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Extraction and purification of nucleic acids from viruses

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Abstract

Research on the diversity and ecology of viruses in the environment has been revolutionized by the ability to detect, fingerprint, and sequence viral genes and genomes. The starting point for these molecular assays is the release and recovery of the viral nucleic acids. The complexity of this task depends in large part on the nature of the starting material and the purity and quality of the nucleic acids one requires for downstream applications. In some cases, simply heating the sample will suffice; in other cases, a series of organic extractions and purification in a buoyant density gradient may be required to achieve adequate purity. Our goal in this chapter is to assist the reader in making informed choices from among the many options available. Toward this end, we briefly review the methods that have been used to harvest and store viruses in preparation for extraction, and the methods by which their nucleic acids may be released and purified. We discuss the general principles upon which various commercial extraction kits are based and conclude with the presentation of four step-by-step protocols. We discuss the advantages and disadvantages of these protocols, and the ways in which they may be adapted to various situations.

The field of viral ecology relies heavily on molecular methods, such as PCR and sequencing, that target viral nucleic acid. The need to extract and purify nucleic acids is therefore nearly universal in the field, and many methods have been described to accomplish these tasks. Which of these methods is most appropriate depends on the nature of the starting material, whether one wishes to purify DNA or RNA, and the purity required of the nucleic acid. Depending on the application, one may be starting with a purified virus, a partially purified virus assemblage, viruses in a complex assemblage of other plankton, or viruses in a complex physical matrix such as sediments. At the time of extraction, the viruses may be captured on a filter, present in a pellet, or suspended in solution. Given the innumerable possible starting materials and the varied requirements for the final product, we do not attempt in this article to provide a direct comparison of methods. Instead, we focus on reviewing the common strategies for nucleic acid extraction and provide a few protocols that have been used in the field of aquatic viral ecology.

We first present some background information on the common methods for harvesting and storing viruses before extraction. We then consider the two basic steps in the extraction and purification of viral nucleic acids: (1) release of the nucleic acid from the virion and (2) separation of the nucleic acids from other viral structural components. After this background information, we present specific step-by-step protocols and assess their advantages and disadvantages. Where appropriate, we also discuss possibilities for adapting these methods to situations other than those explicitly described.

Background information

A. Harvesting viruses for extraction—

Prefiltration: For some applications (e.g., genomic and metagenomic analyses), it may be desirable to prefilter the sample from which viruses will be harvested through a 0.2-µm filter to remove prokaryotic and eukaryotic cells. The advantage of using a 0.2-µm filtered sample is that the majority of the nucleic acid extracted from the sample will be viral. Metagenomic analyses of viruses in whole plankton samples are necessarily limited to the minority of sequences that can be unambiguously recognized as viral (Williamson et al.

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2008). A serious disadvantage to prefiltration is the variable, and sometimes significant, loss of viruses that can occur (Paul et al. 1991; Steward et al. 1992). Sometimes the losses may be minimal (Suttle et al. 1991; Wommack et al. 1995), but when losses do occur, they are likely to be more severe for larger viruses (Brum and Steward, paper accepted 2010). Fractionation in buoyant density gradients is one possible alternative to 0.2-µm filtration for separating cells and viruses (Lawrence and Steward 2010, this volume), since most viruses are more dense than most cells. The separation will not be absolute, however, since the density ranges of cells and viruses overlap.

If one plans to perform virus-specific molecular assays on the sample (e.g., PCR using virus-specific primers), one may be less concerned about the presence of nonviral nucleic acids. In this case, one might consider harvesting the entire microbial assemblage for extraction, especially if one hopes to perform quantitative assays. The advantages of extracting the whole community are that biases from prefiltration can be avoided and all viruses (extra- and intracellular) will be collected. The disadvantages are a reduced detection limit and uncertainties about which sequences are truly of viral origin, since the majority of the DNA will be nonviral.

Filtration: Tangential flow filtration (TFF) using an ultrafiltration membrane (typically 30,000 to 100,000 nominal molecular weight cutoff) is the method most commonly used by microbial ecologists to harvest viral assemblages from natural water samples (Proctor and Fuhrman 1990; Paul et al. 1991; Suttle et al. 1991; Wommack et al. 2010, this volume). TFF allows one to process a wide range of volumes (tens of milliliters to thousands of liters) by scaling the system components, but the cost and complexity of the equipment and the process have usually restricted processing to a single sample at a time. Losses can be significant owing to adsorption of the virus to the membrane, but methods have been suggested for improving recoveries (Gerba 1983). A detailed description of how to concentrate viruses by TFF may be found elsewhere in this volume (Wommack et al. 2010).

Viruses may also be harvested by direct (or normal) flow filtration using membrane filters with a sufficiently small pore size. Direct flow filtration was impractical with early membrane filters because of the very low flow rates (Clive 1967), but newer aluminum oxide filters having a well-defined pore size of 0.02 µm and a high porosity (Furneaux et al. 1989) are well suited for virus capture. These filters are available in a syringe-tip housing with a Luer-Lok inlet fitting (Anotop; Whatman International), making them convenient for field sampling. Numerous samples of whole or prefiltered seawater can be easily processed in parallel using a multichannel peristaltic pump. Depending on the nature of the sample, hundreds of milliliters (eutrophic coastal water) to several liters (oligotrophic oceanic water) can be passed through a single 25-mm-diameter, 0.02-µm-pore-size Anotop filter. This approach has been used successfully for the analysis of both RNA (Culley and Steward 2007) and DNA (Culley et al. 2008) viruses. Existing normal flow ultrafiltration capsules (OptiScale-25, Millipore; Novasip DV20, Pall) might be suitable alternatives. New nanoporous filter materials are also being developed (e.g., Yang et al. 2008) that may provide additional direct flow filtration options in the near future.

