Purification of viral assemblages from seawater in CsCl gradients

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Abstract

OptiPrep gradients, as described for purifying viral isolates, may also be used for purifying marine viral assemblages. There are as yet, however, few descriptions of this application in the literature. CsCl gradients, on the other hand, have been used extensively for purifications of viral isolates and natural viral assemblages. Given the continued popularity of CsCl gradients, and their well-characterized performance, we present the following two-part purification and fractionation protocol for viruses in CsCl gradients. The first part involves relatively quick sedimentation through a step-gradient to remove the bulk of the contaminants. This is followed by a higher resolution continuous gradient to separate the viruses from residual contaminants and to separate viruses having differing buoyant densities from one another. We assume the starting material for the following procedure to be a concentrated suspension of viruses (whether filtered to remove cells or not).

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Guidelines

Materials and Reagents

- Ultracentrifuge
- Swinging-bucket rotor (Beckman Coulter SW 41 Ti or Sorvall TH-641)
- Polyallomer tubes (Beckman Coulter part No. 331372; Sorvall part No. 03669)
- CsCl (molecular biology grade)
- SM Buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris pH 7.5)

Discussion

The point of the step gradient in this two-step protocol is to achieve a quick initial purification of viruses, which can then be purified to greater degree in the subsequent continuous gradient. The spin is kept short to minimize diffusion between layers, which would eventually result in a continuous gradient. Because the spin is short, not all material may reach its equilibrium position in the gradient. Viruses, having relatively high sedimentation coefficients, reach their equilibrium positions more quickly than many other dissolved macromolecules. Since all the material starts at the top of the gradient, viruses can therefore be most efficiently separated from less dense material (e.g., lipids, proteins, and most bacteria) as well as molecules that are more dense, but which have low sedimentation rates (small pieces of DNA or RNA). The subsequent continuous gradient, when centrifuged to equilibrium, provides good separation of viruses from contaminants and different viruses from one another. Since the less dense contaminants are mostly removed in the step

gradient, which is unloaded from the bottom, we recommend unloading the subsequent continuous gradient from the top, thereby minimizing contamination from free nucleic acids, which will either band below the viruses (DNA) or form a pellet at the bottom of the tube (RNA). Viruses have been purified for metagenomic analyses using only a step gradient (Angly et al. 2006; Breitbart et al. 2004; Breitbart et al. 2002; Vega Thurber et al. 2009), or no gradient at all (Bench et al. 2007; Helton and Wommack 2009). In the latter cases 0.2-µm filtration and nuclease digestion are relied upon to remove nonviral nucleic acids. Since many virus genes recovered from the environment may not be recognized as viral based only on their sequence (Edwards and Rohwer 2005), having a highly purified virus preparation increases one's confidence that any novel sequences recovered do indeed derive from viruses rather than from cellular life forms. For that reason, one may find the complete two-part gradient purification protocol especially desirable for viral metagenomic studies. A rigorous gradient purification would be particularly important if the sample had not been first filtered to remove prokaryotes.

Fixed-angle rotors (including vertical, near-vertical, and others) are commonly used for equilibrium buoyant density gradients, especially self-forming gradients, because the centrifugation times required to approach the equilibrium gradient shape can be much shorter. However, the use of a swinging bucket rotor for the continuous gradient, as presented here, has several advantages: 1) only a single rotor is needed for the both the step and the continuous gradients, 2) the open-top tube simplifies gradient unloading from the top, and 3) there is less chance of contaminating the viruses with dissolved nucleic acids or cellular material. Any contaminating nucleic acid, especially RNA, that pellets during the run will be located at the very bottom of the tube where it will not be in contact with the virus bands. Material that is less dense than the least dense portion of the gradient will not pellet, but will float at the top of the gradient. In any type of fixed angle rotor, these potential contaminants will pellet to some degree on the sides of the tube and come in contact with the viral bands during deceleration as the gradient reorients in the tube. This material could contaminate the recovered fractions if it is dislodged or diffuses from the pellets during unloading. This is of particular concern for a sample that has not been first purified through a step gradient.

