Laboratory procedures to generate viral metagenomes

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This collection of laboratory protocols describes the steps to collect viruses from various samples with the specific aim of generating viral metagenome sequence libraries (viromes). Viral metagenomics, the study of uncultured viral nucleic acid sequences from different biomes, relies on several concentration, purification, extraction, sequencing and heuristic bioinformatic methods. No single technique can provide an all-inclusive approach, and therefore the protocols presented here will be discussed in terms of hypothetical projects. However, care must be taken to individualize each step depending on the source and type of viral-particles. This protocol is a description of the processes we have successfully used to: (i) concentrate viral particles from various types of samples, (ii) eliminate contaminating cells and free nucleic acids and (iii) extract, amplify and purify viral nucleic acids. Overall, a sample can be processed to isolate viral nucleic acids suitable for high-throughput sequencing in ~1 week.

INTRODUCTION

Background

Viruses are the most numerous biological entities on the planet, but their small nature, rapid evolution and genomic flexibility make them difficult to study[1,2]. Additionally, the majority of viruses cannot be cultured because their hosts are recalcitrant to isolation. Environmental virology is further complicated by the lack of a single phylogenetic marker that can be used for examination of the diversity and evolutionary history of all viruses, such as the 16S rRNA gene sequence commonly used for Bacteria and Archaea[3]. Alternative approaches are therefore required for the evaluation of viral consortia in environmental samples[4,5].

Several characteristics make viruses well suited for high-throughput sequencing and analysis. For example, the hardiness of many viral capsids makes them ideal for concentration and purification. The small size of viral genomes, particularly of phages, can be advantageous for many bioinformatic techniques and full genome assembly[6,7].

This protocol describes a collection of sequential protocols that can be used to generate viral metagenomes. All of these protocols have been described previously, but we have combined and augmented them to specifically target and enrich for viral particles and nucleic acids with the specific goal of generating a metagenome of a viral consortia or 'virome'[2,8–16]. The first section of this method combines many established protocols for isolating, concentrating and viewing viral particles. We outline the use of tangential-flow filtration (TFF), polyethylene glycol (PEG) precipitation and viral staining. The second portion of this protocol is a derivation of two commonly used protocols for lambda phage particle extraction and nucleic acid purification. The last section discusses nucleic acid amplification using commercially available kits.

Methods comparison

These viral concentration and extraction methods contrast with others in a variety of ways. Centrifugal filter devices can be used as an alternative to TFF, or as an additional step in conjunction with TFF to concentrate viral particles[17–20]. This protocol recommends purchasing individual parts and self-assembling the TFF system; however, an alternative is to purchase a combined and prefabricated system, such as the QuixStand benchtop system. This compact system can quickly process up to 10 liters of sample. Furthermore, during the final steps of TFF, approximately two volumes of filtrate can be recirculated to increase viral recovery. However, this dilutes the final concentrate of the retentate and is the rational for why we do not include this step in our protocol.

Yo-Pro-1, 4,6-diamidino-2-phenylindole and SYBR Green dyes are used for counting viruses either by flow cytometry or direct counts, but SYBR Gold is brighter and more resistant to fading than both of the previously mentioned stains[21–25]. The use of gluteraldehyde as a fixative for accurate viral estimation using epifluorescence can be used as an alternative to paraformaldehyde[26].

The presented method for viral DNA extraction combines the formamide/cetyltrimethylammonium bromide (CTAB) method used for lambda phage extraction and the phenol/chloroform method for bacterial DNA extraction[27]. These techniques were chosen because they are less expensive than kits and because a number of commercially available kits, such as QIAamp MinElute Virus Spin Kit, use carrier RNA that can contaminate samples. However, these types of viral kits can be used for DNA viruses with an additional RNase step to eliminate the carrier RNA.

Potential method applications

With the appropriate modifications, this protocol is potentially applicable to almost any sample type. For example, using derivations of this technique, viral nucleic acids have been successfully isolated from a variety of different samples without any additional nucleic amplification methods (**Table 1**). For many sample types (e.g., human blood viruses), the amount of recovered viral nucleic acid is too small to detect with standard gel electrophoresis or spectrophotometer techniques. Using the below amplification techniques, viral nucleic acids recovered in this protocol can be used for a variety of applications other than metagenome generation, such as conventional and real-time PCR and microarray experiments[28].

The viral metagenomes generated by this protocol can be used to make viral metagenome libraries; these fall into two categories, cloning-dependent or -independent[29]. Each has advantages and

TABLE 1 | Range of viral nucleic acid recovery from different sample types using the methods described in this protocol.

Sample type	Volume or weight	Range of nucleic isolation (ng)	Reference
Seawater DNA viruses	100 liter	20–200	Angly *et al.*[2]
Human feces DNA viruses	500 g	500–3,000	Breitbart *et al.*[9]
Human feces RNA viruses	500 g	140–500	Zhang *et al.*[16]
Marine sediments DNA viruses	1 g	50–100	Breitbart *et al.*[8]
Microbiolites DNA viruses	1 g	50–100	Desnues *et al.*[13]
Coral tissue DNA viruses	1 g	60–100	Vega Thurber *et al.*[55]

disadvantages; clone library production can be expensive, time consuming and requires large amounts of DNA. Also, modified bases and blunt-ending sequences can be unclonable, and inserts are sometimes unstable[30]. Yet cloning is useful for producing very large contiguous sequence or paired end reads that provide better annotation information than many of the cloning-independent methods to date[31].

Virome clone libraries have been generated from randomly amplified sequences. This method is detailed by Rohwer *et al.*[30] and therefore will not be reiterated here. The second derivation of this technique is called a linker amplified shotgun library in which the DNA is randomly sheared using a GeneMachines hydroshearing instrument (GenMachines). Subsequently, *Not*I linkers are ligated to the ends of these random fragments and used as priming sites in a PCR amplification. Sequences are then cloned into pSMART vectors from Lucigen. Most of the steps necessary to generate this library have been conducted for us by Lucigen and are described previously by Breitbart *et al.*[10,11]. This method has been used to make viral metagenomic libraries from water, near-shore marine sediments, human feces and blood and corals[8–10,15,20,32].

In contrast to clone-based libraries, parallel sequencing platforms require no cloning steps and can currently provide sequence reads of up to about 450 base pairs in length with comparably little error[29,33]. Assembly and taxonomic analysis of these data sets, however, is more challenging due to the short size of the reads and the large amount of data generated[33,34]. A majority of our work used 454 Life Sciences technology, mainly on the older GS20 machines, although our more recent libraries are being made on the GS FLEX machine[34].