Centrifugation: For small samples, one can reliably collect viruses by sedimentation in a bucket rotor in an ultracentrifuge. For much larger volumes, samples may be processed by continuous-flow ultracentrifugation, in which a feed stream is passed through a rotor at high speed. The latter procedure has been used to efficiently harvest viruses from very large volumes of seawater (Anderson et al. 1967). In either case, batch or continuous flow, the viruses may be pelleted or they may be banded within a buoyant density gradient in the rotor. The latter method both concentrates and partially purifies viruses while maintaining them in suspension. Although centrifugation is effective at harvesting viruses, ultracentrifuges and rotors are expensive (continuous-flow in particular) and not portable. As a consequence, centrifugation has been used infrequently for the initial harvesting of viruses from environmental samples. Ultracentrifugation is, however, used frequently for the final concentration and purification of harvested viruses (Lawrence and Steward 2010, this volume).

Adsorption-elution: A wide variety of virus adsorption-elution (VIRADEL) concentration methods have been developed for monitoring water quality (Percival et al. 2004), and these are used extensively to screen for low concentrations of known viral pathogens in water. VIRADEL-based methods have not found as much use among aquatic viral ecologists, perhaps because of the need in many of these methods to extensively manipulate the chemistry of the sample to control the adsorption-elution behavior (Sobsey 1976), and the consequence of this for the recovery of total viruses from natural assemblages has been uncertain. The recovery efficiencies of the various VIRADEL methods are virus dependent (Percival et al. 2004), suggesting that these methods would lead to biases if applied to the analysis of complex communities. VIRADEL recoveries, however, are typically measured as infectious units rather than viral particles. The former may be much lower than the latter if some viruses are inactivated by the procedure. For molecular investigations, the loss of infectivity is not a concern, so some of these techniques may turn out to be less biased when evaluated in terms of total recovery of virus particles. Recent work on the precipitation of viruses from seawater by adsorption to iron hydroxide appears to result in very high recoveries of total viruses (John and Sullivan, pers. comm.). Considering the simplicity, low cost, and high capacities of some VIRADEL methods, this approach is likely to become more popular among viral ecologists who need to process many samples under challenging conditions in the field.

Viruses in sediments: Natural viral communities may also be harvested from sediments for molecular assays. Viruses have been separated from unpreserved sediments by squeezing with a press (Steward et al. 1996) or by centrifugation

(Drake et al. 1998). To facilitate the recovery of viruses, sediments have been diluted and agitated in a buffer (Hewson et al. 2001; Labonté et al. 2009). More aggressive treatments with sodium pyrophosphate (Lawrence et al. 2002; Filippini and Middelboe 2007; Helton and Wommack 2009) and sonication (Filippini and Middelboe 2007) have been employed to facilitate recovery viruses more strongly adsorbed to sediment particles. If one wishes to focus exclusively on the viral fraction of the sediment microbial community, additional fractionation such as by filtration (Lawrence et al. 2002; Filippini and Middelboe 2007; Leroy et al. 2008; Helton and Wommack 2009; Labonté et al. 2009) or purification in a density gradient (Filippini and Middelboe 2007; Lawrence and Steward 2010, this volume) would be required to separate the viruses from the other microbes. Once the viruses are suspended in liquid, they may then be further concentrated using one of the various techniques described above. The separation of viruses from sediments is discussed in greater detail elsewhere in this volume (Danovaro and Middelboe 2010).

B. Storing virus samples before extraction—

Freezing: The nucleic acids within harvested viruses can be preserved by freezing the concentrated material at –80°C. This can result in physical damage to some viruses, and potentially a loss of infectivity, but both DNA and RNA are well preserved at or below this temperature (Sambrook and Russell 2001). If one has decided on an extraction protocol ahead of time, it may be advisable to freeze the sample in the extraction buffer, as this may help prevent degradation of any nucleic acids extruded from the virions as a result of the freezing and thawing process. The major drawback of relying on ultra-low-temperature storage is the difficulty of maintaining these temperatures in the field.

Preservation solution: Immediate freezing may be unnecessary with the use of a patented nucleic acid preservation solution (RNALater™; Ambion) that appears to offer protection of not only nucleic acids, but also infectivity among the viruses tested (Lader 2001). An equal volume of RNALater added to liquid samples protected viral RNA from degradation when the samples were subjected to freeze-thaw cycles (Forster et al. 2008). This preservation solution, which appears to function primarily through ammonium sulfate precipitation of proteins and nucleic acids, also preserved the infectivity of RNA viruses in samples stored at room temperature for up to 72 h (Uhlenhaut and Kracht 2005). Although infectious titer declined by several logs after 50 days of storage at room temperature, the titers were orders of magnitude greater than a control sample stored in phosphate-buffered saline for the same period. These promising results suggest that it would be possible to preserve concentrates of aquatic viruses (in solution or on filters) under field conditions where immediate freezing is not possible.

Despite the specificity implied by the name, RNALater preserves DNA as well as RNA (Gorokhova 2005), so it should work just as well for the preservation of the genomes of DNA-containing viruses. If the virion structures are preserved, then viruses suspended in the preservation solution should be recoverable by centrifugation or filtration before extraction. Small amounts of RNALater are compatible with a variety of extraction kits and methods (Ambion technical literature), so limited quantities may also be directly extracted.

C. Releasing nucleic acids from viruses—The most common methods used to release nucleic acids from virions involve the use of heat, osmotic shock, detergents, chaotropic salts, or organic solvents, either alone or in combination, all of which lead to denaturation of capsid proteins (Ralph and Bergquist 1967). The buffer Tris(hydroxymethyl)-aminomethane (or simply Tris) is commonly used to maintain nucleic acid solutions at slightly alkaline pH to minimize chemical hydrolysis of the nucleic acids, but acidic eonditions are sometimes prescribed for selective extraction and storage of RNA.

