interface appears at the surface as a prominent 'spike'. The tips of the 'spikes' are the preferred sites for the insertion of foreign sequences for vaccine purposes as they are the most highly exposed regions of the assembled particles. In the tandem-core modification two copies of the HBc protein are covalently linked by a flexible amino acid sequence which allows the fused dimer to fold correctly and assemble into HBc particles. The advantage of the modified structure is that the assembly of the dimeric subunits is defined and not formed by random association. This facilitates the introduction of single, larger sequences at the tip of each surface 'spike', thus overcoming the conformational clashes contingent on insertion of large structures into monomeric HBc proteins.

Differences in inserted sequences influence the assembly characteristics of the modified proteins, and it is important to optimise the design of each novel construct to maximise efficiency of assembly into regular VLPs. In addition to optimisation of the construct, the expression system used can also influence the ability of recombinant structures to assemble into regular isometric particles. Here, we describe the production of recombinant tandem-core particles in bacterial, yeast and plant expression systems.

Key words: Hepatitis B core (HBc), HBc VLPs, Escherichia coli expression, Pichia pastoris expression, plant expression.

1. Introduction

1.1. Production of Tandem-HBc VLPs in E. coli

The ease and speed of production and the large volumes of culture that can be readily handled highlight the key advantages of producing hepatitis B virus (HBV) core protein (HBc) virus-like particles (HBc VLPs) in E. coli. Following transformation with the expression plasmid, the bacteria can be grown to large scale prior to induction of protein expression. After collection by centrifugation, the bacterial cells are ruptured by sonication and the proteins then precipitated from the clarified lysate. The resuspended precipitated material is centrifuged through a sucrose cushion to concentrate and partially purify particulate material. After resuspension, the VLPs are further purified by centrifugation through a sucrose gradient. Gradient fractions are analysed by SDS-PAGE followed by Coomassie Brilliant Blue staining and/or silver staining and relevant fractions pooled, dialysed, and the protein content quantified prior to further analysis (see **Fig. 1** for outline of procedures). The proteins can be produced at 37°C or at lower temperatures to enhance particle assembly if necessary.

1.2. Production of Tandem-HBc VLPs in Yeast

Recombinant expression of protein in yeast offers a cost-effective approach to producing VLPs in a simple eukaryotic expression system. Genetic manipulation of yeast cells is relatively straightforward, and transformed cells can be grown to very high densities before induction of recombinant protein expression. Thus, large quantities of VLPs can be produced in industrial-scale fermenters. This section describes methods used to express tandem-HBc VLPs in Pichia pastoris transformed using a pPICZ C (Invitrogen) vector containing the tandem-HBc sequence. Briefly, transformed cells are grown in a simple medium to a high cell density before

induction by addition of methanol. 3 - 4 days after induction, the cells are harvested, pelleted by centrifugation and lysed by vortex mixing with glass beads. The soluble material is clarified by centrifugation and filtration, and VLPs purified by size exclusion chromatography or by centrifugation through sucrose gradients as for VLPs produced in E. coli (see **Fig. 2** for outline of procedures). Yields of up to 40 mg HBc VLP have been achieved from 1 litre culture.

1.3. Production of Tandem-HBc VLPs in Nicotiana benthamiana

Plants are a useful alternative for the production of tandem-HBc particles, both for the quality of the particles obtained and for the wide-ranging modifications to the basic tandem-HBc particle that are compatible with correct assembly (2). The expression host used is the solanaceous plant Nicotiana benthamiana, and the particles are expressed using transient expression initiated by Agrobacterium-mediated gene transfer. The pEAQ vector system (3) is the expression system of choice, and the extraction and purification of tandem core particles is based on the method described in (4). The leaf material is processed using a blender, and after clarification of the crude extract the particles are concentrated and partially purified by sedimentation through a double-layered sucrose cushion. The sample can then be further purified by Nycodenz-based density gradient centrifugation (as described in section 3.3) (see **Fig. 3, 4, 5** for outline of procedures). Yields of 250 mg HBc VLP have been achieved from 1 kg of fresh-weight leaf tissue.

1.4. Characterisation of Tandem-HBc VLPs

The ultimate goal of designing, constructing and expressing VLPs for assessment as potential vaccines is to provide particulate products which are pure, defined, uniform in structure, and which possess desired antigenic features. Hence it is necessary to analyse the expressed VLPs to ensure that they have these desired characteristics. A number of methods are available to analyse VLPs, and the most widely used and suitable are described here.

For downstream characterisation of the tandem cores, it is imperative that the sucrose and solutes in the preparation are removed. The procedure to do this is described in **3.4.1**. We follow

the procedure laid down in (5).

Transmission Electron Microscopy (TEM) is used to examine the size and architecture of the electron dense material, and to check for the presence of T=3 and T=4 HBc particles (see **Fig. 6** and **3.4.2.**).

Western blot analysis can demonstrate the presence of the HBc protein, but to confirm the presence of assembled tandem-HBc particles, immunogold TEM analysis of the sample is required. The sample is applied to the grid and is then incubated with an HBc-specific primary antibody, followed by incubation with a secondary antibody conjugated with gold (see **Fig. 7** and **3.4.3.**).

Should the sample contain electron dense material of unexpected architecture, an immunoadsorbent TEM assay can be used to quickly screen for immunoreactive electron dense material (adapted from (6)). The grid is incubated with the primary antibody, exposed to the sample, and then washed (see **Fig. 8** and **3.4.4.**).

Dynamic Light Scattering (DLS), where the scattered backlight of a beam passing through the solution is analysed, is useful for both confirming the size of the HBc VLPs and calculating the polydispersity of the preparation, and is currently routinely used for analysing VLP preparations (9) (see **3.4.5.**).

2. Materials

All solutions are prepared using deionized water, if not stated otherwise.

2.1. Production of Tandem-HBc VLPs in E. coli

2.1.1. Equipment

1. High speed centrifuge (Avanti J-26XP, Beckman Coulter or Heraeus Megafuge 16 R, Thermo Scientific) with appropriate bucket and rotor (e.g. SW40 or SW32). (see **Note 1**)
2. Sonicator (e.g. Soniprep 150, MSS150, MSE with a MSE-SH100 process

timer). (see **Note 2**)

3. Equipment for SDS-PAGE and staining of the gels. (see **Note 3**)
4. Equipment for western blotting (Membrane: nitrocellulose or PVDF membranes, 0.2µm pore size).
5. Gradient maker (either a Gradient station ip, BIOC MP ~~{ Cat. #: 153-002 }~~ or by using a dual chamber { SG 30 ~~[product code 80619780]~~ Gradient Maker or SG 15 Gradient Maker ~~[SG15]~~ depending on the rotor})

2.1.2. Reagents

1. LB medium: 10 g/Tryptone, 5 g/L yeast extract, 10 g/L NaCl; adjust to pH 7.0 - 7.5 using NaOH. Autoclave; if needed add appropriate antibiotics (30 µg/ml kanamycin) directly before usage.
2. Plasmid: The tandem core constructs in our laboratory are cloned into the protein expressing pET28b backbone, which has a kanamycin resistance selection cassette.
3. Competent bacteria: BL21 DE3, Rosetta II (see **Note 4**)
4. 30 mg/µl kanamycin stock (or other appropriate antibiotics). (see **Note 5**)
5. LB agar: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; adjust to pH 7.0 - 7.5 using NaOH, add 5 g/L agar. Autoclave; add 30 µg/ml kanamycin directly before pouring plates.
6. Lysis buffer: 20 mM HEPES (), 250 mM sodium chloride in deionised water; adjust to pH 7.5 with NaOH and autoclave, then add one protease inhibitor tablet (Roche Complete, Mini, EDTA- free) and 1 µl benzonase nuclease (Novagen, 70746) per 10 ml buffer.
7. Capsid buffer: 20 mM HEPES, 250 mM sodium chloride in deionised water; adjust to pH 7.5 with NaOH and autoclave, then add 2mM dithiothreitol (DTT). (see **Note 6**)
8. 20 % and 60 % (w/v) sucrose() in capsid buffer.

9. 1M IPTG(S02122, Glycon) stock, frozen. (see **Note 7**)
10. 4.32 M ammonium sulphate solution: saturated aqueous solution of ammonium sulphate. (see **Note 8**)
11. 1 X TBS-T: 10mM Tris pH7.5, 150 mM NaCl, 0.1% Tween-20
12. Blocking buffer: 5% (w/v) milk powder in 1 X TBS-T
13. Primary antibody: Mouse mAb IgG1 antibody 10E11 (anti-hepatitis B virus core antigen antibody ab8639, Abcam), diluted 1:1000 in blocking buffer.
14. Secondary antibody: Anti mouse IgG peroxidase, produced in goat (product number A 5278, Sigma Aldrich), diluted 1:2000 in blocking buffer.
15. Reagents for Coomassie Brilliant Blue and/or silver staining (ProteoSilver Silver Stain Kit, PROTSIL1-1KT, Sigma) of SDS-PAGE gels. (see **Note 9**)

2.2. Production of Tandem-HBc VLPs in Yeast

2.2.1. Equipment

1. Sterile McCartney bottles.
2. Autoclave.
3. Water baths.
4. Sterile 250 ml and 2 L baffled flasks.
5. Shaking incubator (25°C).
6. Sterile 1.5 ml microtubes.
7. Electroporator (e.g. MicroPulser, Bio Rad).
8. 2mm gap electro-cuvettes.
9. Centrifuge tubes.
10. Superspeed centrifuge (e.g. Beckman Avanti J-25 or equivalent) with appropriate bucket and rotor. (see **Note 1**)
11. Microcentrifuge
12. Vortex mixer (or multi-vortexer – clamped vortex mixer for tube racks).

