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Opti-Prep density separation of viruses for metagenomics

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We use this protocol and it's working

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Abstract

Density purification of viruses from liquid suspensions to remove cells, cell debris, and contaminants related to growth media. Purified viruses can be used in a variety of downstream applications, including sequencing with lower likelihood of cellular contamination and cleaner electron microscopy images.



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- 1 Based on Kauffman, K., Hussain, F., Yang, J. *et al.* A major lineage of non-tailed dsDNA viruses as unrecognized killers of marine bacteria. *Nature* **554**, 118–122 (2018).
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Materials

- 2
 1. OptiPrep Density Gradient Medium, Millipore Sigma, SKU:D1556
 2. Sterile virus-free water (autoclaved or 0.02µm filtered) OR Sterile Salt Mix buffer
 3. Ultraclear thin wall ultracentrifugation tubes (e.g. Fisher Scientific 14-810-54A; will vary depending on centrifuge and rotor)
 4. 1mL syringes (at least two per density step used in the gradient)
 5. 1.5-2inch hypodermic needles, gauge 23, 22, 21, or 20 (at least one per density step used in the gradient)
 6. Beveled hypodermic needles of any length, gauge 23
 7. Falcon tubes/microcentrifuge tubes as needed

Preparation of OptiPrep gradient

- 3 Prepare OptiPrep solutions at your chosen densities (OptiPrep Density Gradient Medium, Millipore Sigma, SKU:D1556). OptiPrep is a 60% w/v solution of iodixanol (density 1.32 g/mL). It comes sterile, with a rubber stopper in the top. Wipe the stopper with 70% ethanol and use a needle and syringe to draw the desired amount through the stopper. Do not remove the cap and stopper.

40% solution: 4mL OptiPrep in 2mL sterile water/salt mix buffer

35% solution: 3.5mL OptiPrep in 2.5mL sterile water/salt mix buffer

30% solution: 3mL OptiPrep in 3mL sterile water/salt mix buffer

25% solution: 2.5mL OptiPrep in 3.5mL sterile water/salt mix buffer

We make 30-40mL of the working solutions and store them in 50mL Falcon tubes at 4°C. As long as you are careful when using it, it should not become contaminated. We also use salt mix buffer for improved viral stability, though sterile water may be sufficient depending on the environment from which the viruses were obtained.

The OptiPrep stock solution should be kept at room temperature.

- 4 Stand 4 ultraclear, thin-wall ultracentrifugation tubes up between the pegs of a peg tube rack (e.g. Fisher Scientific 14-810-54A) . The tube you purchase will depend on your ultracentrifuge and rotor, but you definitely want thin-walled and disposable ones (e.g.

Beckman Coulter C14282) because you will need to puncture the tube with a needle after centrifugation is complete.

You might need more than 4 tubes, depending on your rotor and the requirements for balance. Our ultracentrifuge accepts a maximum of 4 tubes, so that is the highest number of samples we can work with at once.

Setting up the gradient

5 **Optional:** In autoclaved 1.5mL microcentrifuge tubes, aliquot 1mL of each solution for every ultracentrifuge tube you are using. This will help avoid contamination of the stock solutions. We will present the remainder of this protocol according to our normal practices: 4 ultracentrifuge tubes and 4 different densities (25%, 30%, 35%, 40%). You will want to aliquot each of the 4 solutions 4 times, in individual 1.5mL tubes.

6 You will need sterile 1mL leur-lock syringes and sterile 1.5 inch hypodermic needles.

7 Attach a needle to the end of a syringe and slowly draw up 1mL of the **lowest** density solution. In this protocol, that is the 25% solution. Turn the needle so that the point is facing upwards and gently tap it against the edge of the bench to force any air bubbles towards the tip.

It may help to draw the plunger back slightly to pull in any liquid still left in the needle before tapping.

8 Slowly push the air out of the syringe until a tiny bead of solution appears at the end of the needle.

This step is very important to prevent air bubbles from being pushed into the gradient. If air bubbles enter, they will slowly float to the top through the layers of different densities, potentially mixing them and disrupting the gradient.

9 Place the syringe needle end down into the first ultracentrifuge tube and slowly push the solution out. Once the plunger reaches the bottom of the syringe, lift the needle out slowly.


Once you have multiple layers in place, it's extremely important to keep your hands as steady as possible while drawing the needle out to avoid disturbing the boundaries between layers.

**Note**

DO NOT try to extrude every last drop from the syringe. Once the plunger has reached the bottom, you can stop. Any solution left in the needle can stay there. You don't want air bubbles disrupting the gradient.

By tapping out all air bubbles in step 6, and assuming you use needles of a uniform length and gauge every time, you should always have the same volume of solution being dispensed.

- 10 Use a fine-tip black or blue pen to draw a line on the ultracentrifuge tube, marking the height of the solution in the tube.
- 11 Repeat steps 5-8 for the remainder of the ultracentrifuge tubes for the 25% solution.
- 12 Now repeat steps 5-9 for each of the other densities. Still moving from lowest to highest density (30%, 35%, then 40%), layer the solutions by placing the needle all the way at the bottom of the tube and slowly pushing the solution out.