Method limitations

Metagenomics is the sequencing of all nucleic acids isolated from an environmental sample; therefore, any remaining cells, nuclei and free nucleic acids will contaminate the resulting virome. Eukaryotic and most microbial genomes are significantly larger than viral genomes (with a few exceptions, such as Mimivirus and Mamavirus) and can provide a disproportionately large amount of the sequence data within a virome. Therefore, researchers should verify that eukaryotic and microbial cells are destroyed before viral nucleic acid extraction. Free nucleic acids can also contaminate viral metagenomes. DNase I treatment before capsid lysis can reduce, but not completely eliminate, free DNA[35]. Some RNA viruses contain RNA in the nucleocapsid structure. RNase treatment to rid samples of free and cellular RNA may therefore result in the loss of those viral particles[36]. After the nucleic acid extraction steps, routine testing for the presence of contaminating cellular DNA should be conducted by attempting to PCR-amplify small subunit rRNA (ssuRNA) genes using universal primers for 16S and 18S (see ref. 10). As viral genomes do not contain either 16S or 18S genes, the presence of these sequences will provide a semiquantitative measure of microbial and eukaryotic contamination within the virome.

Although this protocol describes the techniques to generate an environmental virome, it does not discuss the data analysis necessary for sequence evaluation. The field of metagenomic analysis is rapidly advancing with more techniques and tools available for scientists from all fields. Several recent reviews have described these techniques[37–51]. There have also been numerous tools created for viral metagenome sequence analysis[3,6,7,52,53]. Furthermore, many informative websites have been developed to help process metagenomic data:

http://www.nature.com/nrmicro/focus/metagenomics/index.html
http://metagenomics.theseed.org/
http://annotathon.univ-mrs.fr/
http://dels.nas.edu/metagenomics/
http://www-ab.informatik.uni-tuebingen.de/software/megan/welcome.html
http://camera.calit2.net/index.php
http://img.jgi.doe.gov/cgi-bin/m/main.cgi

Experimental design

Here, we describe two methods for isolating and concentrating viruses from various environments, such as seawater and tissues. For aqueous samples, optional TFF steps are used to isolate viral particles, including from very turbid environments, such as fish pond effluent, hypersaline water from solar salterns as well as clinical samples, such as liquefied feces[9,11,12,17]. In contrast, other tissue-like samples require modifications of the protocol to ensure the correct buffering of the media and adequate concentration of viruses from the solid-type substrate[8,16,54,55]. Finally, depending on the type of viruses present, each sample will have a unique buoyancy and sensitivity to different compounds, temperatures and acidity. For example, virions of the *Guttaviridae*, a family of Archaeal viruses, lyse in cesium chloride and therefore must be purified using a sucrose gradient[56]. A generalized list of observed viral buoyancies and sensitivities to various compounds are listed in **Table 2**. These data were extrapolated from the 8th Report of the International Committee on the Taxonomy of Viruses[57]. If researchers are interested in a particular viral family, they should consult the literature first before proceeding. Importantly, although this generalized protocol can be used for almost any environmental sample, several issues must be addressed before researchers attempt this method. Below is a series of concerns that each scientist must take into account before virome generation.

The composition of the sample will dictate the viral concentration and extraction protocol. DNA and RNA have been successfully

isolated from viral particles in a variety of biomes, including liquids (saline/fresh waters, media), animal and plant tissues, microbial mats, medical specimens (human blood, feces and sputum) and various types of sediments (terrestrial and marine soils, rocks)[13,14,32,54,58]. The amount of sample required depends on the ease of the viral recovery. There are $\sim 10^7$ viruses per ml of seawater, and the average phage, which accounts for most of the viruses, contains $\sim 10^{-17}$ grams of DNA[25,59]. For a standard pyrosequencing metagenome (approximately 1–5 µg of DNA), this requires isolation of $\sim 3 \times 10^{11}$ viruses. Without any amplification step, this requires that 10 liters of water be filtered with 100% viral recovery. However, virions are lost at every step; viral particles can become immobilized on filters, destroyed in storage and adsorbed to larger particles. Another example is human feces that can require up to 500 g of sample for adequate viral nucleic acid recovery. It is therefore essential that researchers check their samples at every step for viral-particle loss and modify the protocol to suit their needs, especially if they are attempting quantitative analysis. Using this below protocol, we typically isolate and purify approximately 20–200 ng of viral DNA from 10 liters of seawater, a range of 10–50% recovery.

The majority of viruses are small, but recent research has shown the presence of viruses larger (~720 nm particle) than many bacteria[60–63]. These giant viruses or 'giruses'[64] will be lost in this protocol, as will other viruses, such as filamentous viruses, which can reach lengths in excess of 2 µm (see ref. 57). Viruses can also be enveloped and/or have various modifications to their structures that can be sensitive to various steps in the protocols (**Table 2**). These biases must be taken into account for every viral metagenome project.

With all of these precautions in mind, the basic steps for generating a viral metagenome are presented below. This overall strategy aims to ensure adequate viral-particle concentration and elimination of contaminating cells from a variety of sample types (**Fig. 1**).

Tangential-flow filtration

Tangential-flow filtration has been used to isolate viral particles from a variety of environments[20,65]. Particles smaller than the filter pore sizes are pushed out through the filters (filtrate) (**Fig. 2**). A backpressure

TABLE 2 | Ranges of virion buoyant densities (cesium chloride, sucrose and cesium sulfate) and virion sensitivities.

	Range of bouyant density (g cm^{-2})			
Viral family or unassigned genus	**CsCl**	**Sucrose**	**Cs_2SO_4**	**Sensitivities**
dsDNA				
Myoviridae	1.5			
Siphoviridae	1.5			
Podoviridae	1.5			
Tectiviridae	1.29			
Corticoviridae	1.26			E, C, CS, D
Plasmaviridae	?			E, C, D, H
Lipothrixviridae	1.25			
Rudiviridae	1.36			
Fuselloviridae	1.24			
Salterprovirus	1.28			
Guttaviridae	Lyse in CsCl	1.16		CC
Poxviridae	1.25			
Asfaviridae	1.19–1.24			E, C, DC
Iridoviridae	1.26–1.6			
Phycodnaviridae		1.17–1.18		
Baculoviridae	1.18–1.47			D, OS
Nimaviridae	1.22–1.31			D
Herpesviridae	1.22–1.28			
Adenoviridae	1.30–1.37			
Rhizidiovirus	1.31	1.2		
Polyomaviridae	1.20–1.35	1.2		
Papillomaviridae	1.34–1.35			
Polydnaviridae				
Ascoviridae				D, OS
Mimivirus	1.36			
ssDNA				
Inoviridae	1.28–1.3			C
Microviridae	1.30–1.41			
Geminiviridae				
Circoviridae	1.33–1.35			
Anellovirus	1.27–1.33			
Nanovirus	1.34		1.24–1.30	CC
Parvoviridae	1.39–1.43			
rtRNA				
Hepadnaviridae	1.18–1.36			
Caulimoviridae	1.37		1.31	
Pseudoviridae				
Metaviridae				
Retroviridae		1.13–1.18		D, F, H
dsRNA				
Cystoviridae	1.27	1.24		E,C,D
Reoviridae	1.36–1.39			
Birnaviridae	1.30–1.33			
Totiviridae	1.33–1.43			
Partitiviridae	1.34–1.39			
Chrysoviridae	1.34–1.39			
Hypoviridae	1.27–1.30			
Endoviridae				
– ssRNA				
Bornaviridae	1.16–1.22	1.22		OS, D, UV
Rhabdoviridae	1.19–1.20	1.17–1.19		

(continued)

TABLE 2 | Ranges of virion buoyant densities (cesium chloride, sucrose and cesium sulfate) and virion sensitivities (continued).