13. Glass beads - Retsch 0.75µm-1.00µm (VWR).
14. 0.22 PES filter.
15. MegaDalton cross-flow filtration cartridge: Pelicon XL Ultrafiltration Module, Biomax 1000 kDa.
16. Bio Rad NGC / Akta FPLC system.
17. GE HiPrep 16/60 Sephacryl S-500 HR column.
18. Centrifugal concentration device (optional).

2.2.2. Reagents

1. Pichia pastoris KM71H cells
2. Plasmid: pPICZC-HBcAg
3. Restriction Enzyme**:** Pme I
4. HEPES stock solution (pH 4.0)
5. YEPD broth (1 L): 20 g bacto-peptone, 10 g yeast extract; adjust to pH 4.0 with HCl, then add further deionised water to 900 ml. Autoclave, then add 100 ml sterilised 20 % (w/v) glucose (200 mg/L).
6. YEPD agar (1 L): 20 g bacto-peptone, 10 g yeast extract; Add deionized water to 800ml adjust to pH 7.0 with NaOH if necessary, then add 16 g agar and deionised water to 900 ml. Autoclave, then add 100 ml sterilised 20 % (w/v) glucose (200 mg/L). Add 100 mg Zeocin^{TM} (100 µg / µl) at 50 °C just prior to pouring plates.
7. YEPDS broth and agar: supplement YEPD broth and agar with 0.5 M sorbitol (91 g/L sorbitol).
8. 1M DTT in deionized water. (see **Note 6**)
9. 70 % (v/v) Ethanol.
10. Isopropanol.
11. 99.9% (v/v) Methanol.
12. 1 M sorbitol.

13. BMMY induction medium (1 L): 10 g yeast extract, 20 g peptone, 2.0 g yeast nitrogen base (DIFCO 239210), add deionised water to 890 ml. Autoclave and then add 100 ml sterile 1 M potassium phosphate (pH 6.0), 2.0 ml 0.02 % biotin (w/v), 10 ml methanol.
14. BMGY expression medium (1 L): 10 g yeast extract, 20 g peptone, 2.0 g yeast nitrogen base (DIFCO 239210), add deionised water to 800 ml. Autoclave, then add 100 ml sterile 1 M potassium phosphate (pH 6.0), 2.0 ml 0.02% biotin, 100 ml filter-sterilised 10 % glycerol.
15. Lysis buffer: 20 mM Tris (pH 8.5), 5 mM EDTA, 5 mM DTT, Pierce protease inhibitor tablets (EDTA-free). (see **Note 6**)
16. 1 X PBS (pH 7.4): Life Technologies PBS tablets (ThermoFisher #003002) prepared according to manufacturer's instructions.

2.3. Production of Tandem-HBc VLPs in Nicotiana benthamiana

2.3.1. Equipment

1. Razor blade or scalpel.
2. Waring blender or equivalent.
3. Miracloth (Merck Millipore) or equivalent.
4. Needle
5. 0.45 µm and 0.2 µm syringe filters.
6. Long needle (e.g. Sigma steel 304 syringe needle, or equivalent).
7. 1 ml needle-less syringe
8. Ultracentrifuge (Thermo Scientific Sorvall WX floor ultracentrifuge or equivalent). (see **Note 10**)
9. Ultracentrifuge swing-out rotor (e.g. TH641 or Surespin 630/36 from Thermo Scientific). (see **Note 11**)
10. Ultra-Clear ultracentrifuge tubes (Beckman Coulter).
11. 13 ml ultracentrifuge tubes (Ultra-Clear 14 x 89 mm)

12. Dialysis equipment (such as 100 kDa molecular-weight cut-off Float-a-Lyzer from Spectrum Labs)
13. SpeedVac vacuum concentrator() or equivalent.
14. Equipment for SDS-PAGE and staining of the gels. (see **Note 3**)
15. Equipment for western blotting (Membrane: Amersham nitrocellulose membrane from GE Life Sciences).
16. Optional: Equipment for TEM

2.3.2. Reagents

1. Competent strain of E. coli (such as TOP 10) and suitable growth medium (such as LB broth or agar, see **2.1.2. Item 1 or 5**).
2. Competent A. tumefaciens strain LBA4404 and suitable growth medium: LB broth or agar, see **2.1.2. Item 1 or 5 complemented with rifampicin,** or 2 X YT: (31 g of 2 X YT broth from Formedium in 1 l de-ionised water, pH adjusted to 7.4 with NaOH).
3. pEAQ-HT system (3) or other suitable plant expression vector.
4. Kanamycin (use at 50 µg/ml from a 50 mg/ml stock made in water) and rifampicin (use at 50 µg/ml from a 50 mg/ml stock made in DMSO) for selection of transformed bacteria.
5. N. benthamiana plants (3 – 4 weeks after pricking out, typically one week before the start of flowering).
6. MMA buffer: 10 mM MES (pH 5.6), 10 mM magnesium chloride, 0.1 mM acetosyringone.
7. 0.1 M sodium phosphate buffer: 3.1 g/L $NaH_2PO_4*H_2O$, 10.9 g/L Na_2HPO_4; which will give sodium phosphate at pH 7.2 – 7.5. Chill to 4°C prior to usage.
8. Protease inhibitor (such as cOmplete EDTA-free protease inhibitor cocktail tablets from Roche)
9. 25 % and 70 % (w/v) sucrose in sodium phosphate buffer.
10. 20 mM ammonium bicarbonate solution, pH 8.5.

11. 20 %, 30 %, 40 %, 50 % and 60 % (w/v) Nycodenz (Axis-Shield PoC AS) solutions in sodium phosphate buffer or PBS (see **2.2.2. Item 17**).
12. 1 X PBS (pH 7.4): Life Technologies PBS tablets (ThermoFisher #003002) prepared according to manufacturer's instructions.
13. PBS-T: PBS supplemented with tween-20 at 0.1% (v/v).
14. Blocking buffer: 5 % (w/v) milk powder in 1 X TBS-T
15. Primary antibody: Mouse mAb IgG1 antibody 10E11 (anti-hepatitis B virus core antigen antibody ab8639, Abcam), diluted 1:6000 in blocking buffer.
16. Secondary antibody: Anti mouse IgG peroxidase, produced in goat (product number A 5278, Sigma Aldrich), diluted 1:10000 in blocking buffer.
17. Reagents for Coomassie Brilliant Blue and/or silver staining (ProteoSilver Silver Stain Kit, PROTSIL1-1KT, Sigma) of SDS-PAGE gels. (see **Note 9**)

2.4. Characterisation of Tandem-HBc VLPs

2.4.1. Removal of Sucrose and Salts by Dialysis of Tandem-HBc VLPs

1. Spectra/Por1 dialysis membrane standard RC tubing (MWCO: 6-8 kD, part number 132655).
2. Dialysis clips.
3. Foam floaters.
4. Dialysis buffer: 10mM HEPES, 100 mM sodium chloride 1 mM Ethylenediaminetetraacetic acid (EDTA) (see **Note 12**). Adjust pH to 7.2 with NaOH and autoclave, then add 1 mM dithiothreitol (DTT). (see **Note 6 and 13**)

2.4.2. Examination of Tandem-HBc VLPs by Transmission Electron Microscopy (TEM)

1. Freshly glow-discharged formvar/carbon-coated 400 mesh grids (copper) ((S162-4, Agar Scientific) (see **Note 14**).
2. Parafilm.

3. Grid box.
4. Tweezers (either antiparallel or regular with an O-ring, dumoxel or titanium number 7 or equivalent). (see **Note 15**)
5. Whatman No1 filter paper ((L4164, Agar Scientific), torn into small triangles.
6. Automatic pipettes.
7. Stop watch (or watch with second hand).
8. Transmission electron microscope.
9. Wash buffer: 20mM Tris(hydroxymethyl)aminomethane (Tris), adjusted to pH 8.0 with HCl.
10. 2% (w/v) aqueous solution of uranyl acetate (see **Note 16 and 17**)

2.4.3. Immunogold TEM Analysis

1. Tweezers (either antiparallel or regular with an O-ring, dumoxel or titanium number 7 or equivalent). (see **Note 15**)
2. Freshly glow-discharged carbon/formvar-coated 400 mesh nickel grids ((S162-4/S162N-4, Agar Scientific) (see **Note 14 and 18**)
3. Automatic pipettes.
4. Stop watch (or watch with second hand).
5. Grid box.
6. Whatman No1 filter paper ((L4164, Agar Scientific), torn into small triangles.
7. Fine-tipped Pasteur pipettes.
8. Parafilm.
9. Sample dilution buffer: 20mM Tris(hydroxymethyl)aminomethane (Tris), adjusted to pH 8.0 with HCl.
10. TBS: 10 mM Tris pH 7.5, 150 mM NaCl (24.2 g Tris, 80g NaCl adjusted to pH 7.5 with HCl for 1 litre of 10 X
11. Immunogold block buffer (IBB): 0.05 % (w/v) cold water fish skin gelatine (G7765,

Sigma), 0.025 % Tween-20 (w/v) ()in TBS.

