CASE 7

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Molecular Epidemiology
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Fundamentals of Ultracentrifugal Virus Purification

In recent years, in virus research, it has become a standard practice to purify and analyze genomes and identify viruses from samples using commercial kits. Since for the established viruses their genomes have already been known, virus identification is possible even in a mixed state. However, to carry out detailed investigation on the nature of viruses, it is first necessary to refine the virus particles in order to yield a high level of purified materials. These days, our company is also receiving an increasing number of queries regarding information on the use of ultracentrifugation for virus purification. In this context, we asked Dr. Osamu Nakagomi, Professor at Nagasaki University, on the fundamentals of the virus purification by ultracentrifugation, who is an expert on the subject matter. Here is the excerpt from the interview with Professor Nakagomi.

Please tell us about your research background.

My field of research and expertise is the molecular epidemiology of rotavirus. *Rotavirus A* is one of the important viruses since it has been the single most important cause of death due to acute dehydrating diarrhea in infants and children worldwide. Until 1970s, the causes of infantile diarrhea were almost entirely unknown. With the discovery of rotavirus in 1973, it was finally established as the etiological agent accountable for half of the patients admitted to the pediatric wards with diarrhea. This was an epochal discovery in virus research. Rotavirus vaccines were developed and licensed in 2006 after overcoming many hurdles, and the World Health Organization recommended in 2009 that rotavirus vaccines be introduced in the national immunization program of every country in the world.

To tell briefly about the virus; rotavirus is a non-enveloped virus which consists of genome that comprises 11 segments of double stranded RNA (**Fig. 1**). Like other RNA viruses, it evolves rapidly and displays rich genomic diversity. Not only it infects humans but also a variety of other mammals and birds. In the 1980's, for the first time, employing a molecular epidemiological technique that allows analysis at the whole genome level, we were able to establish that animal rotavirus can be pathogenic and can in fact cause disease in humans. Elucidating how rotavirus evolves while it uses humans and other animals as hosts, and how the infection spreads, is the core of our research work. We are also investigating what changes are likely to occur with the wider use of vaccines. Basically, the viral genome analysis lies in the heart of molecular epidemiological study of rotavirus.

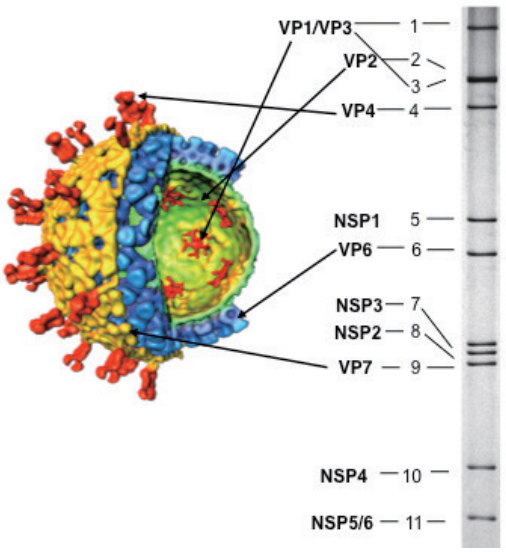


Fig. 1. Rotavirus particle structure and genome RNA
Left: The intact particle has an outer shell made of red and blue proteins and has a three-layered structure, so it is called a triple-layered particle (TLP); if the outer shell is peeled away, a double-layered particle (DLP) with blue proteins remains. Right: The genome RNA of rotavirus is separated into 11 segments with characteristic patterns shown by electrophoresis.
The diagram of the rotavirus particle was kindly provided by Professor Prasad of Baylor University.

Please discuss the necessity of ultracentrifugation in virus research.

When extracting virus genome using the classical method, the virus particles must first be purified. Then the virus genome extracted from the particles is examined. Ultracentrifugation plays an important role in the process. Purifying the virus particles makes it possible from the beginning to ensure that we are dealing with the rotavirus genomes in the virus particles. Currently such analysis is performed almost all the time after hastily extracting the genome without actually purifying the specimen. This practice is common since the genome

of rotavirus is well established and it is a common knowledge that if the genome (**Fig. 1**) characteristic of rotavirus is present, there is no doubt that the genome is present in rotavirus particles as well. However, suppose, for example, that we are dealing with the problem of determining what kind of host cell organelles or virus proteins and genomes are aggregated in an infected cell, ultracentrifugation becomes indispensable.