	Range of bouyant density (g cm^{-2})			
Viral family or unassigned genus	**CsCl**	**Sucrose**	**Cs_2SO_4**	**Sensitivities**
Filoviridae	1.32			
Paramyxoviridae		1.18–1.20		H,D, F, S, OX
Varicosavirus			1.27	
Ophiovirus		1.19		
Orthomyxoviridae				CC, D, H, S, F, UV, OX
Bunyaviridae	1.20–1.21	1.16–1.18		H, S, D, F
Tenuivirus	1.28			
Arenaviridae	1.19–1.20	1.17–1.18		
Deltavirus				
+ ssRNA				
Leviviridae	1.46			R
Narnaviridae				
Picornaviridae	1.33–1.45			pH
Iflavirus	1.33–1.38			
Dicistroviridae	1.34–1.39			
Marnaviridae				
Sequiviridae				
Sadwavirus	1.43–1.46			
Cheravirus	1.41–1.45			
Comoviridae	1.28–1.53			
Potyviridae	1.29–1.31			
Caliciviridae	1.33–1.41			
Hepevirus	1.35			
Astroviridae	1.36–1.39			
Nodaviridae	1.31–1.36			
Tetraviridae	1.28–1.33			
Sobemovirus	1.36			
Luteoviridae	1.42			S
Umbravirus	1.15–1.45			
Tombusviridae	1.34–1.36	1.16, 1.18,1.14		EDTA
Coronaviridae	1.23–1.24	1.15–1.20		D, H, S, F, OX, UV
Arteriviridae		1.13–1.17		
Roniviridae		1.18–1.20		D
Flaviviridae	Various			H, OS, D
Togaviridae				pH, OS
Tobamovirus	1.325			
Tobravirus	1.306–1.324			EDTA
Hordeivirus				
Furovirus				
Pomovirus				
Pecluvirus	1.32			
Benyvirus				
Bromoviridae				R, OS, D
Ourmiavirus	1.375			
Idaeovirus	1.37			SDS, CS
Tymoviridae	1.26–1.46			SDS
Closteroviridae	1.30–1.34			
Flexiviridae	1.31			
Barnaviridae			1.32	pH

C, chloroform; CC, cesium chloride; CS, chloride salt; D, detergent; DC, deoyxchloate; E, ether; EDTA, ethylenediaminetetraacetic acid; F, formaldehyde; H, heat; OS, organic solvent, OX, oxidation compound; UV, ultraviolet light; R, RNase; SDS, sodium dodecyl sulfate. Adapted from ref. 57.

is used to force the filtrate through the holes. The remaining sample (retentate) is then collected into a reservoir basin and repeatedly cycled through the filters. Recirculation therefore concentrates the large particles. Reservoirs can be a variety of basins, such as 1-liter glass Erlenmeyer flasks or 10-, 20- and 80-liter-size plastic carboys. These reservoirs should be sterilized before use. Beneficial aspects of this filtration approach are (i) the large surface area that allows large volumes of filtrate to pass through rapidly and (ii) the tangential-flow that prevents clogging of the system (e.g., as with an impact filter)[66,67].

Tangential-flow filtration filters are available in several pore and surface area sizes (see EQUIPMENT). For the purposes of this method, we will focus on the 0.2-μm (**Fig. 2a**) and 100-kDa filters (**Fig. 2b**). Tubing is attached to either end of the TFF (see **Fig. 2**), and the sample is run from the collection reservoir through a peristaltic pump and a pressure gauge and then across the TFF, concentrating the microbes (0.2 μm; **Fig. 2a**) or microbes and viruses (100-kDa TFFs; **Fig. 2b**) in the 'retentate.' It is important to always keep the pressure within the tubes less than 10 p.s.i. (~62 kPa), as higher pressures can compromise the viral particles. If samples contain particulate matter that can clog the filter, first pass the sample through a Nitex mesh (e.g., pore size ~100 μm). Avoid exposing filters to organic solvents (e.g., chloroform), as these compounds will ruin the filters.

PEG precipitation

Polyethylene glycol precipitation can also be incorporated into the protocol if sample volumes are too large and need to be concentrated even further before CsCl centrifugation or DNA extraction[27]. This is often done when large viral lysates or filtrates are greater than 50–100 ml after being filtered or when diluted for DNase treatment.

Purification of viral particles by CsCl density centrifugation

Viral particles can be purified using density gradient ultracentrifugation, which is based on the physical properties of various virions[57]. The solvent and speed of the centrifugation, as well the types and number of gradient layers chosen, is entirely dependent on the density of target viruses (**Table 2**). Researchers should first carefully evaluate numerous layers to determine the density of the viruses within the sample. It is also important to note that gradients should be made from the same buffer as the samples (e.g., seawater, PBS), and that this buffer has been purified with a 0.02-μm filter to ensure that external viruses do not contaminate the resulting fractions. For example, for seawater phages, density

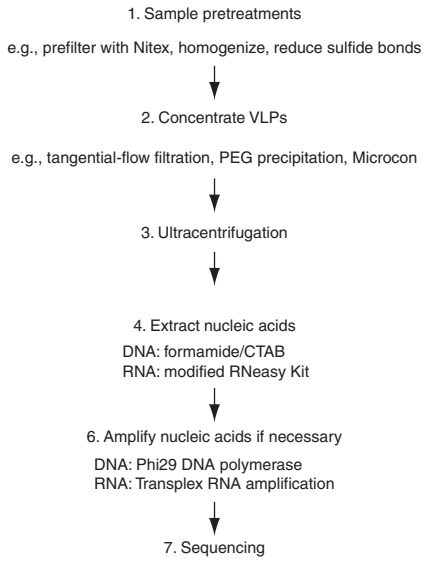


Figure 1 | Flowchart for viral metagenome generation.

layers of 1.7, 1.5 and 1.35 g ml^{-1} CsCl are prepared using 100-kDa filtered seawater (**Fig. 3**). The sample is also brought up to 1.12 g ml^{-1} CsCl and then loaded onto the heavier CsCl layers and centrifuged at 22,000 r.p.m. (~60,000*g* to 82,000*g* maximum) at 4 °C for 2 h in a swinging bucket rotor, such as Beckman SW41 Ti. In contrast, due to the lower buoyant density of many human-associated viruses, 1.7, 1.5 and 1.2 g ml^{-1} layers are made in 1× PBS[9]. These types of subtleties are necessary for each research project.

For making gradients, different researchers prefer different pouring methods (**Fig. 3a**). Pipetmen and tips can be used for pouring every density layer. However, graduated pipettes can also be used for either some or all of the layers. In addition, syringes and needles can be used to pour the layers. Generally, we first load the CsCl layers with pipetmen and then add the sample with a graduated pipette by slowly dripping the sample along the side of the column so that it does not disturb the lightest density.