12. Immunogold diluent (ID): 0.005 % (w/v) cold water fish skin gelatine (G7765, Sigma) in PBS (see **2.2.2. Item 17**, 2 % (w/v) aqueous solution of uranyl acetate(). (see **Note 16**)
13. Primary antibody: mouse mAab IgG1 antibody 10E11 (anti-hepatitis B virus core antigen antibody ab8639, Abcam), diluted in 1:25 in ID. (see **Note 19 and 20**)
14. Secondary antibody: goat anti-mouse IgG colloidal gold conjugate antibody 10 nM gold (EM.GMHL 10, British Biocell) diluted 1:25 in ID. (see **Note 19**)
15. Deionized water.
16. Negative stain: 2% (w/v) aqueous solution of uranyl acetate. (see **Note 16 and 17**)
17. Transmission electron microscope.

2.4.4. Immunoadsorbent TEM Assay

1. Tweezers (either antiparallel or regular with an O-ring, dumoxel or titanium number 7 or equivalent). (see **Note 15**)
2. Freshly glow-discharged carbon/formvarcoated 400 mesh nickel (or copper) grids (S162-4/S162N-4, Agar Scientific) (see **Note 14 and 18**)
3. Automatic pipettes.
4. Grid box.
5. Whatman No1 Filter paper (L4164, Agar Scientific), torn into small triangles.
6. Fine-tipped Pasteur pipettes.
7. Parafilm.
8. Sample dilution buffer: 20mM Tris(hydroxymethyl)aminomethane (Tris) (adjusted to pH 8.0 with HCl.
9. Immunogold block buffer (IBB): 0.05 % (w/v) cold water fish skin gelatine (G7765, Sigma), 0.025 % (w/v) Tween-20 ()in TBS (see **3.4.3. Item 10**).
10. Immunogold diluent (ID): 0.005 % (w/v) cold water fish skin gelatin (G7765, Sigma)

in PBS (see **2.2.2. Item 17**), 2 % (w/v) aqueous solution of uranyl acetate. (see **Note 16**)

11. Primary antibody: mouse Mab IgG1 antibody 10E11 (anti-hepatitis B virus coreantigen antibody ab8639, Abcam), diluted in 1:25 in ID. (see **Note 19 and 20**)
12. Negative Stain: 2% (w/v) aqueous solution of uranyl acetate. (see **Note 16 and 17**)
13. Transmission electron microscope.

2.4.5. Dynamic Light Scattering (DLS)

1. Malvern Zetasizer Nano series. (see **Note 21**)
2. DTS0012 cuvette.
3. Sample to be measured (minimum total volume 1 ml, minimum concentration 2.5 ng/µl, diluted in 1 X PBS (see **2.2.2. Item 17**)). (see **Note 22**)

3. Methods

Always use appropriate personal protective equipment when handling bacteria and antibiotics.

3.1. Production of Tandem-HBc VLPs in E. coli

1. Transform the plasmid into competent bacteria for protein expression . Unlike the standard process for streaking transformed bacteria, plate the culture as for transformation of a ligation reaction (with an incubation time of at least one hour in SOC or LB medium). Incubate transformants on LB agar supplemented with the appropriate antibiotic (typically kanamycin) overnight at 37°C.
2. Pick a colony and grow as a starter culture in 10 ml LB medium with the appropriate antibiotic (30 µg/ml for kanamycin-R plasmid) overnight (37°C, 180 rpm shaking).
3. Add 2.5 ml of starter culture to 250 ml LB medium containing the antibiotic (30µg/ml for kanamycin-R plasmid)(see **Note 23**). Measure optical density (OD) at 600 nm every hour after inoculation (see **Note 24**); use LB medium as blank.. When the OD 600 is 0.6-0.8 (see **Note 25**), remove 25 ml and culture separately overnight (16°C) or for 3 h (37°C). This is the uninduced control culture. To the remainder, add 1 mM IPTG and

incubate at 37°C for 3 h or 16°C overnight. This is the induced culture.

4. Chill the cultures on ice, then pellet the bacteria by centrifuging at 7000 x g for 20 min. Pellets may be stored at -20°C as cell paste; otherwise resuspend in lysis buffer (4 ml lysis buffer to the pellet obtained from 100 ml bacterial culture). Sonicate the resuspended pellet on ice for 30 s with 30 s intervals at 10 mA, repeating the cycle until the solution clarifies (see **Note 26 and 27**). Centrifuge the sonicated culture at 7000 x g for 20 min to separate cell debris from soluble protein and re-centrifuge the collected supernatant at 7000 x g for 20 min to further clarify. Slowly add saturated ammonium sulphate solution (to a final concentration of 40 % saturated) to the supernatant over ice before incubating at 4°C overnight to allow precipitation. Analyse samples of the uninduced and induced pellets and supernatants by PAGE to check for expression and solubility of the required protein.

5. Centrifuge at 7000 x g for 20 min, then discard the supernatant and resuspend the pellet in capsid buffer (1 ml capsid buffer to 100 ml bacterial culture). Clarify the suspension by further centrifugation at 17,000 ×g for 10 min. Layer the suspension over a 60 % sucrose cushion (sucrose in capsid buffer), then centrifuge at 151,000 ×g for 3 h (see **Note 1**). Discard the supernatant and resuspend the pellet overnight in capsid buffer.

6. Layer the suspension over a 20 – 60% continuous sucrose gradient (sucrose in capsid buffer) and centrifuge at 150,000 ×g for 3 h using the appropriate bucket and rotor (see **Note 1**). Collect 1 ml (SW40) or 2 ml (SW32) fractions. Separate the fractions by SDS-PAGE on a gel of appropriate acrylamide percentage (12.5 or 15% depending on the size of the recombinant tandem core construct being analysed), then stain with Coomassie Blue. The same gel can be destained and used for silver staining according to the manufacturer's instructions. Analyse appropriate fractions by Western blotting: following transfer onto a membrane, block with 5% milk powder in 1 X TBS-T prior to application of the primary and secondary antibody.

3.2. Production of Tandem-HBc VLPs in Yeast

1. For transformation of P. pastoris with pPICZC-HBcAg, linearise at least 1 µg of plasmid with restriction enzyme Pme I to completion. Precipitate the linearised plasmid by adding 0.7 volumes of isopropanol and place at 4°C for at least 30 min. Centrifuge at ≥ 12,000 × g for 10 min. Remove the liquid very carefully, taking care not to disturb the very small, translucent pellet that will have formed on the outer wall of the bottom of the microtube. Wash the pellet with 1 ml 70 % ethanol (do not aspirate the pellet) and repeat centrifugation as previously. Carefully remove the ethanol and allow the pellet to dry, then resuspend the dried pellet in 10 µl ddH_2O.
2. To prepare Pichia cells for transformation, inoculate 5 ml YEPD medium with a single colony of P. pastoris KM71H cells and grow overnight at 25°C. The following afternoon, inoculate 40 ml YEPD medium with a range of volumes of the overnight culture from 0.1 – 0.7 ml (e.g. set up four flasks and inoculate with 0.1, 0.3, 0.5 and 0.7 ml) to ensure that the correct cell density is available for transformation. Again, incubate overnight. For transformation, select a flask from the overnight culture that does not contain flocculated yeast with an OD_{600} of 1.3-1.5. Pellet the cells by centrifugation at 4,000 × g for 3 min and resuspend in freshly prepared YEPDS + 20 mM HEPES (pH 4). Transfer cells to a 1.5 ml microtube, add 35 µl 1M DTT, mix gently by inversion and incubate at room temperature for 30 min. Pulse spin (10-15 s) in a microcentrifuge at full speed to pellet cells. Remove supernatant and resuspend in 750 µl 1 M sorbitol by vortex mixing. Repeat the sorbitol wash three times. After the final centrifugation step, resuspend cells in a volume equal to the cell pellet (80-200 µl) 1 M sorbitol. Cells are now ready for electroporation.
3. Mix 40 µl of the Pichia cells with 100-600 ng of linearised plasmid DNA and incubate for 15 min at room temperature. Transfer the cells and DNA to a 2 mm gap electro-cuvettes to a pre-chilled cuvette (+4^{o}C) taking care to tap the yeast/DNA mix to the

bottom of the cuvette gently and dry the sides of the cuvette of moisture. Electroporate at 2.0 kV and immediately add 1 ml YEPDS medium, transfer to a sterile microtube and incubate at room temperature for 1 h. Plate 200 µl of transformed yeast and 20 µl of control yeast (no DNA) on separate YEPDS agar + Zeocin plates. Incubate at room temperature for 2 h, invert and transfer to 25°C for a further three-day incubation. Compare colonies on control and transformed cell plates to evaluate the success of transformation.

4. To express the HBV core VLPs, pick a colony of pPICZC-HBcAg-transformed P. pastoris KM71H cells and grow as a starter culture in 10 ml YEPD medium (see **Note 28**) in McCartney bottles overnight (25°C, 180 rpm shaking). Use a 0.8 ml aliquot of the starter culture to inoculate 800 ml BMGY expression medium in 2 L baffled conical flasks, incubating on an orbital shaker (180 rpm) at 25°C for 3 days (see **Note 29 and 30**). Harvest cells by centrifugation at 2000 × g for 4 min and transfer to 500 ml BMMY induction medium in 2 L baffled conical flasks. Incubate at 25°C and 180 rpm on an orbital shaker, supplementing the cultures with a further 4 ml methanol after 24, 48, and 72 h. After completion of the 96 h induction period, harvest cells by centrifugation at 2000 × g for 4 min (see **Note 31**). Remove the supernatant, then resuspend the cell pellet in water (5 ml water to 50 ml culture). Transfer to 50 ml Falcon tubes and store at –80°C.