Thermal destabilization in the presence of chelators: The simplest method by far to release nucleic acids from virions is to heat the sample (typically to 45–100°C). This alone is sufficient for some applications (Richardson et al. 1988), in particular for obtaining nucleic acids from purified viruses where nuclease contamination is expected to be minimal. If nucleases are expected to be present, then heating should be carried out in the presence of a chelating agent such as ethylenediamine-tetraacetic acid (EDTA). Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) may also be added depending on the circumstances. EDTA, and sometimes EGTA, is included in buffers used with DNA, because they chelate divalent and trivalent cations, which are a required cofactor for certain nucleases (Adams et al. 1992). Divalent cations (particularly Mg^{2+} and Ca^{2+}) also contribute to the stabilization of viral capsids (Brakke 1963; Brady et al. 1977; Ruiz et al. 2007). The presence of these chelators therefore simultaneously facilitates disintegration of the capsid and protects DNA from degradation. DNase is itself irreversibly inactivated by heating to 65°C, but the disintegration of viral capsids can occur faster. Viral DNA can therefore be lost if viruses are heated to 65°C in the presence of DNase and the absence of EDTA (G. F. Steward, pers. observation). For experiments that rely solely on heat to inactivate DNase in the presence of viruses (e.g., Fuller et al. 1998), viral DNA concentration is likely to be underestimated. It should be kept in mind that EDTA and EGTA can affect some downstream reactions (such as PCR) that use Mg^{2+}-dependent enzymes if they are carried over at a concentration that is a significant fraction of the Mg^{2+} concentration in the reaction buffer.

Neither EDTA nor EGTA inactivates RNase, so other RNase-inhibiting agents may be required if one plans to extract RNA-containing viruses by simple thermal destabilization. RNase inhibitors include ribonucleoside-vanadyl complex (Berger and Birkenmier 1979) and RNasin (Blackburn et al. 1977), as well as other commercially available proprietary reagents (e.g., RNAsecure, Ambion; RNase Out, Invitrogen). The ribonucleoside-vanadyl complex will also inhibit downstream reactions

such as in vitro translation and reverse transcription, so is not recommended (Farrell 2005).

Osmotic shock: Osmotic shock can be used to disintegrate the capsids of some viruses, but others are resistant to this treatment (Anderson 1950; Anderson et al. 1953). This phenomenon may therefore facilitate some extraction protocols, but is generally not relied on. The sensitivity of some viruses to osmotic shock should be kept in mind when harvesting and purifying viruses, however, since exchanges of buffers having very different osmolarities may lead to unintentional release and potential loss of nucleic acids.

Detergent: Sodium dodecyl sulfate (SDS) is an ionic detergent frequently added to extraction buffers. SDS solubilizes capsids by disrupting inter- and intraprotein hydrophobic interactions (Putnam 1948; Reynolds and Tanford 1970). SDS may be used alone (Sreenivasaya and Pirie 1938), but is typically used in combination with heating (Fraenkel-Conrat et al. 1957) and enzymatic digestion of proteins (Sambrook and Russell 2001) to effect the release of nucleic acids. Formamide will also disrupt phage capsids and has been used as a rapid, simple, but perhaps less effective (Sambrook and Russell 2001), alternative to treatment with heat, SDS, and proteinase K digestion for extraction of DNA from viruses (Vega Thurber et al. 2009).

Chaotropic salts: Chaotropic salts such as sodium iodide (NaI) or guanidinium thiocyanate (GTC) can also disrupt capsids by denaturing proteins. The guanidinium and thiocyanate ions of GTC are particularly strong denaturants (Mason et al. 2003) and consequently facilitate disintegration of viral capsids while simultaneously inactivating nucleases. Because GTC so effectively inactivates RNase, it remains a common ingredient in RNA extraction protocols since its first use in this capacity thirty years ago (Chirgwin et al. 1979). The nonpolar organic solvent, phenol, also has a long history of use in nucleic acid extractions (Kirby 1956; Kirby 1957). Although typically used for its ability to extract proteins from nucleic acid solutions (Sambrook and Russell 2001), phenol will simultaneously effect the disruption of viral capsids by denaturing the proteins (Faulkner 1962).

The above methods and reagents are among the most common, but a wide range of other strategies have been used to release nucleic acids from viruses. The interested reader will find a comprehensive review of these earlier efforts in *Methods in Virology*, vol. 2 (Ralph and Bergquist 1967).

D. Separating nucleic acids from other macromolecules—Once nucleic acids have been released from the viruses, it may be necessary to separate the nucleic acids from other macromolecules in the lysate. This may be achieved by exploiting differences in solubility or buoyant density among macromolecules. We will consider five general approaches to this task: (1) organic extraction, (2) differential precipitation, (3) solid-phase extraction, (4) density gradient fractionation, and (5) electrophoresis.

Organic extraction: In organic extraction, proteins and lipids are extracted from a nucleic acid solution using an alkaline buffer–saturated phenol (Kirby 1957) or phenol plus chloroform (1:1). A small amount of isoamyl alcohol (IAA) is also commonly added to the chloroform as an antifoaming agent (phenol:chloroform:IAA; 25:24:1). After emulsification, the aqueous and organic phases are separated by centrifugation. Nucleic acids remain soluble in the upper aqueous phase, which is harvested, whereas lipids and proteins partition to the organic phase or the interface of the organic and aqueous phases (interphase). Traces of phenol, which can interfere with downstream enzymatic reactions or assays, are removed from the aqueous phase by extraction with chloroform:IAA, and traces of chloroform can be removed by extraction with water-saturated ether or by alcohol precipitation of the nucleic acids (Sambrook and Russell 2001). In ether extractions, the aqueous phase is on the bottom. After removing the bulk of the ether by pipetting, residual amounts can be easily removed by evaporation by warming the sample with the lid open.