It is extremely important not to disturb the boundaries of the layers before centrifugation. To practice accurate density layer formation, use different CsCl densities labeled with a different food coloring. This makes it easy to visualize layer mixing. Also, although the amount of sample added will depend on the size of the tube and rotor used, it is important to completely fill the column tube, or it can collapse during centrifugation.

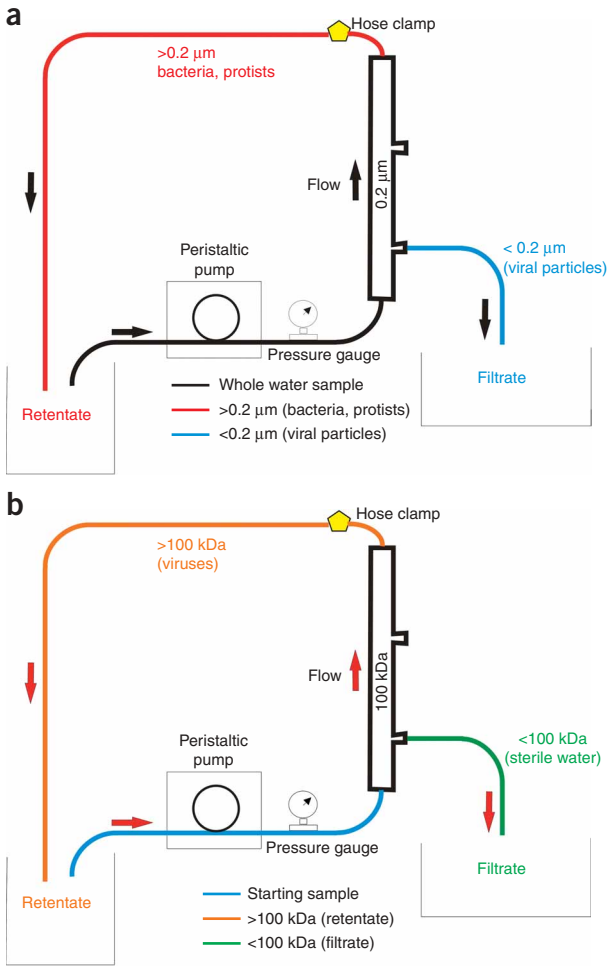
Cesium chloride gradient centrifugation is the main type of viral purification performed in our laboratory. The procedure outlined in this protocol is specifically designed for phage particle concentration. These viral particles are located at 1.5–1.35 g ml^{-1} in CsCl density boundaries after ultracentrifugation.

Figure 2 | Tangential-flow filter setup for viral-particle concentration. (**a**) The original sample is passed through the filter using a peristaltic pump and pressure gauge. The retentate is recirculated to concentrate microbes (A retentate). (**b**) The filtrate containing the viruses is expelled (A filtrate) or saved for further viral-particle concentration using a 100-kDa filter. Viral particles are concentrated in the final 100-kDa retentate.

Verification of virome purity

Column filtration (**Fig. 4**) and epifluorescence microscopy (**Fig. 5**) are used to verify that viral samples do not contain contaminating nuclei or microbial cells (see **Box 1**). For more details on this method, see the review by Patel *et al.*[59]. All equipments necessary to conduct this protocol are listed in MATERIALS.

In brief, aliquots of the viral concentration layers are diluted in 5 ml of buffer solution and then slowly (<10 p.s.i. or ~62 kPa) filtered though 0.02-µm filters. Filters are then removed from the tower glass frits and stained with SYBR Gold. The precise dilution of the SYBR dye is at the user's discretion. Concentrations greater than 1× will result in brighter and longer-lasting slides but is more expensive and may result in higher background staining. This nucleic acid stain will work on both live and fixed samples. If working with live samples, it is critical to use an isotonic buffer instead of water in the protocol. For fixing samples, prepare a fresh solution of 4% (wt/vol) paraformaldehyde in buffer (e.g., 4% paraformaldehyde in sterile seawater) and then dilute the sample with an equal volume of fixative, to give a final concentration of 2%. Viral-particle enumeration has been shown to be significantly affected by the storage time and fixation method used[26]. It is therefore absolutely critical that fixed sample slides are made as quickly as possible (preferably immediately) after viral concentration.



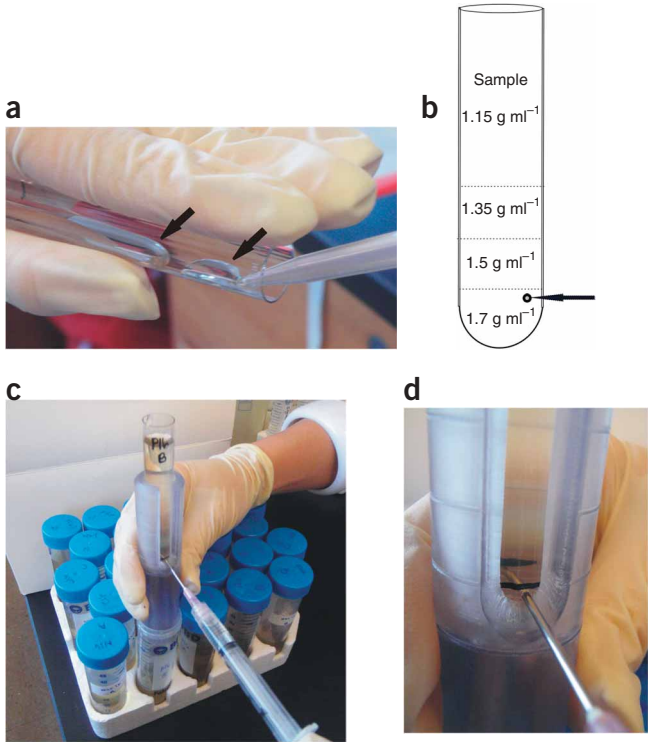


Figure 3 | Buoyant density centrifugation setup and methods. Viruses are isolated using different densities of CsCl and ultracentrifugation. (**a**) The CsCl density gradient is poured by adding 1 ml of the heaviest density (left arrow) and then carefully floating the next density (right arrow) by tilting the tube and slowly adding in the lighter density. (**b**) Phages are concentrated in a four-layer gradient. The resulting gradient localizes the phage between the 1.5 and 1.35 g ml^{-1} densities. A sterile 18-gauge needle is inserted just before the 1.5 g ml^{-1} density to remove the concentrated phages (see arrow). (**c**) After centrifugation, virions are extracted by placing the tube over a reservoir and piercing the tube with an 18-gauge needle and sterile syringe. (**d**) The fraction just above the 1.7 g ml^{-1} step is removed (marker line).

