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Lionel Bertaux, Audrey Lartigue and Sandra Jeudy*

Aix–Marseille Univ., Centre National de la Recherche Scientifique, Information Génomique & Structurale, Unité Mixte de Recherche 7256 (Institut de Microbiologie de la Méditerranée, FR3479), 13288 Marseille Cedex 9, France
*For correspondence: sandra.jeudy@igs.cnrs-mrs.fr

[Abstract] While different giant viruses' purification protocols are available (La Scola *et al.*, 2003; Byrne *et al.*, 2009; Arslan *et al.*, 2011; Philippe *et al.*, 2013; Campos *et al.*, 2014; Andrade *et al.*, 2017), they are not fully described and they use sucrose gradient that does not reach an equilibrium. Here, we report a protocol for the purification of members of the *Mimiviridae* family virions resulting from *Acanthamoeaba castellanii* infections. Viruses are harvested after cell lysis and purified through a high density CsCl gradient to optimize the isolation of the virus from the cell debris or other potential contaminants. Due to the large size of the virion capsids, reaching half a micrometer diameter, the quality of the process can be monitored by light microscopy. The resulting purified particles can then be used to perform new infections, DNA extraction, structural studies, sugar composition analyses, sub-compartment characterization or proteomic experiments.
Keywords: Giant viruses, Mimivirus, CsCl density gradient, Virion purification, Ultracentrifugation

[Background] The discovery of Mimivirus, the first virus visible under a light microscope, overlapping in size and genome complexity with unicellular organisms, initiated a new research area in virology (La Scola *et al.*, 2003; Raoult *et al.*, 2004). Over the past 15 years, many additional members of the Mimiviridae family have been isolated from various environments and several protocols have been published to purify the virions. Different approaches were developed, mostly involving sucrose cushion (Campos *et al.*, 2014; Andrade *et al.*, 2017), or sucrose discontinuous gradients (Arslan *et al.*, 2011). However, these protocols are not optimal since the density of the virus is higher than the maximum density of a sucrose solution (1.36 g/cm^3 and 1.3 g/cm^3, respectively - ICTV 9th report, 2011), meaning the equilibrium cannot be reached. Thus, a long time or high speed centrifugation results in a viral pellet instead of a ring or both depending on the conditions used. We recently reported (Jeudy *et al.*, 2019) an optimized version of our previously published protocol using a CsCl discontinuous gradient (Byrne *et al.*, 2009). Here, we provide the detailed protocol for the purification of Mimiviridae particles.

Materiel and reagents

General use

1. Standard pipette filter tips (StarLab, models: TipOne filter tips, catalog number: S1120-3810,

S1120-1810, S1120-8810 and S1112-1720 or equivalent)
2. Pipettes (Sarstedt, model: 10 mL serological pipette, catalog number: 86.1254.001)
3. Ultrapure water

Cell culture

1. *Acanthamoeba castellanii* (Douglas) Page (ATCC, catalog number: 30010)
2. 175 cm^2 flasks (Greiner Bio-One, catalog number: 660175, or equivalent)
3. D-(+)-glucose (Sigma, catalog number: G8270)
4. Sodium citrate tribasic trihydrate (Sigma, catalogue number: C7254)
5. Proteose Peptone Yeast Extract medium (PPYG; see recipes)
6. Proteose-Peptone (Sigma, catalog number: 82450)
7. Yeast extract (Fisher BioReagent, catalog number: BP1422)
8. Ampicillin Sodium Salt, Cell Culture/Molecular Biology Grade (Euromedex, catalog number: EU0400)
9. Kanamycin Sulfate, Cell Culture Grade (Euromedex, catalog number: UK0010)
10. Filtropur S 0.2 (Sarstedt, catalog number: 83.1826.001)
11. Stericup Quick Release Express Plus 0.22 µm PES (Merck Millipore, catalog number: S2GPU11RE)
12. 36% Glucose solution (Sigma, catalog number: G8270; see Recipes)
13. Ampicillin stock solution (100 mg/mL) (see Recipes)
14. Kanamycin stock solution (25 mg/mL) (see Recipes)