A modified organic extraction procedure using a mixture of phenol and guanidine thiocyanate was developed for the extraction and recovery of RNA, DNA, and protein from the same sample (Chomczynski 1993). In this case, RNA is selectively partitioned to an acidic aqueous phase (Kirby 1956) while DNA and protein partition to the interphase and organic phase. RNA is precipitated from the harvested aqueous phase, and the organic phase is back-extracted with aqueous solution at a higher pH to solubilize the DNA. DNA is then precipitated from the back-extracted aqueous phase and protein is precipitated from the organic phase with acetone. The organic extraction mixture and other materials for this procedure are commercially available (TRI reagent, Molecular Research Center; TRIzol, Invitrogen) along with detailed protocols (e.g., www.mrcgene.com/tri.htm).

Differential precipitation: When separating proteins and nucleic acids by differential precipitation, the proteins can be "salted out" directly with ammonium sulfate or precipitated as SDS–protein complexes by the addition of salt to SDS-containing lysates (Miller et al. 1988). In either case, the proteins are removed by centrifugation followed by recovery of the DNA-containing supernatant. Note that if nucleic acids are not first liberated from viral capsids, ammonium sulfate can result in the precipitation of the intact virions. This method of concentrating viruses has useful applications in molecular biology (Ziai et al. 1988) and viral ecology (Steward et al. 1992) and appears to be the basis of viral preservation in RNALater, as mentioned above.

Instead of precipitating protein, DNA can be selectively precipitated from buffers of low ionic strength with the cationic surfactant cetyltrimethylammonium bromide (CTAB) (Jones 1953). In this case, the proteins are discarded with the supernatant, and the DNA in the pellet is resuspended in a high-ionic-strength buffer (Sambrook and Russell 2001). Note that in high-ionic-strength buffers, CTAB forms complexes with proteins and polysaccharides (but not DNA), which has been used to facilitate the removal of these contaminants by organic extractions with phenol and chloroform (Jones and Walker 1963).

Solid-phase extraction: One of the most common extraction and purification techniques in use today is a solid-phase extraction, which exploits the selective binding of nucleic acids to silica under conditions of high salt concentration and low pH, and their subsequent elution at low salt concentrations (Vogelstein and Gillespie 1979; Boom et al. 1990). This phenomenon is the basis for a wide variety of commercial nucleic acid extraction kits, in which the silica is supplied as a fine particle suspension ("glass milk") or a silica-impregnated membrane.

Density gradients: Nucleic acids can be very effectively and cleanly separated from other macromolecules using density gradient centrifugation. DNA, RNA, proteins, and lipids have sufficiently different buoyant densities that they can be separated in equilibrium buoyant density gradients in an ultracentrifuge (Rickwood 1989). CsCl and CsTFA are commonly used as gradient media for this purpose. DNA can be banded in gradients of either salt. RNA, because of its high buoyant density, will pellet in CsCl gradients (Glisin et al. 1974) but can be banded in CsTFA (Rickwood 1989). Isopycnic banding results in very pure nucleic acids, and this is a reliable way to obtain nucleic acids free of contaminants that can inhibit enzymatic reactions such as PCR. The disadvantages of ultracentrifugation are the relatively long centrifugation times, the limited number of samples that can be processed simultaneously, and the effort needed to recover the nucleic acids from the gradient.

Electrophoretic separation: A novel electrophoresis technique has recently been described that, like the density gradients described above, appears to result in highly purified DNA. This method, referred to as synchronous coefficient of drag alteration (SCODA), very effectively separates nucleic acids from contaminants and simultaneously concentrates them based on their nonlinear response to variable electric fields (Marziali et al. 2005). At present, the instrument used for this technique can process only one sample at a time, so throughput is very limited. However, low-throughput, high-purity methods such as SCODA and ultracentrifugation are invaluable for special applications in which one must extract nucleic acids from challenging matrices rich in PCR inhibitors (Juniper et al. 2001; Pel et al. 2009).

E. Commercial extraction kits and reagents—Commercial purification kits or reagents are available that rely on the extraction principles outlined above of selective precipitation (e.g., MasterPure™, Epicenter; Gentra® Puregene®, Qiagen), selective adsorption (e.g., UltraClean® Microbial DNA isolation kit, Mo Bio Laboratories; QIAamp® MinElute®, Qiagen; AllPrep®, Qiagen) or selective solubility (e.g., TRI Reagent®, Molecular Research Center; TRIzol®, Invitrogen). Although some kits are specifically marketed for extraction of nucleic acids from viruses (e.g., ChargeSwitch® EasyPlex™ Viral RNA/DNA Kit, Invitrogen; QIAamp® UltraSens™ Virus Kit, Qiagen; Arc*Pure*™ Viral DNA [or RNA] Isolation and Sample Preparation Kit, Arcxis Biotechnologies), these kits are not inherently selective for viruses; rather, they assume a cell-free virus-containing fluid as the starting material. The underlying extraction principles are the same as for cell and tissue extraction kits, but the protocols can be simpler, because of the relative ease with which nucleic acids can be released from viral capsids.

Some kits or reagents discriminate between RNA and DNA (e.g., AllPrep, TRI reagent, and TRIzol) and allow separate purification of both types of nucleic acid from the same sample. Other kits result in purification of total nucleic acids (e.g., MasterPure and Puregene), with RNA and DNA being discriminated only by selective nuclease digestion. The latter approach is less desirable if one has a limited amount of material, since a significant portion of the DNA and RNA must be destroyed to get pure fractions of each. Compensating for this drawback are the simplicity (no special columns) and low toxicity (no organic solvents) of the approach. One should be aware that, although the names of some kits suggest specificity for DNA or RNA, the procedure may not be selective. One of the authors (A. I. Culley) has found, for example, that a kit marketed for RNA virus extraction (QIamp viral RNA Mini kit, Qiagen) works as well for extracting viral DNA. The product literature should be consulted to be sure of the limits and selectivity of each kit.