SYBR Gold is viewed in the FITC channel, excited with blue light and fluorescent in the green. Viruses appear as pinpoints of light on unconcentrated water samples (**Fig. 5a**, arrows). Nuclei and microbial cells are much brighter and larger (**Fig. 5c,d**, arrows). For samples with large volume, it is easy to overload a filter with viruses. If the background appears milky or grainy, then it is most likely that the volume of viral concentrate added to the slide was too high (**Fig. 5d**). Dilute another aliquot and remake the slide until individual virions are visible (**Fig. 5b**). Purity verification (**Box 1**) using the direct count approach with SYBR Gold, however, can be laborious if many samples are being collected simultaneously or if the samples are from difficult environmental materials (**Fig. 5**). Sometimes as many as ten slides need to be prepared from each gradient layer to ensure that the virions were concentrated and free of contaminating cells (R.V. Thurber, personal communication).

If there are any contaminating nuclei or microbes remaining, several additional steps should be added to the protocol. For example, in some samples large numbers of microbes (**Fig. 5c**) remain after ultracentrifugation, so it is necessary to perform an additional ultracentrifugation step. Also, prefiltration steps can be conducted before gradient ultracentrifugation, such as passages though 8.0-, 0.45- or 0.22-μm filters, or if you are working with very small volumes, similar pore size Sterivex cartridge filters can be used. Finally, for difficult samples a sucrose cushion step can be added[68].

Nucleic acid extraction

The viral DNA extraction protocol described is a derivation of a standard protocol[69]. It is important that every solution in this protocol be virus free, so compounds must be molecular biology grade or passed through a 0.02 μm filter. Formamide and phenol/chloroform/isoamyl should be kept cold and correctly buffered before use. Test the pH of the phenol/chloroform/isoamyl often or purchase new reagents on a regular basis to ensure they are not degraded.

Although a large majority of our work is on DNA viruses, metagenomes have been generated for RNA viruses, particularly from medical samples and seawater[16,70,71]. For sequencing, researchers must generate sufficient quantities of cDNA from the viral RNA. When isolating RNA viruses, always use RNase-free and viral-free solutions. Also, some viruses incorporate RNA into their coat structures, and treatment with RNase will destroy those particular RNAs[36]. In such cases, it may be preferable to accept some level of contaminating host RNA.

Nucleic acid amplification

The abundance of viral nucleic acids obtained through these protocols is often sufficient (see **Table 1**) for small-scale cloning or PCR-based analysis[72]. For small, rare or difficult-to-process samples, however, the purification of sufficient quantities (approximately 3–5 μg) of viral nucleic acids for sequencing may be impossible. If adequate quantities of DNA cannot be prepared directly from the samples, the DNA can be amplified using multiple displacement amplification. This method makes use of Phi29 DNA polymerase, an enzyme with strand-displacement activity that enables it to amplify genomic DNA using random primers and a single denaturation step[73–76]. A single 20-μl Genomiphi reaction (containing 10–100 ng of template DNA) will produce ~4 μg of

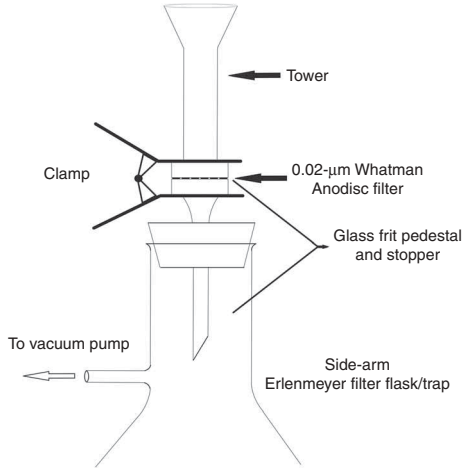


Figure 4 | Filtration setup for viral enumeration and verification of sample purity. To verify that only virus particles were collected from TFF and/or ultracentrifugation, construct filtration device. Glass frit and filter pedestal is placed within an Erlenmeyer filter flask/trap. Trap is attached to vacuum pump. Viral 0.02-μm filter is placed atop glass frit. The glass tower is attached to the glass frit pedestal with a stainless steel clamp. The sample is poured into the graduated glass tower.

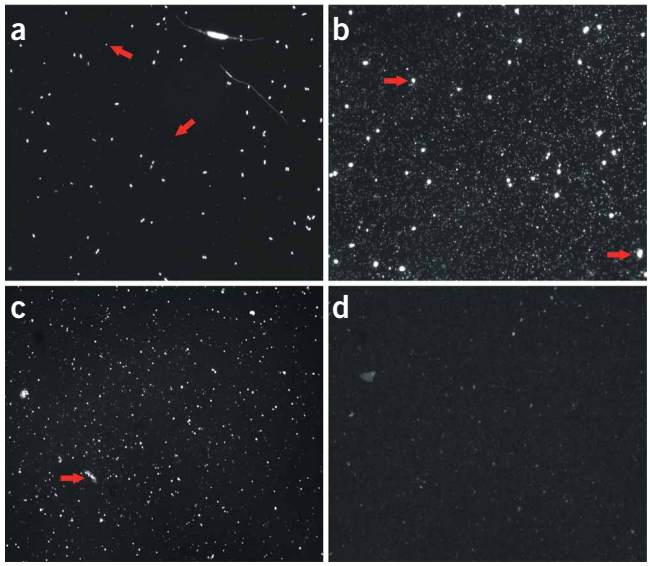


Figure 5 | Epifluorescent microscopy to determine viral-particle concentration and purity. (**a**) Seawater sample with characteristic pinpoint fluorescence. (**b**) Viral particles appear as dim pinpoints of light next to larger and brighter microbial cells. Concentrated virions and microbial cells (arrow) from 100-kDa TFF retentate. (**c**) Virus concentrate after CsCl ultracentrifugation containing contaminating eukaryotic and microbial debris (arrow). (**d**) Fluorescence image showing the characteristic milky appearance of a filter overloaded with virus particles. All images were taken at 600× magnification with an oil immersion objective.

amplified DNA. DNA produced using this technique has been shown to be underrepresented in the 500–1,000 bp near the termini of linear templates[73]. To reduce sequence bias and ensure high yield, it is recommended that several independent amplification reactions be conducted for each sample. Recent reports have also suggested that two reactions be performed for each sample, one with and one without the denaturation step to reduce certain types of sequence bias[77]. The amplification can be verified by running an aliquot on a 1% (wt/vol) agarose gel or by taking an optical density reading on a spectrophotometer. The optical density ratio of A_{260}/A_{230} indicates nucleic acid purity, and should ideally be in the range of 1.8–2.2. Values less than 1.0, often obtained with environmental samples, indicate the presence of contaminants that may interfere with enzymatic reactions. However, for an optical density measurement to reflect polymerized DNA, it is necessary to repurify the DNA by using silica columns intended for genomic DNA so that primer oligomers and dNTPs are removed. We use a modification of the DNeasy extraction method to suit our cleanup purposes.

The amplification process not only increases total DNA abundance, but also effectively purifies the DNA. Viral DNA samples (depending on the source) often contain PCR or other enzyme inhibitors. This step, if successful, essentially eliminates this issue.