Moreover, while studying new viruses, it becomes increasingly necessary to investigate whether or not the genome is present in the particle. In such cases, purification with an ultracentrifuge becomes a necessity. Information on the buoyant density, size and sedimentation coefficient (Svedberg value, S value), all of which are taken into consideration in ultracentrifugation, is in fact the fundamental aspect of virology which taken together are called the physiochemical properties of viruses.

Please give us outlines on the purification of viruses by ultracentrifugation.

While purifying rotavirus in samples obtained from patients, we must separate cell organelles, biological macromolecules, and the like from the virus. This is achievable since these particles have a variety of sizes and densities. The purification of viruses by ultracentrifugation utilizes density gaps into which other particles do not come to rest by using a combination of "sucrose density gradient centrifugation, which is affected by the S value of particles" and "cesium chloride density gradient equilibrium centrifugation (density equilibrium ultracentrifugation), which is affected by the buoyant density of particles in a solvent." **Fig. 2** shows the relationship between banding density (the density at which bands appear during density gradient centrifugation) and S value. The virus is located exactly in gaps between a variety of cell particles, including microsomes and ribosomes. Viruses are complexes of proteins (mostly with a buoyant density of 1.3 g/cm^3) and nucleic acids (mostly with a buoyant density of 1.7 g/cm^3), so the buoyant density ranges from 1.35–1.4 g/cm^3. On the other hand, the size of viruses reaches S values of 100–1,000 S. The S values of microsome fractions overlap with those of viruses (horizontal axis), but separation by density is possible (vertical axis). Thus, separation is performed using density gradient centrifugation of sucrose, cesium chloride, and the like. Virus fractions have a higher density than microsome fractions because they include nucleic acids. Moreover, the larger the virus, the larger its S value. Ultracentrifugation involves purification by combining these two parameters, so it is apparent that it is closely tied to physicochemical properties.

The centrifuge is also extremely important to discern these physiochemical properties. Size can also be reliably ascertained with an electron microscope, but if a new strain of virus is present, the discernment of where the virus will come to rest when centrifuged is important. For instance, influenza and the rotavirus with a moderate size of 100 nm, will sink if spun overnight for about 10,000 + rotations using an Avanti high-speed refrigerated centrifuge. However, norovirus measuring 30 nm in size will not sink with the Avanti centrifuge and in such cases an Optima ultracentrifuge becomes necessary. To make 30nm particles sink with an Avanti centrifuge, purification is performed after reducing the volume following procedures such as salting out.

Moreover, when it comes to the processing volumes following methods are considered in the order of the largest to the smallest: differential centrifugation method (normal pelleting), cushion method and density gradient method. The cushion method allows 60-70% of the tube to be filled with the sample, but the volume is reduced to 10-15% in density gradient centrifugation. Thus, after ascertaining the behavioral characteristics of the particles using the density gradient method, the cushion method is used to enable a greater volume of samples to be processed. For instance, the rotavirus particle passes through a 30% sucrose layer, but does not pass through a 70% sucrose layer, so the virus can be concentrated at the interface between 30% and 70% sucrose layers (**Fig. 3**).

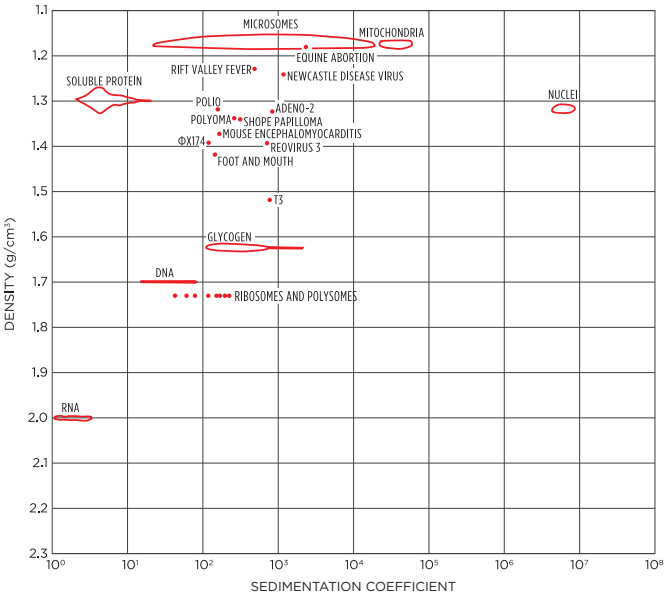


Fig. 2. Virus/cell organelle densities and S values[1]

Please tell us about ultracentrifugal purification of a specific virus.