Some kits are specifically designed to remove inhibitors that may be found in more complex matrices such as soil. This is not an issue for many aquatic viral ecology applications, but in some cases (e.g., extracting viral nucleic acids from total plankton concentrates or from sediments), a kit designed for soil (e.g., Power Soil Kits, Mo Bio Laboratories) may help remove substances that can inhibit PCR.

Another option for simultaneously extracting and preserving small-volume virus samples is to spot them on FTA cards (Whatman). These cards are impregnated with buffer, chelating agent, detergent, and uric acid that serve to lyse microbes and protect the nucleic acids (Burgoyne 1996). These cards have been used for preserving nucleic acids from a wide variety of microorganisms (Rajendram et al. 2006), including RNA-containing (Li et al. 2004) and DNA-containing (Sudhakaran et al. 2009) viruses. Nucleic acids have been detected from samples stored for more than 4 years at room temperature with minimal decay (Li et al. 2004). One limitation of this approach is that the volume that can be applied is relatively small (≤500 μL), since the sample must be absorbed by the paper without excessively diluting the reagents and then be dried completely.

To decide which commercial kits or reagents are most appropriate, one needs to consider the type (DNA, RNA), mass, and size of the nucleic acids to be extracted and the final purity required. Most kits, particularly those based on selective adsorption, have limitations on the mass and size of the nucleic acids that can be efficiently recovered. We do not cover the protocols of these kits here, since the brands are numerous and the protocols are supplied with each kit. Instead we present a few manual purification protocols for situations not covered by the kits.

Protocols

A. Phenol-chloroform extraction with ethanol precipitation—The traditional phenol extraction procedure, based on early work by Kirby (1957) and elaborated in most modern protocol compendia, yields very clean nucleic acids suitable for a variety of downstream applications. This extraction method is coupled with a routine alcohol precipitation step to allow buffer exchange, removal of trace amounts of chloroform, and concentration of nucleic acids (Sambrook and Russell 2001).

Materials and equipment:

- fume hood
- microcentrifuge (refrigerated if possible)
- pipettes and sterile, disposable tips
- safety gear (gloves, lab coat, safety glasses)
- sterile microcentrifuge tubes
- TE buffer (10 mM Tris, 1 mM EDTA; pH 8)
- Tris-saturated phenol, pH 8 (*see* "Warning" below)
- CI (chloroform:IAA, 24:1, vol:vol) (*see* "Warning")
- PCI (phenol:chloroform:IAA, 25:24:1) (*see* "Warning")
- sodium acetate, 3 M, pH 5.2 (*see* Sambrook and Russell [2001] for a discussion of alternative salts that may be used for nucleic acid precipitation and their advantages and disadvantages)
- Optional: polyacryl carrier (Molecular Research Center)
- ethanol, 70% and 100%

Warning: Phenol can cause chemical burns if it comes in contact with bare skin. Phenol and chloroform are volatile and carcinogenic and must be used in a fume hood with proper protection (gloves, lab coat, and safety glasses). PCI and CI preparations that are ready to use can be purchased from a variety of scientific chemical suppliers. Details of how to prepare these solutions for oneself can be found elsewhere (Sambrook and Russell 2001).

Steps:

1. To the viral suspension (≤0.6 mL per 2-mL microcentrifuge tube, scale up for larger volumes) add an equal volume of PCI and shake to emulsify.
2. Centrifuge at 10,000*g* for 5 min to facilitate separation of the organic and aqueous phases.
3. Transfer the DNA-containing aqueous phase (upper) to a new tube by aspiration with a pipette, being careful to avoid material at the interface.
4. Repeat steps 1–3 as needed until the interface appears to be free of extracted material (one extraction may suffice for relatively pure viral preparations).
5. Add an equal volume of CI to the aqueous phase and shake to emulsify.
6. Centrifuge as in step 2 to separate phases.
7. Transfer the aqueous phase (upper) to a new tube.
8. Add 1 µL polyacryl carrier (this optional step is unnecessary when working with tens of nanograms or more of DNA, but can improve yields when working with nanogram to subnanogram quantities).
9. Add 1/10 of a volume of sodium acetate and invert tube or vortex to mix.
10. Add 2 volumes of ethanol and invert tube to mix.
11. Incubate sample on ice for 10 min.
12. Centrifuge for 10 to 30 min, at 0–4°C if possible.
13. Aspirate or decant the supernatant, being careful not to disturb the pellet (a pellet may not be visible if the amount of DNA is low and no carrier has been added).
14. Add 500 µL ice-cold 70% ethanol.
15. Centrifuge at 10,000*g* for 10 min.
16. Decant or aspirate supernatant as completely as possible, being careful not to disturb the pellet.
17. Allow residual liquid in the tube to evaporate by air-drying with the cap open and the tube upside down or by placing briefly in a centrifugal vacuum concentrator (e.g., SpeedVac concentrator, Thermo Scientific; Concentrator *plus*, Eppendorf). Note that excessive drying will make the nucleic acid more difficult to dissolve.
18. Resuspend the dried pellet in a small volume of Tris (10 mM, pH 8) or TE buffer. Note that some of the material will be on the side of tube, so the appropriate side of the tube should be exposed to the liquid used for resuspension to maximize recovery.
19. The purified, solubilized DNA may be stored at 4°C for short periods of time, at –20°C for long periods of time, and at –80°C indefinitely. For long-term storage, one might also consider storing the dried DNA pellet, which should remain stable at room temperature or below if kept dry.