A similar technique has been created for the amplification of RNA. One particular method is called TransPlex for Whole Transcriptome Amplification of Intact and Degraded RNA. This kit can amplify total RNA in less than 4 h without any terminal bias[78]. The RNA template is primed with quasirandom primers. Reverse transcriptase and displaced strands are used as additional templates for further amplification. These reactions can then be purified using PCR cleanup kits.

MATERIALS

REAGENTS

- CsCl (Fisher Scientific, cat. no. BP1595)
- Formamide 'Super Pure' (Fisher Scientific, cat. no. BP228) **! CAUTION** Formamide is toxic and should be used in the fume hood and with personal safety equipment, such as gloves and goggles.
- Chloroform, molecular biology grade (Fisher Scientific, cat. no. BP1145) **! CAUTION** Chloroform is toxic and should be used in the fume hood and with personal safety equipment, such as gloves and goggles.
- Phenol:chloroform:isoamyl alcohol 25:24:1 mixture, pH 6.7 or 8.0 (Fisher Scientific, cat. no. BP1752I-100) **! CAUTION** Phenol is both toxic and a burn hazard. Use in a fume hood and wear personal protection, such as gloves and goggles.
- Bleach (Fisher Scientific, cat. no. 23-279355) **! CAUTION** Bleach is poisonous. Wear personal protection, such as gloves and goggles.
- Hexadecyltrimethyl-ammonium bromide (Sigma Aldrich, cat. no. H5882)
- DNase I (Sigma Aldrich, cat. no. AMPD1)
- 32% (wt/vol) paraformaldehyde (Electron Microscopy Sciences, cat. no. 15714) **! CAUTION** Paraformaldedye is toxic. Use in fume hood and wear personal protection, such as gloves and goggles.
- 10× PBS, pH 7.4 (Fisher Scientific, cat. no. BP399)
- NaCl, molecular biology grade (Sigma Aldrich, cat. no. S3014)
- $MgSO_4$, anhydrous (Fisher Scientific, cat. no. M65)
- Tris (Fisher Scientific, cat. no. BP152)
- Ascorbic acid (Sigma Aldrich, cat. no. A7631)
- 0.5 M EDTA (Sigma Aldrich, cat. no. E7889)
- Proteinase K (Fisher Scientific, cat. no. AC61182-0500)
- Glycerol (Fisher Scientific, cat. no. BP229)
- Ethanol 200 proof 99.5% (Fisher Scientific, cat. no. 64-17-5) **! CAUTION** Ethanol is flammable.
- Isopropanol 99% HPLC grade (Fisher Scientific, cat. no. 67-63-0) **! CAUTION** Ethanol is flammable and an irritant. Keep away from flame and wear personal protection, such as gloves and goggles.
- 2-Mercaptoethanol (β-ME) (Sigma Aldrich, cat. no. M6250) **! CAUTION** β-ME is toxic. Use in fume hood and wear personal protection, such as gloves and goggles.
- DNeasy Kit (Qiagen, cat. no. 69506)
- RNeasy Plus Kit (Qiagen, cat. no. 74104)
- Genomiphi DNA amplification (GE Healthcare, cat. no. 25-6600)
- TransPlex for whole transcriptome amplification of intact and degraded RNA (Sigma Aldrich, cat. no. WTA2)
- SYBR Gold (Invitrogen/Molecular Probes, cat. no. S-11494) **! CAUTION** SYBR is a suspected carcinogen. Wear personal protection, such as gloves and goggles.
- Sigma water (Sigma Aldrich, cat. no. W4502)
- Gelatin (Fisher Scientific, cat. no. G7)

EQUIPMENT

- TFF filters (GE Healthcare)
  - 0.84 m^2, 0.02-μm filter (cat. no. CFP-2-E-9A)
  - 1.15 m^2, 100-kDa filter (cat. no. UFP-100-C-9A)
  - 0.042 m^2, 0.02-μm filter (cat. no. CFP-2-E-4A)
  - 0.065 m^2, 100-kDa filter (cat. no. UFP-100-C-4A)
- Tubing from MASTERFLEX (Cole Parmer)
  - Size 82 for large filters (cat. no. U-96440-82)
  - Size 15 medium filters (cat. no. U-96440-15)
  - Size 73 (adapt medium filters to large pump heads; cat. no. U-96440-73)
- Peristaltic pump, for use with size 82 or 73 tubing large filters (Cole Parmer)
  - Masterflex pump, I/P Brushless Drive (cat. no. EW-77410-10)

- Masterflex pump head, Easy-load PSF/SS (cat. no. FF-77601-10)
- Masterflex hardware for additional pump heads (cat. no. FF-77601-96)
- Peristaltic pump for use with size 15 tubing medium filters (Cole Parmer)
- Masterflex pump, L/S Easy-load (cat. no. FF-77521-40)
- Masterflex pump head, Easy-load (cat. no. FF-07518-12)
- Mounting hardware for additional pump heads (cat. no. FF-07013-05)

To avoid having to buy both pumps, you can use the large pump for both filter sizes by threading the size 15 tubing through the size 73 where it goes into the pump head.

- Pressure gauges and adaptors (Cole Parmer)
- 0–30 p.s.i. stainless steel gauges (cat. no. P-68007-03)
- Gauge guards (cat. no. U-07359-08)
- 1/4 female–female Teflon adaptor (cat. no. P-06376-31)
- Kynar T-adaptors (cat. no. P-30704-69 for size 15 or 26 tubing), (cat. no. P-30704-70 for size 73 tubing), (cat. no. P-30704-71 for size 82 tubing)
- Mineral oil (cat. no. U037359-50)
- Hose Adaptor Kit (GE Healthcare, cat. no. KA12 4PS)
- Other supplies
- 136-µm Nitex Mesh (Coastal Supplies, cat. no. 3-123-70)
- PowerGen 125 Hand Homogenizer (Fisher Scientific, cat. no. 14-261-02)

List of supplies for CsCl gradients

- Luerlok sterile 0.02-µm Anotop 25-mm Whatman filters (cat. no. 6809-2102)
- Beckman SW41 Ti rotor package (Beckman Coulter, cat. no. 331336)
- Ultracentrifuge tubes (Fisher Scientific, cat. no. NC9194790)

List of supplies for viral counts/SYBR Gold staining

- GAST manufacturing oil-less filtration vacuum pump (Fisher Scientific, cat. no. S63086)
- Milipore 25-mm glass microanalysis vacuum filter holder and support (Fisher Scientific, cat. no. XX1002505)
- Millipore flat forceps (Fisher Scientific, cat. no. XX6200006)
- 0.02-µm Al_2O_3 anodisc Whatman filters 25 mm (Whatman, cat. no. 6809-6002)
- 8-µm nucleopore polycarbonate 25-mm Whatman filters (Fisher Scientific, cat. no. 110405)
- Polypropylene 25-mm syringe adapters (Advantec, cat. no. MFS 540100)
- Millipore Sterivex 0.22-µm filter unit (Fisher Scientific, cat. no. SVGP01050)

REAGENT SETUP

Saline magnesium buffer Mix 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris (pH 7.5) and 0.002% gelatin (wt/vol). Keep sterile and store at room temperature (20–24 °C) for a maximum of 1 month.