Fixed-angle versus swinging bucket rotors

Generally, if rotors that produce the same centrifugal force are compared, the fixed-angle rotor allows a greater volume to be processed and is easier to use. However, because top-loading swinging bucket rotors such as the SW 32 Ti allow buckets to be loaded from the top, the difference in ease of use may be eliminated. In fact, as the swinging bucket rotors require a lid on the tube, it could be argued that they are much easier to handle.

If you want to neatly remove bands produced by density gradient centrifugation, swinging bucket rotors are preferred. Pellets leave trails on the surface of the wall with fixed-angle rotors, which some people may consider problematic. In particular, recently, the detection sensitivity of real-time PCR has increased, making it necessary to consider levels of contamination that could have previously been ignored.

In general, then, I think fixed-angle rotors should be selected if larger volumes are required, and swinging bucket rotors should be used if contamination is a major concern.

Sample preparation

I shall explain sample preparation step using the purification of rotavirus from the infectious culture fluid (ICF) as an example. Since the yield of purified virus particles is roughly proportional to the starting volume, it is preferable to use 1 liter of ICF. After a certain degree of concentration has been achieved using a high-speed refrigerated centrifuge, it is further concentrated and purified with a floor-type ultracentrifuge using a swinging bucket rotor SW 32 Ti or SW 55 Ti. In my opinion, the SW 32 Ti is a "dream rotor" because its design makes it easy to use. We use this rotor in Optima ultracentrifuge from Beckman Coulter Life Sciences we have in our laboratory. I consider Optima a trusted partner, especially every time I run it overnight. I know I can always trust it and rely on it the next day to get expected outcomes. And I have never been disappointed.

Fig. 3 is our protocol for purifying rotavirus from ICF.

At first, to eliminate cellular debris, 1 liter of ICF is divided into 6 bottles which is centrifuged for 10 minutes at 15,000 xg using a JA-14 fixed-angle rotor. This step can be omitted, but if done so, there will be more impurities during ultracentrifugation with the cushion method in the next step. The more the pretreatment, the easier and cleaner will be the subsequent operations. Moreover, if there is a large quantity of impurities, the virus gets enmeshed in them, which might decrease the yield.

After removing cellular debris, the remaining supernatant is subjected to further centrifugation for 14-16 hours at 30,000 xg using a JA-14 fixed-angle rotor to get the pellets where viruses are. If you are in hurry, you can use an ultracentrifuge instead, and process 300 mL at a time with a Type 45 Ti fixed-angle rotor. You can rapidly centrifuge these samples either at 30,000 rpm for 2 hours or 40,000 rpm for 1.5 hours.

Next, suspend the pellets in 26 mL of phosphate buffered saline (PBS). Deciding in what volume of PBS the pellets are to be suspended requires experience and intuition. In general, never concentrate thicker than 1 to 10 at each step of purification. For instance, if your starting material is 1 L, you should not concentrate below 100 mL; if you concentrate to say, 10 mL, the resulting product will be muddy. For ease of handling you may want to opt for higher concentrations, but you will regret later. However, this is only a rule of thumb.