Gradient preparation and recovery

1. Plastic Syringes (Terumo, model: 5 mL syringe, catalog number: SS*05SE1)
2. 21G x $1^{1/2}$" needle (Terumo, catalog number: AN*2138R1 or equivalent)
3. Sterile 50 mL conical tubes (Sarstedt, catalog number: 62.547.254, or equivalent)
4. Polyallomer Tubes, 38.5 mL (Beckman, catalog number: 326823)
5. UltraPure Cesium chloride Optical Grade (Invitrogen, catalog number: 15507-023)
6. Cesium chloride solutions (1.2 g/cm^3, 1.3 g/cm^3, 1.4 g/cm^3 and 1.5 g/cm^3 ; See Recipes)
7. K36 buffer (see Recipes)

Purification quality control

1. Glass microscope slide (Biosigma, catalog number: VBS653/A)
2. Coverslip (Biosigma, catalog number: VBS636)
3. UVette 220-1600 nm (Eppendorf, catalog number: 952010051)
4. Formvar and carbon coated 200 mesh copper/rhodium grids (Electron Microscopy Sciences, catalog number: FCF200-Cu)
5. Uranyl acetate 1% in water

Note: Uranyl acetate is a radioactive chemical. It is provided by electron microscopy facilities

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as we are not allowed to use it in the lab.

Equipment

1. Microbiological safety hood (ADS laminaire, model: Optimal 12, or equivalent)
2. Incubator Bio Performance (Froilabo, catalog number: BP240 or equivalent)
3. Centrifuge (Beckman Coulter, model: JXN-30)
4. Swinging-Bucket Rotor (Beckman Coulter, model: JS24-38)
5. Spectrophotometer (Eppendorf, model: Biophotometer)
6. Inverted microscope (Zeiss, model: Axio Observer.Z1)
7. Transmission Electron Microscope (FEI, model: Tecnai G2)
8. Autoclave (SHP Steriltechnik SG, model: Laboklav 135)
9. Peristaltic pump (KNF Lab, model: Laboport)

Procedure

1. Seed 20 175 cm^2 flasks with 1,000 *Acanthamoeba castellanii* cells per cm^2 in 20 mL of PPYG medium. Incubate at 32 °C for two days.
   Note: You can add ampicillin and kanamycin at final concentrations of 0.1 mg/mL and 0.025 mg/mL, respectively, in each flask to avoid bacterial contamination.
2. After two days, the cell confluence should be between 130,000 and 180,000 cells/cm^2. Infect the cells with a Mimivirus stock at a multiplicity of infection of 0.2.
   Incubate at 32 °C for two days or until cell lysis is complete.
   Note: There is no need to change the medium before infection or to remove the virus from the flasks.
3. Harvest the cell debris-containing supernatants from the flasks and pool them into 50 mL conical tubes.
   Note: For 20 175 cm^2 flasks containing 20 mL of medium you will need 7 tubes.
4. Centrifuge at 500 *x g* for 10 min at room temperature (~25 °C, RT) to remove the cell debris.
5. Decant the supernatant into new 50 conical tubes.
6. Centrifuge at 10,000 *x g* for 25 min at RT to pellet the virus.
7. Discard the supernatant.
8. Resuspend the resulting virus pellets in 10mL K36 buffer by pipeting and pool in two new 50 mL conical tubes (35 mL per tube). Complete the volume to 50 mL with K36 buffer.
9. Centrifuge at 10,000 *x g* for 25 min at RT to pellet the virus.
10. During the centrifugation, prepare four CsCl gradients in Beckman polyallomer tubes. Add 7.5 mL of CsCl solution density 1.5 g/cm^3 at the bottom of each tube, then carefully overlay with 9 mL of 1.4 g/cm^3 CsCl solution and 9 mL of 1.3 g/cm^3 CsCl solution, dropwise. Hold for the final virus-containing 1.2 g/cm^3 density layer.

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11. Discard the supernatant from the centrifugation in Step 9.
12. Resuspend the two viral pellets with 16 mL of 1.2 g/cm^3 CsCl solution for each pellet by pipeting.
13. Overlay 8 mL of resuspended virus pellets on top of each of the four gradients, dropwise (Figure1A).
14. Centrifuge overnight (between 16 and 18h) at 100,000 *x g* at 20 °C in a Beckman Coulter JS24-38 rotor.
15. After centrifugation, harvest the white ring corresponding to the virus fraction (Figure 1B) by carefully pipetting through the gradient or by puncturing the polyallomer tube and aspirating with a needle and a syringe (Figure 2). Transfer the virus fraction to two new 50 mL conical tubes, add K36 buffer up to 50 mL in each tube and mix by inverting the tubes.