5. Purification procedures are outlined in **Fig. 5**. Thaw frozen cells rapidly in warm (approximately 37°C) water and centrifuge at 2000 × g for 3 min in 50 ml Falcon tubes. Discard the supernatant, then add 2 g glass beads per tube and resuspend the pellet to 11 ml with ice cold lysis buffer. Vortex mix for 15 min on a multi-vortexer and cool on ice for 5 min to prevent overheating. Repeat this cycle four times, then centrifuge the tubes at 2000 × g for 30 min. Warm the supernatant in a water bath (60°C) for 30 min (see **Note 32**) before clarification by centrifugation at 20,000 × g for 30 min. Remove,

pool, and retain the supernatant (lysate), putting aside a 100 µl aliquot for future analysis, then filter the pooled lysate through a 0.22 PES filter. Put aside a 100 µl aliquot of filtered lysate for future analysis.

6. Using a MegaDalton cross-flow filtration cartridge, concentrate the equivalent lysate of 200 ml culture to 5 ml. Dilute this to 40 ml in PBS, then concentrate again to 5 ml. Load the cross-flow retentate onto a GE HiPrep 16/60 Sephacryl S-500 HR column and elute with PBS at a flow rate of 1 ml / minute (see **Note 33**). Collect 2.5 ml fractions in order to capture the peak of VLPs (see **Note 34 and 35**). If necessary, concentrate the eluted VLPs using a centrifugal concentration device.

3.3. Production of Tandem-HBc VLPs in Nicotiana benthamiana

1. Transform the expression plasmid (pEAQ-HT) into a suitable strain of E. coli (TOP10) to allow for sequencing, then transform into A. tumefaciens (LBA4404). Allow both to grow: E. coli at 37 °C overnight on LB agar or broth containing kanamycin (50 µg/ml) for plasmid selection; A. tumefaciens at 28 °C for 2-3 days on LB agar, broth, or 2 X YT containing kanamycin (50 µg/ml) for plasmid selection and rifampicin (50 µg/ml) for A. tumefaciens selection.
2. Prepare liquid cultures of positive clones of A. tumefaciens by growing them to stationary phase at 28°C in either LB or 2 X YT supplemented with kanamycin and rifampicin (50 µg/ml each) as described in (3) (see **Note 36**).
3. Centrifuge the cultures at 1100 × g for 20 min, then resuspend the pellet in MMA buffer to an OD_{600} of 0.4.
4. Carry out syringe agroinfiltration of prepared N. benthamiana plants (3 – 4 weeks after pricking out, typically one week before the start of flowering) by lightly scratching the surface of a leaf with a needle, then using a 1 ml needle-less syringe to infiltrate the intercellular space of the leaf with the A. tumefaciens suspension. Agroinfiltration of 3 – 4 leaves from 5 plants is typically sufficient for small-scale experiments. Leave the plants

to grow in a glasshouse or growth cabinet at 25°C for 6 – 8 days, providing water and 16 h of daylight (artificial if necessary) daily (see **Fig. 3).**

5. Harvest the agroinfiltrated leaves, using a razor blade or scalpel to remove the areas of the leaves that were not agroinfiltrated (see **Note 37**). Weigh the remaining leaf material, then use a blender to homogenise the leaf tissue with three volumes of chilled sodium phosphate buffer supplemented with protease inhibitor (e.g. 60 g of leaf tissue would require 180 ml buffer. If using cOmplete EDTA-free protease inhibitor cocktail tablets from Roche, use one tablet for every 50 ml of buffer). Filter the homogenate through a layer of Miracloth, then centrifuge the primary filtrate at 15,000 ×g for 20 min at 4°C. Retain the pellet (insoluble fraction) for further analysis if desired, then filter the supernatant through a 0.45 μm syringe filter (see **Note 38**).

6. Purification is carried out using a double-layer sucrose cushion. Pour the plant extract into a suitable ultracentrifuge tube, then use a long needle to underlay a volume of 25 % sucrose solution approximately equal to 1/6 of the volume of plant extract. Below that, underlay a smaller volume of 70 % sucrose solution (1/5 – 1/10 of the volume of the 25 % sucrose solution, see Note 11 for precise volumes). Ultracentrifuge at maximum speed for 2.5 – 3 h at 4°C, after which a thick green band is visible at the interface between the sucrose layers. Pierce the bottom of the tube with a needle and allow the sample to drip into a collection tube. The 70 % sucrose and the interface fractions combined should include all of the VLPs present in the sample, whereas collection of the 70 % fraction only will result in a cleaner sample with a lower yield (see **Fig. 4**). In both cases, dialyse the recovered sample against 20 mM ammonium bicarbonate (pH 8.5) (see **Note 39**). Clarify the dialysate by centrifugation at 15,000 ×g for 20 min at 4°C, then filter through a 0.2 μm syringe filter and concentrate to 2 ml in a vacuum concentrator, being careful not to concentrate the sample to dryness (see **Note 40, 41 and 42**).

7. Layer 2 ml fractions of 20 %, 30 %, 40 %, 50 %, and 60 % solutions of Nycodenz in a

13 ml ultracentrifuge tube then overlay with the concentrated sample (see **Note 43**). Using a TH641 ultracentrifuge rotor, centrifuge at maximum speed (~ 274,000 × g) for a minimum of three hours at 4°C. VLPs will appear as either a distinct iridescent band or a more diffuse brown-grey band below the sedimented green impurities (see **Fig. 5**) (see **Note 44**). Recover the VLP fractions by piercing the bottom of the tube with a needle and collecting 1 ml fractions, or by piercing the side of the tube just below the desired band with a needle and aspirating the band with a syringe. Analyse the fractions by SDS-PAGE or Western blotting (see Notes). Fractions containing recombinant protein can be examined for the presence of VLPs by transmission electron microscopy (TEM), or dialysed against sodium phosphate buffer or PBS (using 100 kDA MWCO Float-a-Lyzer dialysis cassettes from Spectrum Labs) for long term storage in a refrigerator or cold room.

3.4. Characterisation of Tandem-HBc VLPs

3.4.1. Removal of Sucrose and Salts by Dialysis of Tandem-HBc VLPs

1. Cut the required length of membrane and allow it to soak in the dialysis buffer for 5 min before teasing it open and folding one of the ends thrice. Seal the membrane with one dialysis clip at one end, then pipette in the VLP solution through the other (see **Note 45**). Press out any bubbles, then seal the distal end by folding it thrice and clipping with a dialysis clip. Connect the floats at either end and dialyse for 1 h at room temperature, using 1.5 L of dialysis buffer per dialysis tube (see **Note 46**).
2. Exchange the buffer with new dialysis buffer, then dialyse for 2 h at room temperature. Exchange the buffer with new dialysis buffer, then dialyse for an additional 5–6 h at room temperature (or overnight at 4°C). Unclip one of the dialysis clips and carefully pipette out the solution, which may then be stored at 4°C.

3.4.2. Examination of Tandem-HBc VLPs by Transmission Electron Microscopy (TEM)

1. Secure Parafilm on a designated surface, then, in a row, pipette out 10 µl of sample (see **Note 47**), 20 µl 20 mM Triswash buffer, 20 µl uranyl acetate solution, another 20 µl uranyl acetate solution, and 20 µl wash buffer. Using a pair of forceps, incubate a freshly glow-discharged grid on the surface of the sample for 30 s, then dip the grid once in the first droplet of uranyl acetate solution and touch dry on blotting paper (see **Note 48**). Dip and incubate the grid for 20 s in the second droplet of the uranyl acetate solution and touch dry on blotting paper, then dip into the second droplet of wash buffer and touch dry on blotting paper. Place the grid on labelled blotting paper to dry, then store the grid in a sample box, recording the number of the alcove where the grid is stored.
2. Examine the grid according to the instructions of the TEM microscope, following the local guidelines.

3.4.3. Immunogold TEM Analysis

1. If necessary, dilute the sample in an 10 mM Tris pH 7.5. As controls, incubate the sample with an unrelated primary antibody of the same species or with only ID, followed by incubation with the secondary antibody. Ensure grids are freshly glow-discharged before starting.
2. Place a strip of Parafilm on a smooth surface (see **Note 49**), then place an appropriate number (for the number of samples to be labelled) of 20–30 µl droplets of IBB onto the Parafilm. Using the tweezers, carefully take a freshly glow-discharged grid and, using an automatic pipette, load a 5–10 µl droplet of sample onto the grid and incubate for 30 – 60 s. With a triangle of filter paper in one hand and a minimum of 40 µl of deionised water in a Pasteur pipette in the other, carefully add the water to the grid and wash by touching the filter paper to the side of the droplet, being sure not to touch the grid except at the very edge. As soon as the droplet is removed, place the grid sample side down onto a droplet of IBB and incubate for 45–60 min.
3. Place an appropriate number of 20–30 µl droplets of ID on the Parafilm, then lift the

grid and blot as before, by carefully touching the filter paper to the side of the remaining IBB droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of ID. Place an appropriate number of 20–30 μl droplets of diluted primary antibody on the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of primary antibody and incubate for 1.5–2 h.

4. Place an appropriate number of 20–30 μl droplets of ID (5 droplets for each grid) on the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining primary antibody droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of ID. Incubate for 3–5 min, then blot and transfer to the second droplet (see **Note 50**). Repeat this process for a total of five washes. Following the final wash, place an appropriate number of 20–30 μl droplets of diluted secondary antibody onto the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of secondary antibody and incubate for 45–60 min.

5. Wash the grid as before, in five droplets of ID. Then, with a triangle of filter paper in one hand and a minimum of 40 μl of negative stain in a Pasteur pipette in the other, blot the grid as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place approximately 10 μl of negative stain onto the grid and blot without incubation. Immediately repeat this process, leaving the second negative stain droplet on the grid for 15– 20 s, then blot the grid as before, by carefully touching the filter paper to the side of the remaining negative stain droplet. Ensure that any additional stain is blotted from the tweezers before proceeding, then carefully place the grid sample side up on a piece of filter paper and allow to air dry for

at least 2 min before transferring the grid to a sample box, recording the number of the alcove where the grid is stored.