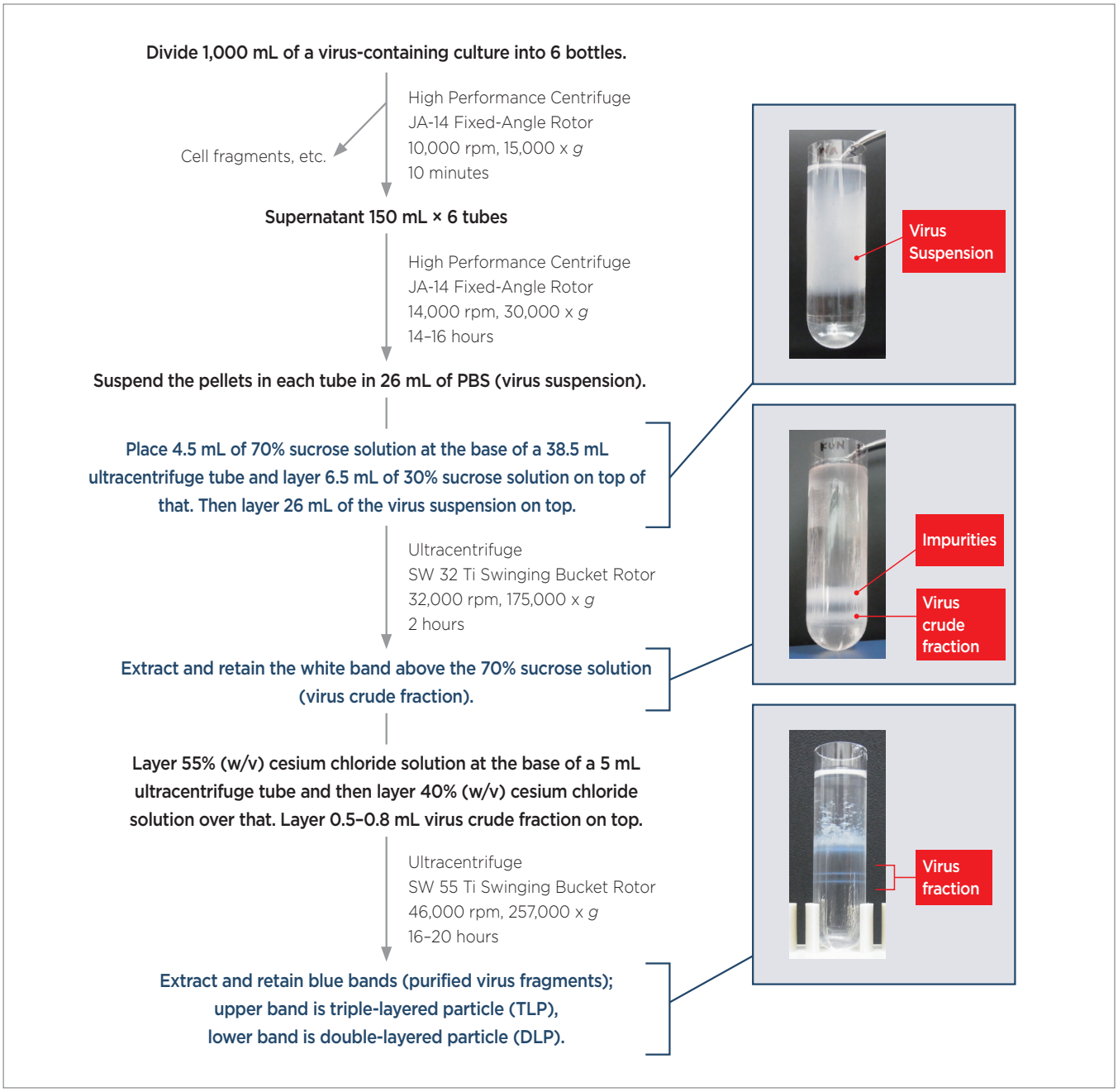


Fig. 3. Rotavirus purification

Purification by Ultracentrifugation

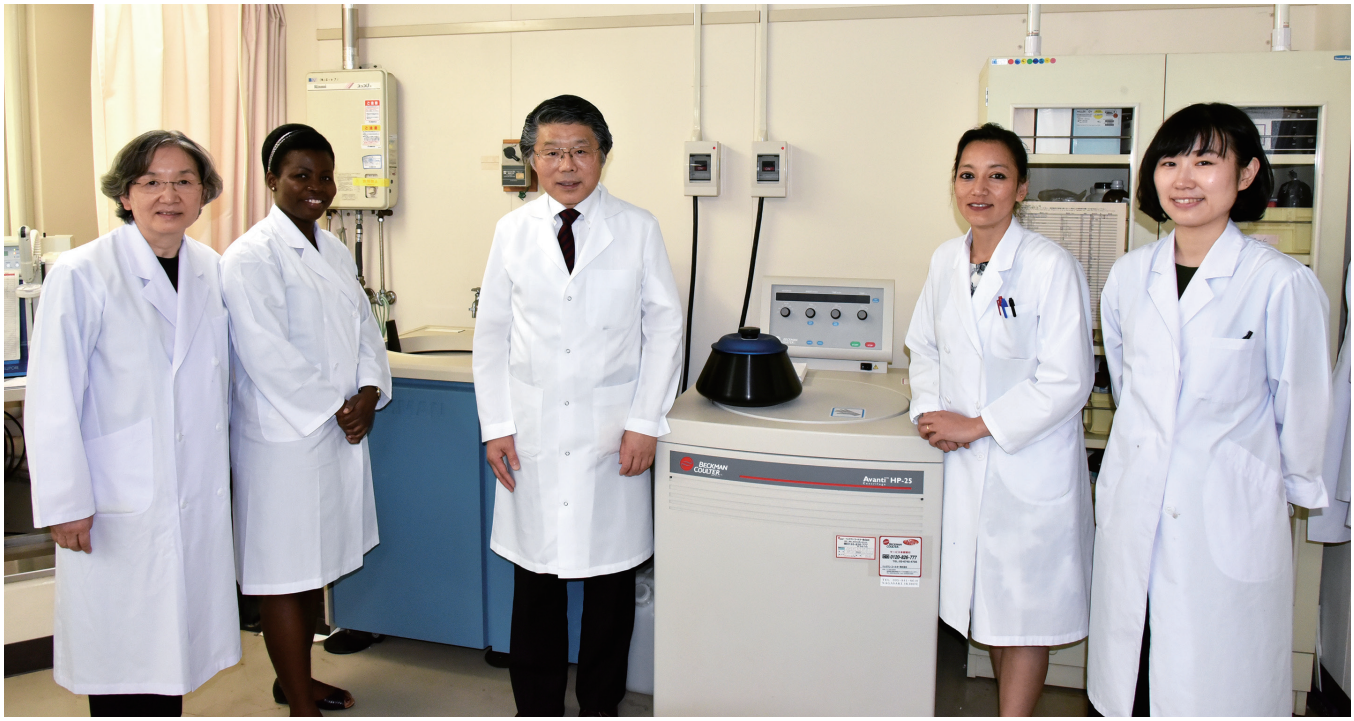
The impurities can be further removed using sucrose cushion method with a large capacity SW 32 Ti swinging bucket rotor. The rotavirus particles can be concentrated at the interface between the 30% (w/v) and 70% (w/v) sucrose layers. For this, dispense 4.5 mL of 70% sucrose solution at the base of a 38.5 mL ultracentrifuge tube and layer 6.5 mL of 30% sucrose solution on top, which is then overlaid with 26 mL of the virus suspension in PBS. When ultracentrifugation is finished under the conditions given in **Fig. 3**, impurities will pool in larger quantities above the 30% sucrose solution layer and the rotavirus particles will pool between the 70% and 30% sucrose cushion and appear as a white band. The white band can be collected by one of the three different ways; directly inserting a syringe into the band to extract the white band; collecting the white band after removing the unnecessary liquid layers just above it, or by separating the fractions by inserting a needle through the base of the tube.

In order to further increase the degree of purity, density gradient centrifugation can be performed using a small- capacity SW 55 Ti swinging bucket rotor. For this, dispense 55 % (w/v) cesium chloride solution at the base of a 5 mL ultracentrifuge tube and then overlay it with same quantity of 40% (w/v) cesium chloride solution. Density gradations are produced by layering solutions with different densities, and this procedure is often considered time-consuming. However, a density gradient