Assessment: This traditional method of extraction is most commonly used to extract DNA. When phenol is saturated with alkaline buffer (e.g., Tris, pH 8), however, both RNA and DNA will partition to the aqueous phase, so the method can be used for total nucleic acid extraction. Either DNA or RNA can be specifically selected by digestion of the recovered total nucleic acids with RNase or DNase. If targeting RNA, an RNase inhibitor may be included to help ensure stability. If one wishes to isolate both RNA and DNA but in separate fractions by organic extraction, we recommend the use of the commercially available reagents TRI Reagent and TRIzol (*see* above).

Although still in use, the popularity of organic extraction has waned somewhat as new extraction procedures have been developed that do not require the use of toxic organic compounds. In addition to the extra precautions that must be taken when handling phenol and chloroform during extraction, the disposal of the resulting organic waste is costly.

B. Release of nucleic acids with heat, chelator, and detergent—If one has a purified stock of viruses obtained, for example, by banding in a buoyant density gradient, or even a relatively pure viral concentrate obtained by size fractionation, it is possible to release the DNA in a high molecular weight form suitable for some applications (e.g., pulsed-field gel electrophoresis [PFGE] for sizing or probing, or nucleic acid quantification

by fluorescence) relatively simply. This method involves exchanging the buffer in which the viruses are suspended with one containing EDTA and SDS, followed by heating. The method is similar to that described previously (Steward 2001), but with the optional addition of detergent to facilitate disintegration of the viral capsid.

Materials and equipment:

- Centrifugal ultrafiltration device (30,000 molecular weight cutoff, e.g., Millipore Ultracel YM-30, cat no. 42410)
- TE buffer (10 mM Tris, 1 mM EDTA, pH 8) or TEGED buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA)
- Optional: 6× SDS-EDTA loading buffer (1% SDS, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol)

Steps:

1. Concentrate the viruses by centrifuging in the centrifugal ultrafiltration device at 1000*g* until only a small volume (ca. 10 µL) remains.
2. Add 100 µL TE (or TEGED).
3. Concentrate the sample again to ca. 10 µL.
4. Repeat steps 2 and 3 once more.
5. Recover the final concentrate.
6. Rinse the membrane in the device by adding a small volume of TE or TEGED (5–10 µL).
7. Recover the rinse and pool with the concentrate.
8. Optional: If conducting electrophoresis on the sample, add SDS-EDTA loading dye to a final concentration of 1×.
9. Heat the recovered sample (with or without loading buffer) to 60°C for 10 min to release the nucleic acid.

Assessment: This method is similar in strategy to the simple protocol for assaying the DNA content of bacteriophage λ stocks described by Sambrook and Russell (2001, p. 2.45–2.46). SDS is added to facilitate the release of DNA from the capsids and to minimize DNA–protein interactions during electrophoresis. The protocol described here, but without SDS, has been used in a number of studies to investigate genome size distributions in viral communities (e.g., Steward and Azam 2000; Steward et al. 2000; Riemann and Middelboe 2002; Jiang et al. 2003; Jiang et al. 2004; Filippini and Middelboe 2007). One can carry out essentially the same procedure by pelleting viruses in an ultracentrifuge rather than using centrifugal ultrafiltration. If one assumes a minimum sedimentation coefficient for viruses (e.g., 80S), the time needed to pellet the virus can be determined from the *k*-factor of the rotor being used (Lawrence and Steward 2010, this volume). The centrifugation time required in a swinging bucket rotor, which will produce the most compact pellet, can vary from 30 min (6 × 4 mL sample in a Beckman SW 61 rotor) to 3 h (6 × 38.5 mL sample in a Beckman SW 28 rotor). In this case, the supernatant is drained completely and carefully from the pellet. Residual liquid on the walls can be removed using the tip of a twisted lint-free absorbent wipe (e.g., KimWipe, Kimberly Clark) or sterile cotton swab. TE is added to the pellet, and the tube is sealed with plastic wrap to minimize evaporation and heated to 60°C with occasional gentle agitation for 10 to 15 min.

If the samples are handled carefully (to minimize shearing, pipette slowly, use wide-bore pipette tips, and avoid vortex mixing), the DNA should be of high molecular weight suitable for sizing by PFGE (Steward et al. 2000; Steward 2001). DNA prepared by the centrifugal ultrafiltration method has resulted in no noticeable shearing of bands up to several hundred thousand base pairs. The alternative ultracentrifugation method has sometimes resulted in slight smearing of bands, indicating some shearing. Even in the former case, some small amount of shearing of the higher molecular weight nucleic acids might be expected from handling them in solution.

The sensitivity of viral DNA to shearing will depend on its size, composition, and conformation. Most viral genomes are small enough that they can be extracted in solution without appreciable shearing if handled gently. Bacteriophage genomes up to 100 kb produced crisp bands with no evidence of shearing when extracted using a protocol similar to that described here (Steward et al. 2000). A large algal virus genome (320 kb) was found to be fragmented when subjected to standard phenol-chloroform extraction procedures (Lanka et al. 1993). When treated gently, however, Chlorella viruses ranging in size up to 380 kb tolerated limited pipetting in liquid and produced crisp single bands by PFGE (Rohozinski et al. 1989). If shearing must be minimized to the greatest possible extent, one should consider embedding the viruses before extraction as described in protocol C. Even embedded viral DNA, however, may be susceptible to some degree of fragmentation (Lanka et al. 1993), perhaps due to premature disintegration of viruses during the embedding process (*see* protocol C, "Assessment," below).

It may be possible to obtain intact viral RNA using the same general approach as described here by simply including an RNase inhibitor in the TE buffer, but we have not explicitly tested this. If more purified nucleic acids are required, the simple release step described here can be followed by purification by organic extraction (Cottrell and Suttle 1991; Wilson et al. 1993; Sambrook and Russell 2001) or purification with any of a number of commercially available nucleic acid purification kits. If one wishes to purify both RNA and DNA free from proteins, the appropriate extraction buffer from a suitable kit (QIAamp MinElute or UltraSens virus kits, Qiagen; MasterPure total nucleic acid extraction kit, Epicenter) can be substituted for the TE after concentrating the viruses by centrifugal ultrafiltration or ultracentrifugation.