6. Examine the grid according to the instructions of the TEM microscope, following the local guidelines.

3.4.4. Immunoadsorbent TEM Assay

1. If necessary, dilute the sample in an 10 mM Tris pH 7.5. As controls, incubate the sample with an unrelated primary antibody of the same species or with only ID. Ensure grids are glow-discharged before beginning.
2. Place a strip of Parafilm on a smooth surface (see **Note 49**), then place an appropriate number (for the number of samples to be labelled) of 20-30 µl droplets of IBB onto the Parafilm. Using the tweezers, place the grid sample side down onto one of these droplets and incubate for 45-60 min.
3. Place an appropriate number of 30 µl droplets of ID onto the Parafilm, then carefully blot the grid by touching the filter paper to the side of the IBB droplet, being sure not to touch the grid except at the very edge. As soon as the droplet is removed, briefly place the grid 'sample side' down onto a droplet of ID. Place an appropriate number (one per grid) of 25 µl droplets of diluted primary antibody on the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of primary antibody and incubate for 30 min.
4. Place an appropriate number of 20–30 µl droplets of ID (5 droplets for each grid) on the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining primary antibody droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of ID. Incubate for 3 – 5 min, then blot and transfer to the second droplet (see **Note 50**). Repeat this process for a total of five washes. Following the final wash, place an appropriate number of 20–30 µl droplets of VLP

sample onto the Parafilm, then lift the grid and blot as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place the grid sample side down onto a droplet of VLP sample and incubate for 30 min.

5. Wash the grid as before, in five droplets of ID. Then, with a triangle of filter paper in one hand and a minimum of 40 µl of negative stain in a Pasteur pipette in the other, blot the grid as before, by carefully touching the filter paper to the side of the remaining ID droplet. As soon as the droplet is removed, place approximately 10 µl of negative stain onto the grid and blot without incubation. Immediately repeat this process, leaving the second negative stain droplet on the grid for 15– 20 s, then blot the grid as before, by carefully touching the filter paper to the side of the remaining negative stain droplet. Ensure that any additional stain is blotted from the tweezers before proceeding, then carefully place the grid sample side up on a piece of filter paper and allow to air dry for at least 2 min before transferring the grid to a sample box, recording the number of the alcove where the grid is stored.
6. Examine the grid according to the instructions of the TEM microscope, following the local guidelines.

3.4.5. Dynamic Light Scattering (DLS)

1. Open the Zeta Sizer software (see **Note 21**) and select the following settings: Measure: “Manual”, Temperature: “25°C” (as appropriate), Sample Name: as appropriate, Cuvette: “DTS0012” (as appropriate), Measurement Type: “Size”, Parameter: “Mark Horowitz”, Material: “Protein”, Advanced: “Default Values”, Dispersant: “PBS”, Data Processing: “General” “Protein Analysis" "Repeats".
2. Under “Measurement”, set the measurement angle to “173 Back Scatter”, the number of measurements to “3” (as appropriate), and the delay to “0”. Press the green start button and wait for the machine to collect the data – it will beep upon completion (see **Note 51**).

3. Ensure that “Number” and “PDI” are enabled under the parameters analysed, and then save the data. In the “Results” section, export the data as “Number” and copy it into a spreadsheet for ease of use (see **Note 52**).

4. Notes

1. Ultracentrifuge buckets ought to be weighed accurately to avoid issues caused by imbalance. Depending on the centrifuge tubes used, it is normally recommended to fill them up almost to the brim, to prevent them collapsing.
2. Always wear appropriate ear protection during sonication.
3. Acrylamide is highly toxic and should be handled with utmost care.
4. The efficacy of the bacteria used is crucial; the Rosetta 2 strain seems to work best.
5. Kanamycin is toxic and must be handled appropriately.
6. DTT is toxic and should be handled with care. DTT solution should be freshly prepared or stored in the dark in a -20°C freezer. DTT or solutions containing it, should not be autoclaved.
7. The induction needs to be carried out with good quality IPTG; as IPTG is not stable in the fridge, the stock should be frozen.
8. The ammonium sulphate used is a saturated solution; there will be undissolved ammonium sulphate in the stock solution.
9. Reagents used in silver staining and Coomassie Brilliant Blue staining can be harmful to the worker or the environment; appropriate measures need to be taken to protect the operator and to dispose of the waste in accordance to the rules and regulations of the work place.
10. Always use centrifuges and ultracentrifuges appropriately, ensuring rotors are correctly balanced.

11. For the double sucrose cushion, any swing-out (swinging-bucket) ultracentrifuge rotor may 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), the ideal tubes are Ultra-Clear 13 ml (14x89 mm). The double sucrose cushion is prepared by pouring the plant extract in the tube, then carefully underlaying first with 2 ml of 25% sucrose solution and then 0.25 ml of 70% sucrose solution. The sample is then ultracentrifuged at 40,000 rpm (274,000 x g) for 2.5 hours 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).
    - The larger Surespin 630/36 spin-out rotor (Thermo Scientific) uses Ultra-Clear 36 ml tubes, 25x89 mm. The double sucrose cushion is prepared by pouring the plant extract into the tube, then carefully underlaying first with 5 ml of 25% sucrose, then 1 ml of 70% sucrose and ultracentrifuging at 30,000 rpm (167,000 x g) for 3 hours 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).
12. Using prepared 500 mM EDTA stock solution is recommended.
13. The dialysis buffer, without DTT, can be prepared, autoclaved and stored at 4°C.
14. The ‘sample side’, the side to which the sample is applied.
15. Crossover tweezers (Dumont) are highly recommended for the experiment.
16. Uranyl acetate is an alpha emitter and is highly toxic. Local rules would be in place regarding the use and disposal of anything contaminated with uranyl acetate.
17. In the absence of 2 % uranyl acetate, 1 % uranyl acetate works equally well.
18. Nickel grids are recommended for this procedure.

19. Antibodies should be held on ice and diluted during wash step prior to use.
20. The primary antibody described here binds to the N-terminus of the protein, so any modifications in that region might affect the results.
21. This protocol is written for use with the Malvern Zetasizer Nano series. The protocol will need to be adapted should the user have access only to other DLS machines.
22. Whilst starting the first run, it is recommended to use a serial dilution of the sample to determine the optimal amount needed for accurate measurement. It is also recommended to check the samples first with TEM and silver stain to get a general idea about the purity of the preparation. Should the sample be too impure, then it might be difficult to observe any particles that match the VLP architecture due to the background.
23. This can be scaled up.
24. The initial incubation time of at least an hour seems crucial, given that most of the tandem-HBc plasmids are low copy number plasmids with kanamycin resistance cassettes.
25. Typically, it takes approximately 3 h to reach an OD 600 of 0.6- 0.8.
26. Typically, it takes approximately 6 – 10 cycles until the solution clarifies.
27. Sonication is a crucial step and needs to be carried out on ice. Over-sonication might result in protein aggregation. Depending on the sonicator used, it is advisable to visually check the solution between each round of sonication. The moment the suspension clarifies cell disruption is complete and further sonication is not needed.
28. Antibiotic (Zeocin) is not required after initial selection of transformed Pichia clones on agar plates.
29. With regards to schedule: cultures are typically set up on Thursday to provide overnight cultures for priming BMGY expression cultures on Friday. Cells are then ready for induction on Monday.

30. Incubation of cultures at temperatures above 25°C reduces yields of VLP substantially.
31. Expressions may be carried out for 72 - 96 hours. With a 72 hour culture, the cells can be processed and VLPs purified by the end of the week, but yields may be slightly lower than from a 96 hour culture.
32. Heating lysates to 60°C for 30 minutes prior to purification causes precipitation of host cell proteins, allowing removal by centrifugation. The HBc VLPs form the major component of the clarified, heat treated lysate.
33. While SEC purification of the HBc VLPs is done routinely, anion exchange chromatography on a DEAE column also yields highly purified VLPs.
34. TEM analysis of size exclusion chromatography fractions may reveal VLPs that are slightly smaller in size (~ 20 nm diameter) than HBV core. Western blot analysis of these fractions reveals that these assemblies do not contain HBc and are likely endogenous VLPs.
35. Fractions are analysed routinely by TEM and SDS PAGE. The fractions containing uniformly assembled VLPs of the correct size as identified by TEM are examined on a stained SDS PAGE gel. Only those fractions containing HBc protein are pooled and concentrated.
36. Positive clones of E. coli and A. tumefaciens can be stored as glycerol stocks by supplementing an aliquot of liquid culture with glycerol (to a final concentration of 25 % (v / v)) and snap-freezing in liquid nitrogen prior to storage at –80°C.
37. Take care when cutting leaf tissue with a razor blade or scalpel.
38. Note that large volumes may cause the filters to become clogged with large impurities found in the homogenate, so numerous filters will need to be used for large volumes. Also, syringe filters equipped with pre-filters (such as Sartorius Minisart NML plus

0.45 µm syringe filters with glass-fiber pre-filter) will allow larger volumes to be processed with a single filter.