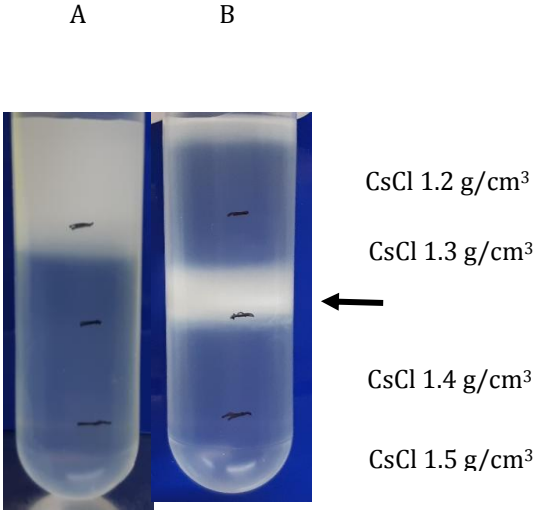

Figure 1: centrifuge tubes before (A) and after (B) separation of Mimivirus particles on CsCl gradient. The white ring indicated by the black arrow corresponds to Mimivirus particles.

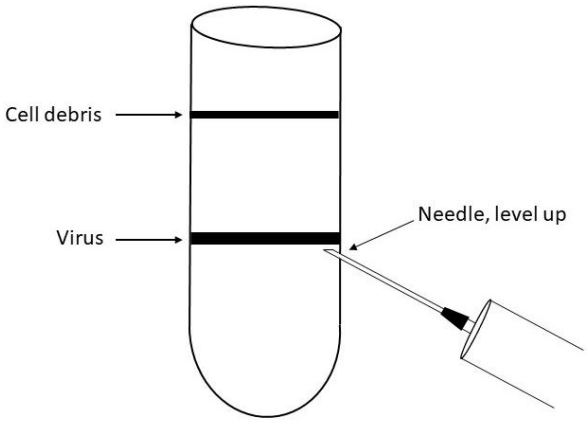

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Figure 2: Collection of the viral fraction by side puncture

16. Centrifuge at 10,000 *x g* for 25 min at RT to pellet the virus.
17. Resuspend the two pellets with 10 mL of K36 buffer each, complete the volume to 50 mL with K36 and centrifuge them at 10,000 *x g* for 25 min at RT to wash the virus.
18. Repeat Step 16 and 17 twice. For the last wash, pool the resuspended virus in a single 50 mL conical tube.
19. Resuspend the final pellet in 10 mL of K36 buffer.
20. The virus is ready to be titrated or stored at -80 °C until further use.

Note: Typically, when purifying Acanthamoeba polyphaga Mimivirus using that protocol, we recover 10 mL of viral solution containing 1 x 10^{10} to 4 x 10^{10} particles/mL
The purified particles are infectious and can be used to performed new infections or any other experiments.

Data analysis

1. Light microscopy
   Drop 4.5 µl of the purified virus suspension on a microscope slide and overlay with a coverslip. Invert for a few hours in order to let the virus settle onto the coverslip for easier focusing. Observe with a light microscope using the 63x objective to confirm the purified virus does not contain any obvious contaminants (Figure 3).

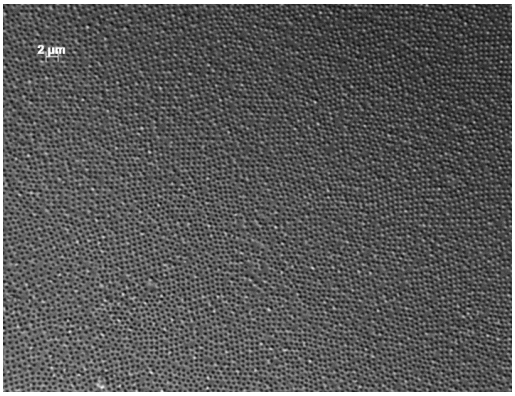

Figure 3. Observation of Mimivirus purified particles with a light microscope (63X objective, 1.6X optovar).

2. Transmission Electron Microscopy (TEM)
   For TEM, dilute the purified Mimivirus sample 1:2 (v/v) in K36 buffer and incubate a 10 µl droplet on a formvar- and carbon-coated 200 mesh copper/rhodium grid for 1 min at RT. Wash the grid with three successive 1% uranyl acetate droplets. Leave the residual uranyl acetate on

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the grid for 1 min and remove by gently touching the edge of the grid with a filter paper. After drying, examine the grid using an electron microscope (Figure 4).