C. Extracting DNA from viruses embedded in agarose—If one wishes to have a stock of high molecular weight viral DNA that can be stored for long periods of time with minimal shearing or degradation, the viruses can be embedded in agarose before extraction. Extraction of embedded cells is the standard procedure for sizing the genomes of bacteria and

yeast by PFGE (Sambrook and Russell 2001), and a similar protocol can be used for embedded viruses (Rohozinski et al. 1989; Lanka et al. 1993; Wommack et al. 1999; Sandaa et al. 2010, this volume).

Materials and equipment:

- Agarose (molecular biology grade, low gelling temperature; InCert® Agarose, Lonza)
- SE buffer (75 mM NaCl, 25 mM EDTA, pH 8)
- TE buffer (10 mM Tris, 1 mM EDTA, pH 8)
- Lysis buffer (TE pH 8; 1% SDS)
- Proteinase K
- Optional: casting wells
- Optional: phenylmethanesulfonylfluoride (PMSF)

Steps:

1. Add agarose to SE buffer for a final concentration of 1.5% (wt/vol).
2. Melt the agarose in a microwave oven, then cool and maintain at 37°C in a water bath.
3. Warm the viral concentrate to 37°C in the water bath, then immediately mix with an equal volume of molten agarose and quickly transfer the mixture to casting molds. Casting in rectangular plug molds is preferred if the embedded DNA is to be analyzed by electrophoresis, since this results in plugs of uniform height and thickness that fit the wells without extensive trimming. Electrophoresis using plugs that are not uniform will result in bands with uneven intensity. Special rectangular plug molds for PFGE are available from BioRad. Alternatively, one can draw the molten mixture into a 1-cc syringe that has had the tip cut off, or simply pipette the mixture as drops onto a sheet of plastic wrap or Parafilm M®.
4. Once the agarose has gelled, transfer the plug (or noodle from the syringe, or buttons from the parafilm), into a tube containing 5 volumes of lysis buffer amended with proteinase K (1 mg mL^{-1} final concentration).
5. Incubate at room temperature overnight.
6. Decant the lysis buffer, being careful not to lose the plugs.
7. Optional: Rinse the plugs twice, each time adding 25 volumes of fresh TE containing 1 mM PMSF, incubating for 1 h with gentle agitation, then decanting the rinse fluid. This step will inactivate the proteinase K, which is recommended if the DNA in the plug is to be further manipulated with enzymes (e.g., digestion with restriction endonucleases).
8. Rinse the plugs twice, each time adding 50 volumes of fresh TE with no PMSF, incubating for 30 min with gentle agitation, then decanting the rinse fluid.
9. Store the plugs at 4°C submerged in TE.

Assessment: One of the main purposes of extracting nucleic acids from embedded viruses or cells is to avoid shearing of high molecular weight DNA. The use of a low-melting/gelling-point agarose in SE buffer is recommended to minimize premature disruption of viral capsids by thermal and osmotic shock. DNA released before the casting of the gel plugs has the potential to be sheared during mixing and pipetting.

Use of a low-gelling-temperature agarose also allows one to recover nucleic acids from the agarose plug using an agarase enzyme (β-agarase, Lonza or New England BioLabs). DNA can be recovered from other types of agarose using silica-based gel extraction methods, by electroelution, or by organic extraction (Sambrook and Russell 2001), so it is possible to use molecular biology–grade agaroses with higher gelling temperatures. In this case, however, the agarose must be maintained at a higher temperature before mixing with the sample. Gelling temperatures for other pulsed-field grade agaroses are around 36–42°C, so maintaining at 50–60°C before mixing with sample should be adequate. One should bear in mind that some viruses may disintegrate at this temperature. For some applications (e.g., shotgun cloning), some fragmentation of the DNA is not an issue. If sheared DNA is not an issue for one's application, then one might consider a less cumbersome extraction protocol that results in DNA in solution.

The release of viral DNA in plugs is commonly used for sizing of large viral genomes either intact (McCluskey et al. 1992) or after digestion with a restriction endonuclease (Rohozinski et al. 1989; Lanka et al. 1993). Variations of the above method have been used for analyses of genome size distributions in viral assemblages using PFGE (e.g., Wommack et al. 1999; Larsen et al. 2001; Øvreås et al. 2003; Sandaa and Larsen 2006; Parada et al. 2008; Sandaa et al. 2010, this volume). Viral community DNA has also been recovered from agarose plugs for subsequent sequence analysis by shotgun cloning (Bench et al. 2007).

The disadvantages of the method for community genome size analyses are that the preparation time is longer and the resolution of bands will typically be lower when performing PFGE from viral DNA in plugs (depending on the thickness of the plug) compared to that achievable with DNA in solution prepared by protocol B (Steward 2001). The considerable advantage of the method is that the DNA appears to be more stable at 4° when embedded in agarose (many months) than when dissolved in buffer (up to a few days), so embedding is recommended for storing extracted viral DNA that will not be used right away. One of the authors (G. F. Steward) has observed that a high molecular weight PFGE standard embedded in agarose that was accidentally frozen on dry ice resulted in a banding pattern that was indistinguishable from that of parallel standard that had never been frozen. Freezing the plugs at –80°C may therefore be useful for long-term archiving of samples. Freezing is not recommended for samples that will be accessed more than once or twice, since repeated freeze-thaw cycles are likely to degrade the DNA.