39. Due to the high osmotic pressure of the sucrose, the volume of the dialysate may increase 2 – 3 fold.
40. During vacuum concentration, the ammonium bicarbonate will decompose to volatile compounds as the water evaporates, meaning that the buffer will not be significantly concentrated during vacuum evaporation.
41. 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 temperature in the vacuum concentrator. To avoid heat shock, the sample should be placed on ice immediately after concentration.
42. Because impurities will concentrate along with the sample, short (10 min) centrifugation in a microcentrifuge is recommended halfway through concentration to pellet some of the impurities, particularly if the sample is being concentrated more than 5-fold.
43. As an alternative to manually layering fractions to form the Nycodenz gradient, the extract can be mixed with a solution of 40 % Nycodenz and ultracentrifuged for 24 h: the gradient will form spontaneously. As Nycodenz forms a density gradient during centrifugation, the components within the sample equilibrate at their isopycnic points and remain there irrespective of duration of centrifugation. As such, the sample cannot be centrifuged for too long. The method described in 3.3. is intended to be time-saving, allowing the gradient to form after only 3 h. However longer centrifugation (16-24 h) may yield better separation of particles from impurities.
44. The position of the particles in the gradient depends on their buoyant density, which is heavily influenced by nucleic acid content of the particles. Heterogeneity in nucleic acid content will result in a diffuse or multiple bands. A high concentration of tandem-

core particles will result in a visible band (or multiple bands) in the Nycodenz gradient, which can easily be recovered by piercing the side of the tube and aspirating the band with a needle and syringe. If concentration is low, bands may not be visible and the entire gradient may need to be fractionated in order to identify which fraction contains the particles.

45. Care should be taken not to fill the entire dialysis tube lest the tube splits during the process.
46. A magnetic stirrer, stirring at low rpm, is recommended for the dialysis.
47. The VLPs should be dialysed prior to TEM analysis, lest the sucrose interfere with the staining. Should the VLPs be resuspended in other buffers (especially those containing phosphates), they should be dialysed into the recommended buffer lest the UA precipitate.
48. The time of the initial incubation is crucial, should the incubation be prolonged, the background would be greater.
49. E.g. the upturned lid of a sandwich box.
50. It is recommended to work in rows when working with multiple grids, ensuring that each grid has sufficient time in ID.
51. Eye protection must be worn whilst using the apparatus.
52. The measurements themselves are saved as “number” because the “intensity” (default measurement) would be proportional to the size of the particles.
53. VLPs may be analysed by SDS-PAGE and western blotting: samples can be mixed 1:1 and in a suitable denaturing loading buffer with reducing agent (such as LDS and 2-mercaptoethanol mixed in a 3:1 ratio) and boiled for 20 minutes before loading onto an SDS-PAGE gel. Proteins can then be transferred to a nitrocellulose membrane, which can then be blocked using blocking buffer then probed with the anti-HBcAg 10E11 primary antibody mixed into blocking buffer. After washing, the secondary

antibody can be added (also mixed in blocking buffer). After washing, the proteins can be detected using film or a digital detector such as a GE Las500 Quant automated chemiluminescent detector.

5. References

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Figure Captions

Fig. 1: The E. coli HBc expression and analysis pipeline scheme.

The tandem-HBc gene is cloned into a bacterial expression vector and propagated in the appropriate strain of E. coli. Protein expression in large scale bacterial cultures is induced by IPTG and the culture is centrifuged to recover the cell pellet. This is resuspended in the capsid buffer, sonicated and the proteins precipitated with ammonium sulphate. The tandem-HBc cores are purified by sucrose cushion and sucrose gradient ultracentrifugation. Fractions of the gradient are taken and analysed for the presence of the VLPs by Coomassie Brilliant Blue staining. The selected fractions are purified by dialysis and the protein is quantified and analysed by other downstream methods.

Fig. 2: P. pastoris expression, purification and analysis pipeline.

After methanol expression, cells are frozen at 80°C. To release VLPs, the thawed cells are induced washed and lysed by glass bead vortexing. The clarified lysate is heat treated to precipitate host protein and centrifuged to clarify. Syringe filtration (0.22 µm) is followed by 1 MegaDalton cross-flow filtration to wash and concentrate the VLPs. The retentate containing VLPs is fractionated by size exclusion chromatography and collected fractions are analysed by SDS PAGE and negative stain TEM prior to pooling.

Fig. 3: The plant transient expression pipeline.

The tandem-HBc gene is cloned into a plant expression vector and propagated in E. coli, then transformed into Agrobacterium tumefaciens for agroinfiltration into N. benthamiana leaves.

VLPs can be extracted from the leaves 5-9 days later. Figure reproduced from Peyret and Lomonossoff, 2013 (7).

Fig. 4: Using a double sucrose cushion to concentrate and partially purify tandem-HBc VLPs. a) UV light (left) and white light (right) photographs of a 14 x 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. Figure reproduced from Peyret, 2015 (4).

Fig. 5: Using a Nycodenz gradient to purify tandem-HBc VLPs. a) UV light (left) and white light (right) photographs of a 14 x 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 (after dialysis against ammonium bicarbonate) 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. 7**). All scale bars are 100 nm. Figure reproduced from Peyret, 2015 (4).

Fig. 6: Scheme of TEM analysis of tandem-HBc VLPs.

The purified VLPs are pipetted on to Parafilm and the freshly glow discharged grids are exposed to them. The grids are washed and exposed to two droplets of the stain (2 % uranyl acetate) and washed a final time and dried prior to analysis.

Fig. 7: Scheme of immunogold staining of tandem-HBc VLPs.

The sample is placed onto the grid and then the grid is wasted prior to blocking. The blocking solution is washed off and the grid is exposed to the primary antibody. After incubation, the grid is washed and exposed to the secondary antibody prior to another wash and exposure to the stain.

Fig. 8: Scheme of immunoabsorbent assay of tandem-HBc VLPs.

The grid is placed in the blocking solution and then washed prior to incubation with the primary antibody. This is then washed off and the grid is exposed to the sample. After washing the grid, it is stained with uranyl acetate.

Fig. 1

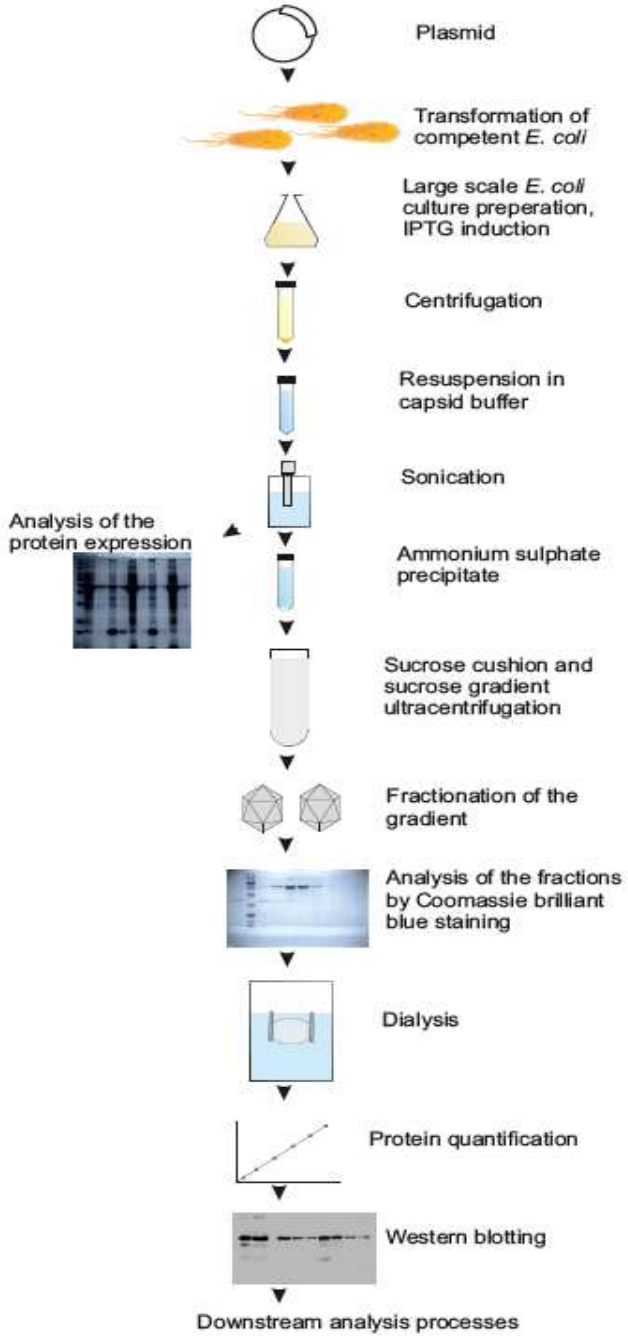
Plasmid
Transformation of competent E. coli
Large scale E. coli culture preperation, IPTG induction
Centrifugation
Resuspension in capsid buffer
Sonication
Analysis of the protein expression
Ammonium sulphate precipitate
Sucrose cushion and sucrose gradient ultracentrifugation
Fractionation of the gradient
Analysis of the fractions by Coomassie brilliant blue staining
Dialysis
Protein quantification
Western blotting
Downstream analysis processes