To partially compensate for the longer centrifugation times required for gradient to reach equilibrium in a swinging bucket rotor, we present a protocol in which a continuous gradient forms by diffusion from an initial step gradient. The modest amount of extra effort needed to prepare the step gradient is compensated by a significantly shorter run time. If one were to start with a homogeneous CsCl solution in an SW 41 or TH-1641 rotor, it could take ≥ 80 h for the gradient, and the constituents within it, to approach equilibrium. Another advantage of the step gradient is that it provides a dense cushion at the bottom of the tube that prevents viruses from pelleting early in the run before the gradient has fully formed.

WARNING! The protocol we present here is tailored to the specified rotors and operating conditions. Many other rotors and centrifugation conditions could be used instead. However, if you wish to change the conditions or adapt the methods to a different rotor, it is critical that you ensure that the new conditions are within the safe limits for centrifugation of CsCl gradients. Centrifugation of CsCl solutions at certain combinations of concentration, temperature, and rotor speed can result in CsCl crystallization at the bottom of the tube. The high density of the crystals will exceed the tolerance of the rotor and could result in catastrophic rotor failure.

Protocol

Step gradient

Step 1.

Prepare CsCl solutions in SM buffer having densities of 1.2, 1.35, and 1.55 g mL^{-1}.

ANNOTATIONS

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See “Gradient preparation” for helpful information.

Step gradient

Step 2.

Sequentially layer the CsCl solutions:

2 mL of 1.55 g mL^{-1}
2 mL of 1.35 g mL^{-1}
1 mL of 1.2 g mL^{-1}

followed by the sample, into an ultracentrifuge tube.

ANNOTATIONS

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To minimize mixing at the interface where viruses will collect, the bottom layer (1.55 g mL^{-1}) can be chilled on ice prior to adding the next layer. The total volume should fill the tube to within 2-3 mm of the top. This procedure will accommodate up to 6.5 mL sample, but smaller sample sizes will ensure the most rapid sedimentation of viruses into the gradient. If necessary, top off the sample with mineral oil. NOTE: mineral oil is compatible with polyallomer tubes, but not Beckman Coulter Ultra-Clear or equivalent tubes.

Step gradient

Step 3.

Prepare a balance tube (containing a second sample or a blank) in the same manner as described above.

Step gradient

Step 4.

Verify that tubes that will be in opposing positions in the rotor are well matched.

ANNOTATIONS

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Opposing tubes should have the same layers that match in density and volume. The total masses of opposing tubes can be matched by adjusting the volume of the top layer. The final total masses should be matched to well within the tolerance of the rotor. Balancing to within 1% of the total mass should be more than adequate. Modern centrifuges have much greater imbalance tolerance (as high as 10%), but consult the manual for your rotor and centrifuge for recommendations and always err on the side of caution.

Step gradient

Step 5.

Centrifuge the sample at 40,000 rpm for 2.5 h at 20°C (4°C is also acceptable).

DURATION

02:30:00

ANNOTATIONS

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Do this as soon as possible after the gradient has been prepared. For older rotors that have been permanently derated to 36,000 rpm, spin at this lower speed, and increase centrifugation time to 3.5 h.

Step gradient

Step 6.

Unload the gradient immediately after centrifugation by puncturing the side of the tube close to the bottom with a needle and stopcock assembly (see "Gradient Fractionation and Sample Collection") and collecting 0.5 mL fractions.

ANNOTATIONS

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A low side puncture is recommended, because some unencapsidated nucleic acids may have pelleted and could contaminate the virus fractions if the tube is punctured at the very bottom.

Step gradient

Step 7.

Measure the densities of the collected fractions (see "Measuring fraction densities" for important considerations). Fractions having densities in the appropriate range can be pooled and subjected to further fractionation and purification.

ANNOTATIONS

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The majority of viruses in seawater have densities > 1.35 and < 1.50, while most bacteria will have densities < 1.35 g mL^{-1}.

Step gradient

Step 8.