D. Extracting nucleic acids from viruses on a filter—This protocol is a minor modification of that reported by Culley and Steward (2007). As the starting point for this protocol, we assume that viruses have been collected on an aluminum

oxide 0.02-µm syringe-tip filter (Anotop, Whatman), but other filters capable of capturing viruses may be substituted.

Materials and equipment:

- Total nucleic acid extraction kit (MasterPure, Epicenter)
- Optional: polyacryl carrier (Molecular Research Center)
- Syringes (sterile, disposable, with Luer-Lok tips)
- Luer-Lok female–female adapter fittings
- Hybridization oven with rotisserie

Steps:

1. Add 1 mL T + C lysis buffer containing 100 µg/ml proteinase K to a low-volume (1–3 cc) syringe that has been fitted with a female–female Luer-Lok adapter (the injection syringe). It is convenient to use a larger syringe (10–20 cc) as an extraction buffer reservoir. The injection syringe can be easily filled with the proper volume by connecting it tip to tip with the reservoir syringe via the adapter. The reservoir can be used to fill multiple injection syringes if more than one sample is to be extracted.
2. Ensure that there is minimal air in the injection syringe-adapter assembly, then connect it to the outlet of the filter. Connect a second low-volume syringe to the filter inlet (the aspiration syringe). Hold the filter-syringe assembly vertically with the injector syringe pushing upward from below. Hold the filter securely to the injection syringe and gently, but firmly, push extraction buffer into the filter housing until liquid just begins to appear in the aspiration syringe.
3. Incubate the assembly (filter with two syringes attached) for 15 min at 65°C in a hybridization oven. It is helpful to connect the syringe-filter assembly to a rotisserie so that the entire filter surface is wetted in the event that bubbles are present in the housing. The syringes on either side of the filter can be secured to the clips of the rotisserie with elastic bands.
4. Allow the syringe-filter assembly to cool briefly; then remove the extract by holding the syringe assembly vertically with the aspiration syringe underneath (and the filter upside down) and gently pulling on the plunger to pull the extract into the aspiration syringe.
5. Detach the aspiration syringe; transfer the extract to a microcentrifuge tube; and chill on ice for 2–3 minutes.
6. Add one-half volume of MPC protein precipitation reagent (supplied in the kit) and vortex for 10 s.
7. Pellet the debris by centrifugation at 10,000*g* for 10 min.
8. Transfer the supernatant (containing the nucleic acids) to a sterile microcentrifuge tube; be very careful to avoid the pellet (containing the SDS-protein complex). All or some of the sample may be archived at this point by freezing at –80°C.
9. Transfer up to 800 µl of the sample to a fresh tube; add 1 µl polyacryl carrier, and vortex briefly (carrier is optional, but can improve yields when working with nanogram to subnanogram quantities of nucleic acid).
10. Add an equal volume of 100% isopropanol and mix by inverting the tube several times.
11. Centrifuge the sample at ≥10,000*g* for 15–45 min (longer centrifugation times can improve the yields for small amounts of nucleic acids, especially in the absence of carrier).
12. Decant or aspirate the supernatant (use caution, the pellet can dislodge easily and be lost).
13. Wash the pellet twice, each time adding 70% ethanol, centrifuging for 1 min, and decanting (or aspirating) the ethanol.
14. Air-dry the pellet, then dissolve in 10 µL of 0.02-filtered, sterile 0.5× TE buffer heated to 50°C.
15. If required, DNA or RNA can be selectively removed from the total nucleic acid precipitate by enzymatic digestion with DNase or RNase.

Assessment: One caveat in extracting from aluminum oxide membrane filters is that they can irreversibly bind DNA under certain conditions (Dames et al. 2006). In particular, guanidinium-containing extraction buffers facilitate the binding of DNA to aluminum oxide (Gerdes et al. 2001) and are likely to result in low yields from the filters. For this reason, we do not recommend extracting from Anotop filters using the lysis buffers from any of the popular silica column–based kits. The Gentra PureGene Kit (Qiagen) is similar to the MasterPure kit and may work as well. These latter kits are based on a published protocol (Miller et al. 1988) that could be adapted for extraction from a filter. A version of that protocol designed for simultaneous DNA and RNA extraction (Yu and Mohn 1999) could also be used by employing heat (65°C, 15 min) instead of bead beating to facilitate lysis. It is worth noting that SDS and phosphate buffer appear to inhibit the binding of nucleic acid to aluminum oxide (Gerdes et al. 2001; Dames et al. 2006) and should aid in recovery. If one wishes to recover RNA using a self-made recipe, then we would recommend including an RNAse inhibitor (e.g., RNASecure, Ambion) in the extraction buffer.

We have recovered both viral RNA and DNA suitable for PCR amplification from aluminum oxide filters using essentially the procedure as described above (Culley and Steward 2007; Culley et al. 2008). A modification added here is the introduction and removal of the extraction buffer in a direction counter to that of filtration during sample collection (i.e., backflushing). According to the Anotop specifications, these filters are not designed to be backflushed, or to be operated at temperatures above 40°C. We have found that the filter can rupture if too much pressure is applied during backflushing, especially after incubating at 65°C. Injecting the extraction buffer slowly and removing the extract by gentle aspiration seem to avoid this problem. Although we have not tested the protocol described here on other filter types, the procedure should work as well for any direct flow filter capsule capable of retaining viruses.

Conclusion

Extraction of nucleic acids from viruses may be achieved by a wide variety of methods. Essentially any protocol that is suitable for extracting nucleic acids from cells should work as well for viruses. Because viruses do not have a cell wall, however, extractions can be much quicker and simpler. The nature of the starting material and final purity of the nucleic acids required will vary widely depending on the application, so it is not possible to provide a single recommended protocol. We hope that the background information and the handful of explicit protocols provided here will arm the reader with the information necessary to select, adapt, or design a protocol best suited to their needs and the materials and equipment they have available.

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