Fig. 2

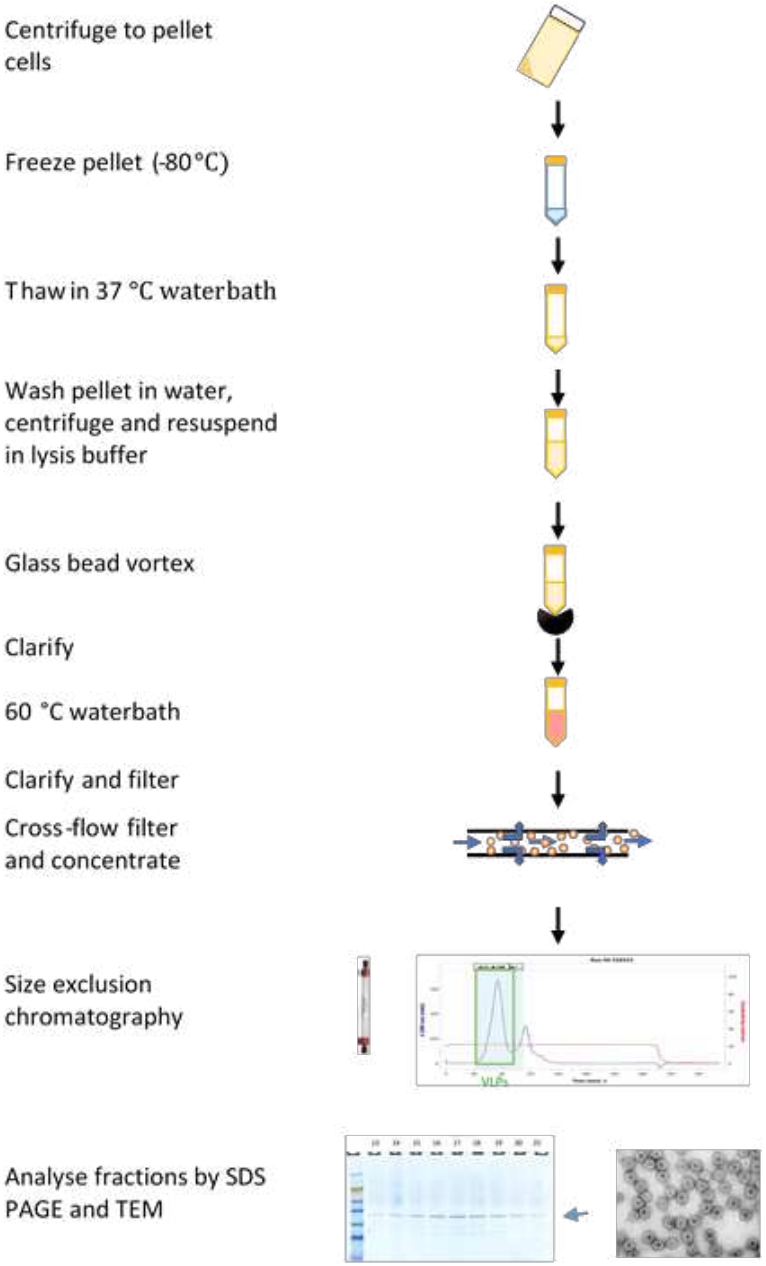
Centrifuge to pellet cells
Freeze pellet (-80°C)
Thaw in 37 °C waterbath
Wash pellet in water, centrifuge and resuspend in lysis buffer
Glass bead vortex
Clarify
60 °C waterbath
Clarify and filter
Cross-flow filter and concentrate
Size exclusion chromatography
VLPs
Analyse fractions by SDS PAGE and TEM

Fig. 3

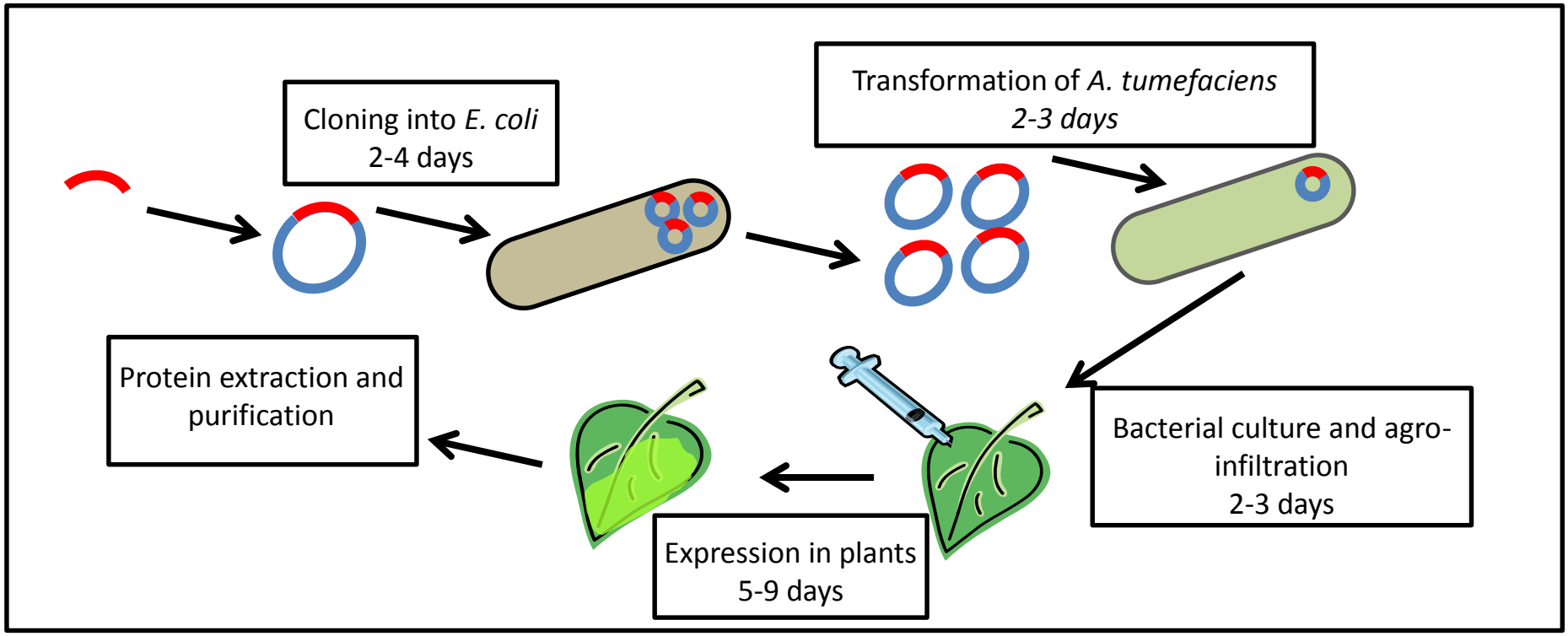
Cloning into *E. coli*
2-4 days
Transformation of *A. tumefaciens*
2-3 days
Protein extraction and purification
Bacterial culture and agro-infiltration
2-3 days
Expression in plants
5-9 days