To ensure that the maximum numbers of viruses are recovered with the minimum contamination, one may wish to count viruses (and bacteria if appropriate) in each fraction by epifluorescence or electron microscopy.

ANNOTATIONS

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One could also assay the fractions for nucleic acid content to determine the distribution of RNA and DNA viruses, or by PCR to determine the location of specific viruses of particular interest.

Step gradient

Step 9.

If proceeding to the subsequent continuous gradient purification, pool the virus-containing fractions of the appropriate density and purity.

Step gradient

Step 10.

Make sure they are well mixed, then determine the initial density of the pooled sample (see "Measuring fraction densities") and proceed with the continuous gradient protocol below.

ANNOTATIONS

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If further purification is not required, proceed to the postgradient cleanup steps described following the continuous gradient protocol.

Continuous gradient

Step 11.

Add CsCl and SM buffer as needed to the sample to achieve a final density of 1.45 g mL^{-1} and a final volume of 4 mL.

Continuous gradient

Step 12.

Make sure all of the CsCl is dissolved, the sample is well mixed and near 20°C, and then verify its density.

ANNOTATIONS

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See "Measuring fraction densities".

Continuous gradient

Step 13.

Prepare solutions of CsCl in SM having a density of 1.20 g mL^{-1} (≥ 4.5 mL per sample) and 1.60 g mL^{-1} (≥ 3 mL per sample).

Continuous gradient

Step 14.

Layer 3 mL of the 1.60 solution, followed by 4 mL sample at 1.45 g mL^{-1}, then 4.5 mL of the 1.20 g mL^{-1} solution into an ultracentrifuge tube.

Continuous gradient

Step 15.

Prepare a balance tube (containing a second sample or a blank) in the same manner as described above.

Continuous gradient

Step 16.

Verify that tubes in opposing positions in the rotor are well matched.

ANNOTATIONS

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Opposing tubes should have the same density and volume and, therefore, total mass. The masses of opposing tubes should be matched to well within the tolerance of the rotor. Consult the manual for your rotor for recommendations, but within 1% of the total mass should be more than adequate (guidelines for modern centrifuges are 10%).

Continuous gradient

Step 17.

Top off the gradients with CsCl (1.20 g mL^{-1}) as needed to achieve balance and to ensure that each tube is filled to within a few millimeters of the top.

Continuous gradient

Step 18.

Centrifuge the samples for ≥ 40 h at 30,000 rpm at 20°C (4°C is also acceptable).

Continuous gradient

Step 19.

Unload the gradient immediately, top end first, by direct unloading with an Auto DensiFlow, a piston fractionator, or by displacement from below (see "Gradient Fractionation and Sample Collection").

Continuous gradient

Step 20.

Screen the fractions for viruses (TEM, epifluorescence, nucleic acid assay, etc.) to determine which contain the viruses of interest.

ANNOTATIONS

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Alternatively, if the desired buoyant density range of the targeted viruses is known, the appropriate fractions can be selected based on measured densities of the fractions (see "Measuring fraction densities" for important considerations when measuring fraction density).

Postgradient cleanup

Step 21.

The CsCl in the relevant virus-containing gradient fractions can be removed by buffer exchange using a centrifugal ultrafiltration unit with a nominal molecular weight cutoff of 30,000 to 100,000 (Microcon, Millipore or Nanosep, Pall Life Sciences).

Postgradient cleanup

Step 22.

In this case, the sample is repeatedly concentrated to a small volume, then resuspended in the desired buffer.

Postgradient cleanup

Step 23.

After several such buffer exchanges, the sample is resuspended and recovered in the final desired volume.

Warnings

The protocol we present here is tailored to the specified rotors and operating conditions. Many other rotors and centrifugation conditions could be used instead. However, if you wish to change the conditions or adapt the methods to a different rotor, it is critical that you ensure that the new conditions are within the safe limits for centrifugation of CsCl gradients. Centrifugation of CsCl solutions at certain combinations of concentration, temperature, and rotor speed can result in CsCl crystallization at the bottom of the tube. The high density of the crystals will exceed the tolerance of the rotor and could result in catastrophic rotor failure.