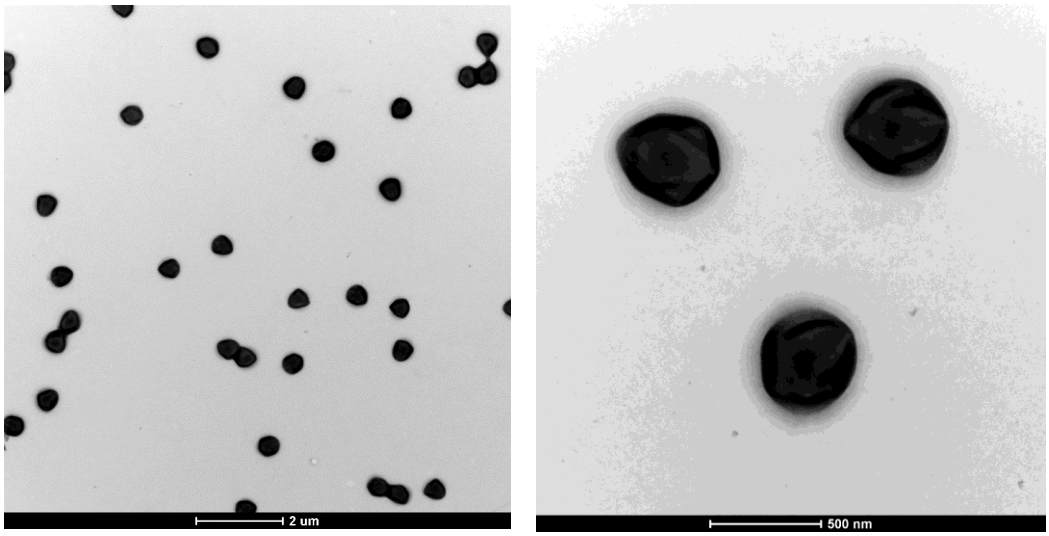

Figure 4. Observations of Mimivirus purified particles by negative staining.

Recipes

1. Glucose solution
   Dissolve 36 g of glucose and 2 g of sodium citrate in 100 mL of slightly warm water.
   When the solution is clear, use it to prepare the complete PPYG medium.
2. Proteose Peptone Yeast Extract (PPYG) medium
   Base medium:
   Autoclave 40 g of proteose peptone and 2 g of yeast extract diluted in 1.8 L of ultrapure water in a 2 L glass bottle.
   Let it cool at room temperature.
   Complete medium:
   a. Add the following to the basal medium
      20 mL of $MgSO_4$ 400 mM
      16 mL of $CaCl_2$ 50 mM
      20 mL of $Fe(NH_4)_2(SO_4)_2$ 5 mM
      20 mL of Na_2HPO_4 250 mM
      20 mL of KH_2PO_4 250 mM
      and 100 mL of glucose solution.
   b. Filter through 0.22 µm Stericups using a peristaltic pump and store at room temperature.
3. Ampicillin stock solution (100 mg/mL)
   a. Dissolve 1 g of ampicillin in 10 mL of ultrapure water

b. Filter through a 0.22 µm filter plugged on a syringe
c. Aliquot in 1.5 mL tubes and store at -20 °C

4. Kanamycin stock solution (25 mg/mL)
   a. Dissolve 250 mg of kanamycin in 10 mL of ultrapure water
   b. Filter through a 0.22 µm filter plugged on a syringe
   c. Aliquot in 1.5 mL tubes and store at -20 °C
5. K36 buffer
   a. Dissolve 2.25 g of KH_2PO_4, 5.8 g of $K_2HPO_4.3H_2O$, 7.4 g of KCl and 0.9 g of NaCl in 1 L of ultrapure water.
   b. Filter through a 1 L 0.22 µm Stericup linked to a peristaltic pump and store at room temperature.
6. Cesium chloride solutions

Density 1.5 g/cm^3: dissolve 22.7 g of CsCl in 27.3 mL of ultra pure water.
Density 1.4 g/cm^3: dissolve 19.4 g of CsCl in 30.6 mL of ultra pure water.
Density 1.3 g/cm^3: dissolve 15.62 g of CsCl in 34.38 mL of ultra pure water.
Density 1.2 g/cm^3: dissolve 11.2 g of CsCl in 38.8 mL of ultra pure water.
Once the solutions appear clear, filter through a 0.22 µm filter plugged on a syringe.

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