Fig. 4

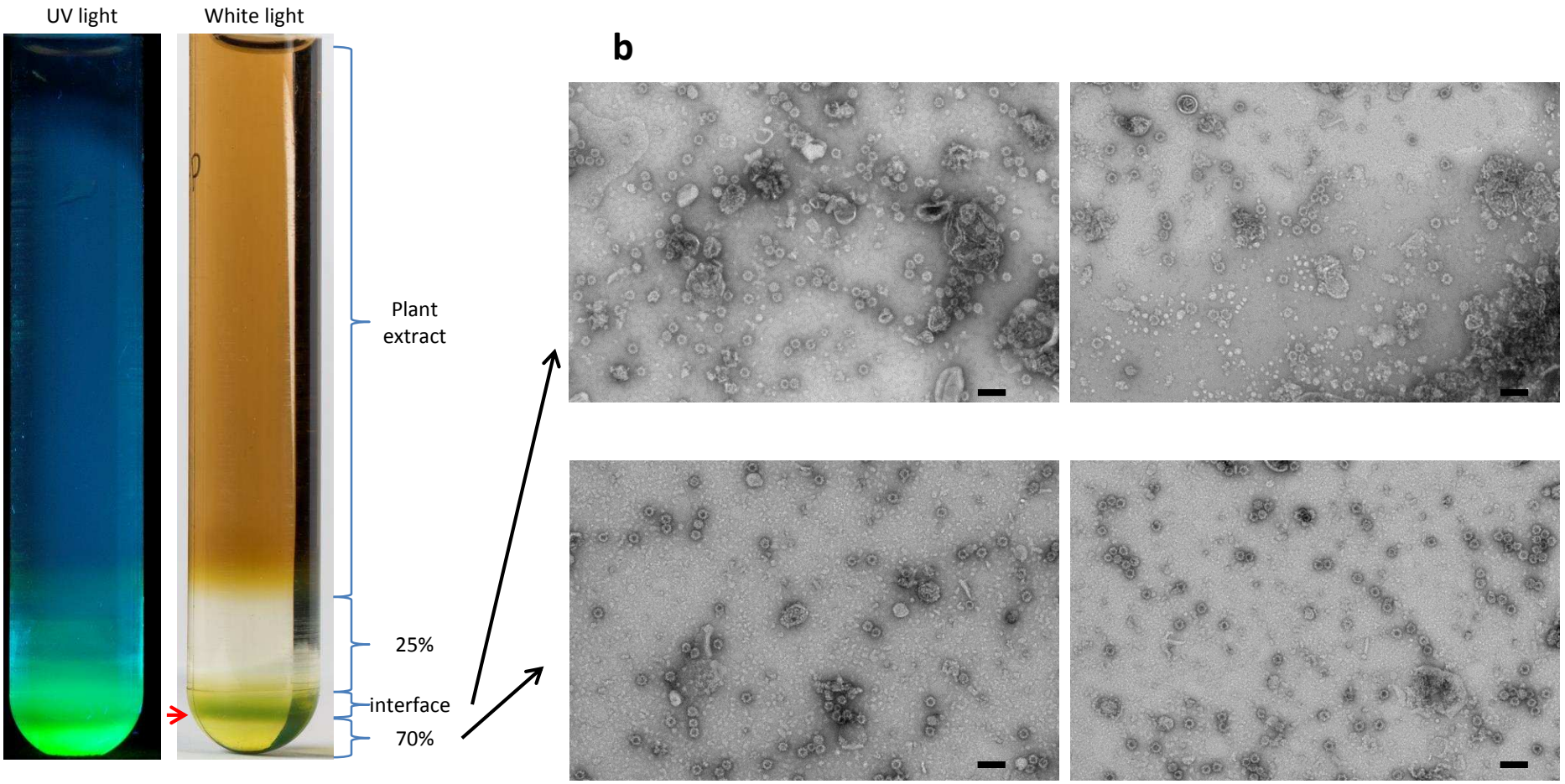

Fig. 5

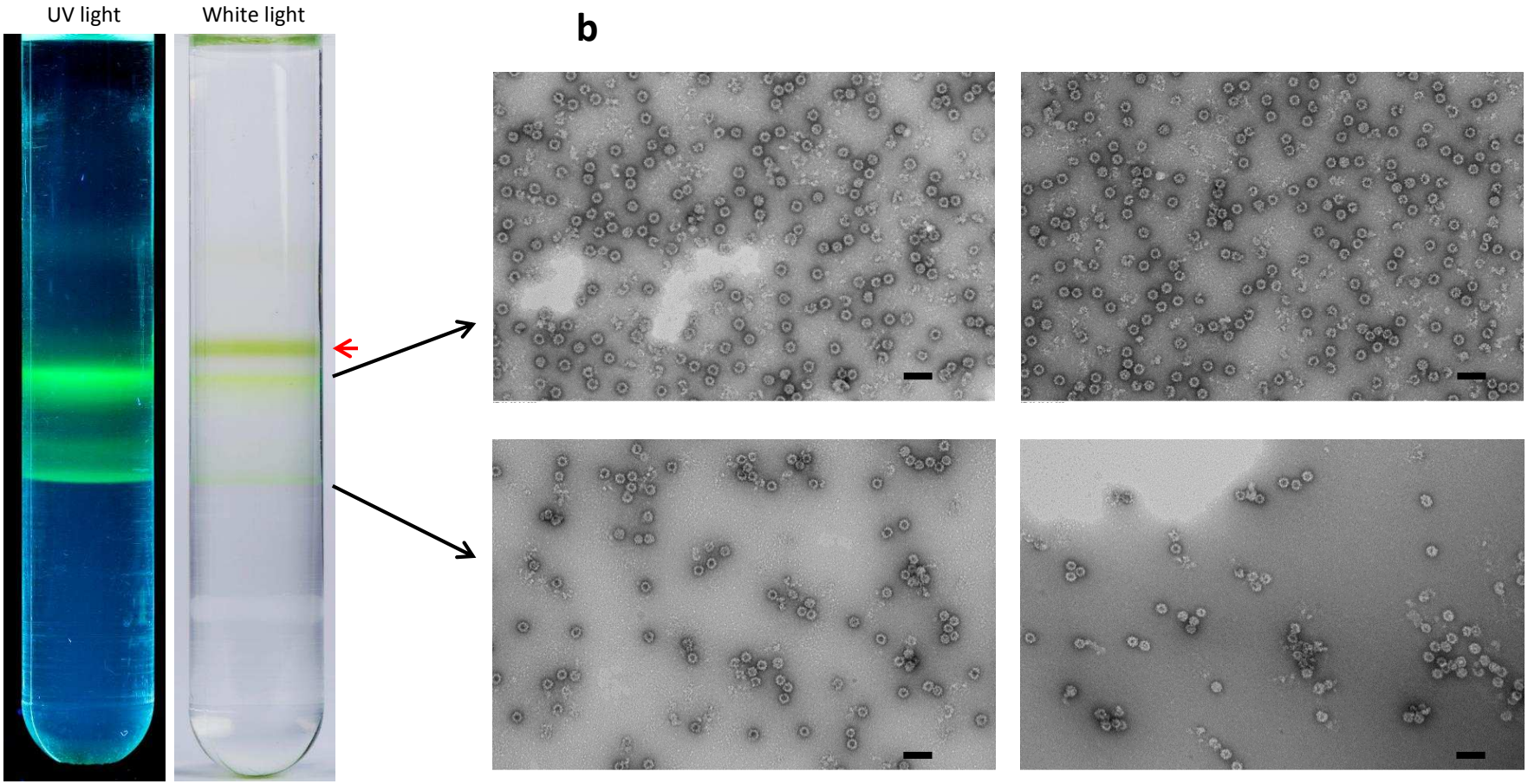

Fig. 6

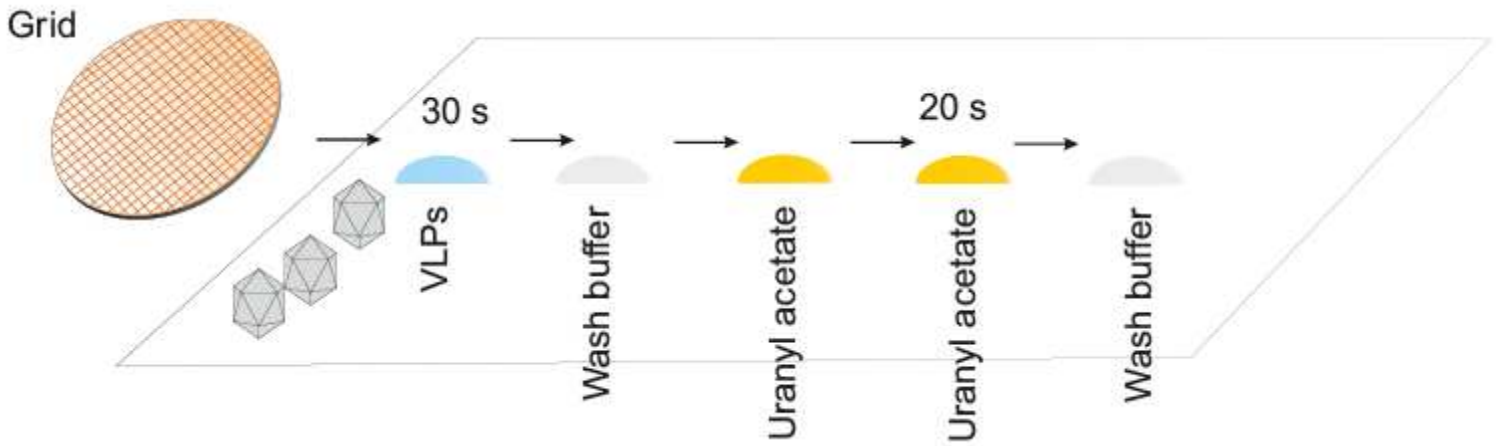
Grid
30 s
20 s
VLPs
Wash buffer
Uranyl acetate
Uranyl acetate
Wash buffer

Fig. 7

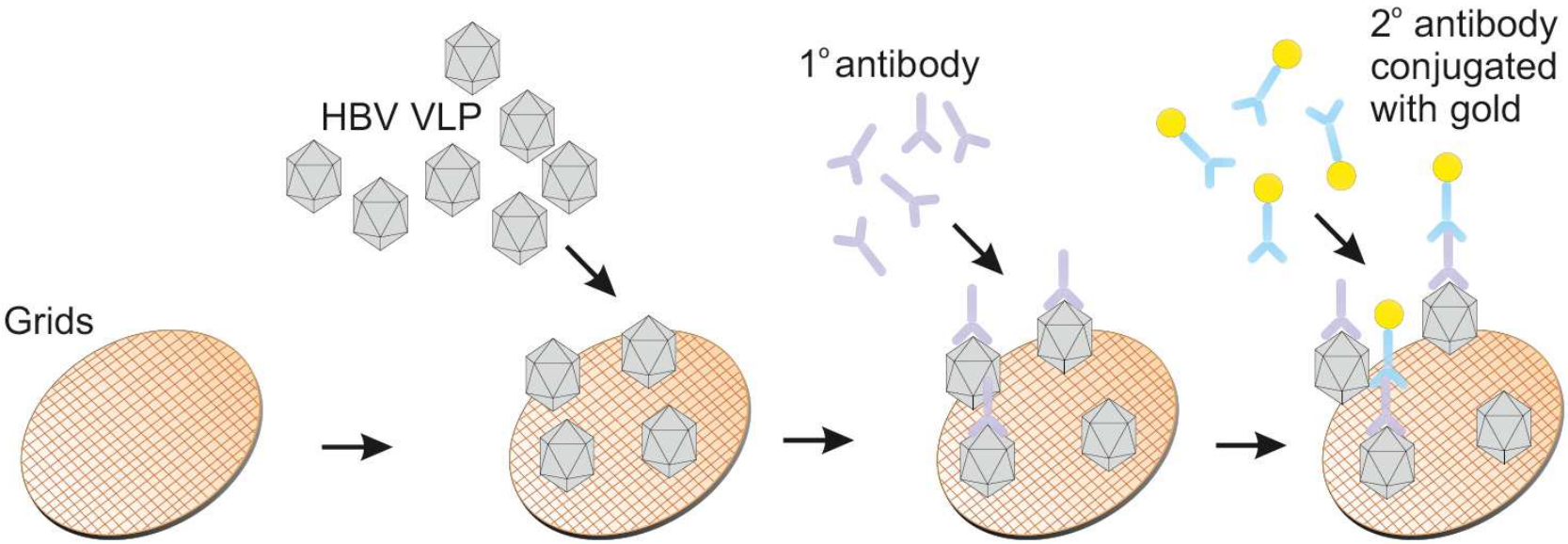

Fig. 8