Cetyltrimethylammonium bromide Add 1 g of NaCl to 15 ml of water. Slowly add 2.5 g of CTAB. If necessary, heat to 65 °C until dissolved. Purify through a 0.02-µm filter. Keep sterile and store at room temperature for a maximum of 1 month.

2 M Tris/0.2 M EDTA, pH 8.5 (TE) Add 24.228 g of Tris base, 40 ml of 0.5 M EDTA (pH 8.0) and 30 ml of H_2O; adjust the pH to 8.5 and add water to make up a final volume of 100 ml.

Mountant Add 100 µl of 10 % ascorbic acid to 4.9 ml of 1× PBS (pH 7.4). Mix well and add 5 ml of 100 % glycerol and mix well. Filter the mountant through a 0.02-µm syringe filter. Aliquot and store at −20 °C in the dark.

PROCEDURE

Viral-particle sample preparations ● TIMING 2–16 h depending on the volume of sample

1| Viral-particle sample preparations can be prepared using option A using TFF or option B using sample preparations from other complex sources (e.g., soil, animal tissues, clinical samples).

(A) Using TFF

(i) Assemble the TFF equipment as described in the Experimental design and shown in **Figure 2b**. This method refers only to the 100-kDa filter apparatus.

(ii) Collect a large volume (e.g., 50–100 liters of seawater for marine viruses) of liquid sample in a sterilized container.

(iii) Sample the water for microscopy before filtration to determine if the sample is concentrated during the procedure (see **Box 1**).

▲ CRITICAL STEP It is important to confirm the initial presence (and concentration) of viruses in the sample to determine the final yield efficiency.

(iv) Place the intake and retentate hoses into the container.

(v) Place the filtrate hose into the sink or accompanying container if collecting viral-free buffer/water.

(vi) Turn on the peristaltic pump at a low speed and in the correct direction to draw sample into the filter.

▲ CRITICAL STEP Make sure the pressure is below 10 p.s.i. or viruses may be destroyed.

(vii) Once the volume of liquid is <100 ml, place the filtrate hose in the container.

(viii) Remove the pressure from retentate hose, allowing the sample to pass through the filter uninhibited.

▲ CRITICAL STEP If pressure is not removed, viral recovery efficiency will be reduced.

(ix) Run at high speed for several minutes until almost the entire volume of liquid is within the filter system. This should allow the particles that are retained on the column to be washed off and into the remaining liquid.

(x) Allow air to be drawn into the filter to void the system of all the liquid that will be the final concentrate.

▲ CRITICAL STEP This will maximize the yield of viruses.

(xi) Check the final retentate with microscopy to verify that the concentration of microbes and viral particles is higher than in the original sample (see **Box 1**).

(xii) Wash the filters in between individual collections as suggested by the manufacturer's protocol.

(xiii) After washing, run deionized water through the filter until a neutral pH (measured with pH paper) is reached in both the filtrate and retentate.

(xiv) If the remaining volume is greater than 50 ml, then add solid PEG 8,000 to the sample at a final concentration of 10% (wt/vol) and store overnight at 4 °C. If the sample is smaller than 50 ml, proceed to Step 2.

(xv) If the sample is in fresh water, add NaCl to a final concentration of 1 M.

(xvi) Centrifuge for 30 min at 13,000*g* and 4 °C to pellet the viruses.

(xvii) Discard the supernatant.

(xviii) Resuspend the pellet in TE for either CsCl purification or DNA extraction.

BOX 1 | ASSESSING THE PRESENCE OF VIRAL PARTICLES USING MICROSCOPY

1. Prepare 1–5× working strength solution of SYBR Gold.
▲ CRITICAL STEP 1× SYBR Gold working solution should be prepared fresh.
2. Dilute 1–10 µl of viral filtrate from Step 10 (this is really dependent on the concentration of your filtrate and the volume should be adjusted accordingly) into 5 ml of sterile viral-free water.
3. Assemble the filtration system as shown in **Figure 4**.
4. Using flat-tipped forceps remove a 0.02-µm filter from its box and inspect for cracks or breaks.
▲ CRITICAL STEP Do not use the filter if it is damaged in any way.
5. Gently place the 0.02-µm filter on a glass frit while the vacuum pump is on at a pressure less than 10 p.s.i. (~62 kPa).
6. Clean the glass-graduated column with deionized water. Clip the graduated column onto the filter and frit.
7. Add the diluted sample from Step 12 onto the column.
8. Apply a vacuum to the filter until all of the liquid has passed through the filter.
9. Remove the clip and column and gently remove the filter whilst the vacuum is still engaged.
10. Pipette a 100-µl aliquot of 1–5× SYBR Gold into a sterile Petri dish.
11. Place the filter on top of the drop of SYBR Gold solution (sample-side up).
12. Incubate in the dark for 15 min at room temperature, e.g., 20–25 °C.
13. Remove the filter and place (sample-side up) on top of a 100-µl drop of sterile water for a few seconds in another Petri dish.
14. Blot the bottom of the filter on a Kimwipe to remove excess water.
15. To mount the filter onto a glass slide for viewing and archiving, pipette 10 µl of mountant onto a microscope slide, then place the filter on top of the mountant.
16. Add another 10 µl of mountant to the center of the filter, and put a coverslip over it. Slightly press on the coverslip with a Kimwipe to depress the filter.
17. View the filter using an epifluorescent microscope within 12 h of filtration.
■ PAUSE POINT Store the slides at −20 °C in the dark until they can be viewed.
? TROUBLESHOOTING

(B) Using sample preparations from other complex sources (e.g., soil, animal tissues, clinical samples)

(i) Place the sample in appropriate buffer, e.g., PBS (pH 7.4) or saline magnesium buffer (pH 7.5). This will depend on the starting pH of the sample.

(ii) Disrupt tissues with a hand-held electric homogenizer at ~5,000 r.p.m. for 30–60 s or use a mortar and pestle.

(iii) Centrifuge the slurry at a low speed (~2,500*g*) at room temperature for 5–10 min to pellet the tissue, cells or sediment.

(iv) Transfer the supernatant to a new tube. Filter the supernatant to remove microbial and eukaryotic cells and nuclei by sequential passage through 0.45 µm and then a 0.22-µm Whatman filter.

(v) Check the filtrate by microscopy to ensure virions were not lost during this step (see **Box 1**) or proceed directly to density gradient ultracentrifugation directly.

(vi) Add 0.2 volumes of molecular biology-grade chloroform to the filtrate.
! CAUTION Chloroform is toxic and should be used in the fume hood and with personal safety equipment, such as gloves and goggles.

(vii) Shake vigorously for several minutes, and place the sample at 4 °C until further processing.
■ PAUSE POINT The sample can be stored at 4 °C for a maximum of 3 months. However, the longer the sample is stored, the lower the viral recovery will be. It is recommended that samples be processed within a week.