can naturally be formed after ultracentrifugation even with only two layers. This is a little trick. Layer 0.5-0.8 mL of crude virus fraction obtained by the cushion method (it is always better to use a small quantity of the solution in this case) on top of a cesium chloride solution and perform ultracentrifugation for 16-20 hours at 257,000 xg. Sixteen hours mean starting centrifugation in the evening of the previous day and collecting the next morning, but there is no need to be strict about the duration.

As you can see in **Fig. 3**, there are two visible bands of rotavirus in the tube. The upper band comprises 1.36 g/cm^3 intact rotavirus triple-layered particles (TLP) and the lower band comprises 1.38 g/cm^3 double layered particles (DLP) in which the outer shell is stripped off. The virus particles contain RNA. The DLP has fewer proteins than TLP; hence, the proportion of nucleic acids is higher, so it is heavier. The blue band above is of the empty particles consisting only of proteins with no nucleic acids and because they are proteins, the buoyant density is around 1.3 g/cm^3. In fact, when the outer shell of the TLP is removed to reveal the DLP, RNA polymerase is activated. And in experiments involving RNA polymerase, DLP rotavirus with the outer shell removed is used.

Rotor cleaning

Since, the high-density cesium chloride is used, it is important to wash the rotor thoroughly with lukewarm water after use to prevent pinhole-like corrosion.



The staff of the Molecular Epidemiology Laboratory

1. Mazzone HM. CRC Handbook of Viruses: Mass-Molecular Weight Value and Related Properties. Boca Raton (FL): CRC Press LLC; 1998.