You should end up with your densest layer on the bottom of the ultracentrifugation tube, followed by the lighter solutions in decreasing order of density. The 25% layer should now have risen to the top of the tube as the heavier solutions were layered below it.
- 13 Allow the gradient to sit at room temperature for 2 hours to blend slightly.  02:00:00

Adding sample and centrifuging

- 14 Viral samples should be filtered to remove most cells and debris. Though density separation is meant to purify your viral particles, excess debris should be removed.
- 15 If each tube will be used for a different sample, use a fine-tip black pen to write the sample ID around the tube.
- 16 Carefully layer 1mL of your viral sample on top of the density gradient, making sure not to disrupt the gradient. You want your sample to float on top.

There are various ways to do this carefully. Some favored by members of the lab:

1. Just pipette very slowly with a P1000. Pushing the pipette tip against the inner wall of the tube helps prevent large droplets from hitting the surface forcefully.



2. Instead of dispensing normally, fill a P1000 and slowly turn the knob that adjusts volume to push the liquid out of the pipette. Again, positioning the tip against the inner wall of the tube can help.

3. Use a 1mL lure lock syringe without a needle, and layer the sample on top of the gradient by gently touching the tip of the syringe to the gradient and depressing the plunger of the syringe slowly. This maintains the surface tension of the gradient very well.

You should now have 5 layers in each tube. From top to bottom: the sample, the 25% layer, 30%, 35%, and 40%


- 17 If you have a swinging bucket rotor, put each tube into its holder; for fixed angled rotors, leave the tubes as they are. Carry your tubes over to a weigh station. Walk slowly, and do not jostle the tubes to the extent possible.
- 18 Weigh each tube (with the holder, for the swinging bucket rotors). Bring lighter tubes up to the weight of the heaviest with the dropwise addition of sample, sterile salt mix buffer, 1X PBS, or water.

Safety information

The tubes must all be the same weight, or you risk catastrophic failure of the ultracentrifuge. At best, this can cause expensive damage; at worse, serious injuries or fatalities to those in the vicinity.

- 19 Place the tubes/tube holders into your rotor. We use a Beckman Coulter Optima MAX-E Ultracentrifuge with an MLS 50 50,000rpm swinging bucket rotor, and the following parameters will be specific to that machine. You will need to find the equivalent parameters for your ultracentrifuge. We usually spin a little below room temperature.

If you are concerned about samples overheating, you can also run your ultracentrifuge at 10°C.

Spin at 50,000rpm (268,000 x g) for 5 hours  50000 rpm, 21°C, 05:00:00

- 20 In the meantime, label a series of 2mL microcentrifuge tubes with the sample name and the density fraction. For example, we might write

Sample 1, top layer

Sample 1, 25%

Sample 1, 30%

Sample 1, 35%

Sample 1, 40%



Sample 2, top layer

Sample 2, 25%

Sample 2, 30%

Sample 2, 35%

Sample 2, 40%

Etc.

Sample removal

21 Once the centrifugation is complete, remove tubes from the rotor and place back onto your peg tube rack. Be careful not to jostle them.

22 Gather more sterile microcentrifuge tubes (recommend 2mL), 1mL syringes, and 23 gauge hypodermic needles. You will need one of each for every horizon (e.g. every density fraction) in every tube.

Note the lines that were drawn on the tube when the density gradient was first made. These lines will now act as guide markers for extracting samples from different density fractions.

If working with cultured viruses at high numbers, you may even see a band indicating where the viruses are. We suggest pulling this band out first, then pulling the rest of the fractions out as outlined below.

23 Gently push the hypodermic needle into the side of the tube just underneath the topmost line. Do not push through the opposite wall. Turn the needle such that the opening faces upwards (these are hypodermic needles, so should have beveled ends).

24 With the needle/syringe angled slightly upward, SLOWLY draw the topmost fraction of the density gradient into the syringe and dispense into the labeled 2mL tube.

25 Repeat with the 25%, 30%, 35%, and 40% fractions, using lower and lower lines as fiduciary markers.

26 Repeat steps 21-23 for the remaining tubes.

Keep in mind that these fractions are no longer at their starting densities due to the centrifugation - the labeling as "25%" or "30%" helps us keep track of what part of the tube the sample was drawn from, but are not true representations of the iodixanol concentration.



- 27 In our experience, the 30% and 35% fractions held almost all of our viruses. There was very little in the topmost watery layer or the 25% fraction, though some samples had viruses in the 40% fraction. The easiest way to determine which fractions contain the viruses is to stain with SYBR Gold or some equivalent nucleic acid stain and image with fluorescence microscopy.

Protocol references

Kauffman, K., Hussain, F., Yang, J. *et al.* A major lineage of non-tailed dsDNA viruses as unrecognized killers of marine bacteria. *Nature* **554**, 118–122 (2018). <https://doi.org/10.1038/nature25474>