Density gradient ultracentrifugation ● TIMING 2–4 h

2| From heaviest to lightest pour each density into clear ultracentrifugation tubes (e.g, Beckman Coulter tubes), marking the outside of the ultracentrifugation tube with an ethanol proof marker to identify each density interface (see **Fig. 3**). For phages use 1 ml of: 1.7, 1.5 and 1.35 g ml^{-1} CsCl densities, respectively (**Fig. 3a**). For other viruses, consult **Table 2** for the appropriate densities and type of gradient (e.g., CsCl versus sucrose).

3| After the layers are successfully poured, slowly add identical volumes of samples to the top of each gradient (**Fig. 3b**).

4| Exactly balance the tubes before centrifugation by weighing one ultracentrifugation tube on an analytical balance. Tare the balance and weigh the partner tube. Add or remove small drops of the sample to the ultracentrifugation tube until the balance reads less than 1 mg.
▲ CRITICAL STEP If the rotor is unbalanced, vibrations can cause the ultracentrifuge to abort the run and/or damage the instrument.

5| Place the corresponding columns into the partnered rotor tubes and screw on the lids for centrifugation.

6| For phages, centrifuge the columns for 2 h at 22,000 r.p.m. (~60,000*g*) and 4 °C in a swinging bucket rotor. Depending on the density of the viruses, different speeds and times will apply. To determine the correct centrifugation speed, consult the literature.

7| To collect the virions, prepare a sterile 18-gauge needle placed on a luer lok 3–10 ml syringe (**Fig. 3c**). Place the ultracentrifugation tube over an appropriate collection vessel (50-ml centrifuge tubes work well) to retain the other gradient fractions for microscopic examination (see **Box 1**) to ensure that viruses are not lost (**Fig. 3c**). Place the needle, with the mouth facing upward, just below the appropriate step (**Fig. 3d**). For phages, this is just below the 1.5 g ml^{-1} step.
! CAUTION If the sample contains any infectious human disease-associated particles, make sure that this is done in a safe manner. We have had custom polycarbonate ultracentrifugation tube holders (**Fig. 3c**) made for us to prevent needle-stick injuries.

8| Pierce the tube slowly, using a screwing motion, until the needle is midway though the tube. Try not to disturb the column.

9| Slowly withdraw the plunger of the syringe and pull the desired volume of the sample into the syringe barrel. Typically, we collect between 1 and 1.5 ml of sample from a 12-ml tube; however, the location of the virions will depend on their density that may be heavier or lighter (see **Table 2**).

10| Transfer the collected virion layers into a sterile 1.5-ml centrifuge tube. Check the sample for the presence of virus particles (see **Box 1**).
▲ CRITICAL STEP Expel the viral fraction from the syringe slowly, to avoid creating shear forces that may disrupt the virions.
? TROUBLESHOOTING

Viral nucleic acid isolation ● TIMING 1–2 d depending on the overnight steps

11| Viral nuclei acids can be isolated using option A for DNA viruses or option B for RNA viruses.

(A) DNA viruses

(i) If required, DNase I–treat the sample by adding 2.5 U of DNase I to each milliliter of sample.
▲ CRITICAL STEP In blood or tissue samples, the amount of DNase I may need to be increased significantly to ensure removal of background host DNA.
(ii) For every 100 µl of sample, incubate for 1 h at 37 °C.
(iii) Extract the virions by adding 0.1 volume of 2 M Tris-HCl (pH 8.5)/0.2 M EDTA, 100 µl of 0.5 M EDTA (pH 8.0) per 10 ml sample, 1 volume of formamide and between 1 and 10 µl of glycogen (20 mg ml^{-1}) (optional).
! CAUTION Formamide is toxic. Use in fume hood and wear personal protection, such as gloves and goggles.
(iv) Incubate at room temperature (20–25 °C) for 30 min.
(v) Add 2 volumes of room-temperature 100% ethanol.
(vi) Pellet the DNA by centrifugation for 20 min at >8,000*g* at 4 °C.
(vii) Wash the pellet twice with 70% ethanol.
(viii) Resuspend the DNA in 567 µl of TE.
■ PAUSE POINT Store at −20 °C for a maximum of 1 month.
(ix) Add 30 µl of 10% (wt/vol) SDS and 3 µl of 20 mg ml^{-1} proteinase K to the sample and mix.
(x) Incubate for 1 h at 37–55 °C.
(xi) Add 100 µl of 5 M NaCl and mix thoroughly.
(xii) Add 80 µl of CTAB/NaCl solution (see REAGENT SETUP), mix and incubate for 10 min at 65 °C.
(xiii) Add an equal volume of chloroform, mix and centrifuge for 5 min at ~8,000*g* at room temperature.
! CAUTION Chloroform is toxic. Use in fume hood and wear personal protection, such as gloves and goggles.
(xiv) Transfer the supernatant to a separate tube and add an equal volume of phenol/chloroform/isoamyl alcohol to the supernatant fraction, mix and centrifuge for 5 min at ~8,000*g* at room temperature.
! CAUTION Phenol/chloroform/isoamyl is both toxic and a chemical burn hazard. Use in fume hood and wear personal protection, such as gloves and goggles.
(xv) Transfer the supernatant to a separate tube avoiding the organic layer.
(xvi) Add an equal volume of chloroform to the supernatant fraction, mix and centrifuge for 5 min at ~8,000*g* at room temperature.
(xvii) Transfer the supernatant to a separate tube and add 0.7 volumes of isopropanol, and mix gently until the DNA precipitates (maximum of 2 h at −20 °C or overnight at 4 °C).
(xviii) Centrifuge for 15 min at a maximum of 13,000*g* and 4 °C.
(xix) Remove the supernatant and wash with ~500 µl of cold 70% ethanol.
(xx) Remove the ethanol and air-dry the pellet (~20 min) but do not overdry the pellet.
(xxi) Resuspend in 50 µl of water (certified viral free) or TE.

(xxii) Check the optical density of the sample to estimate the DNA concentration and purity.
■ PAUSE POINT Sample can be stored for a long term at −80 °C or up to 1 year at −20 °C.
? TROUBLESHOOTING
(xxiii) To combine duplicate tubes or to further concentrate the DNA samples, add 10 µl of 3 M sodium acetate (pH 5.2) to 100 µl of sample. Add 1–2 µl of glycogen (20 mg ml^{-1}) (optional) and 240 µl of 100% ethanol and incubate overnight at −20 °C.
(xxiv) Pellet the DNA as described in Step 1A(xxviii).
(xxv) Remove the ethanol and air-dry the pellet (do not overdry or the DNA will not resuspend). Add 15 µl of water or TE (virus free).
■ PAUSE POINT Sample can be stored for a long term at −80 °C or up to 1 year at −20 °C.
(xxvi) Validate bacterial and eukaryotic RNA removal using primers for PCR or reverse transcriptase-PCR amplification of the 16S and 18S rDNA genes, respectively[10].

(B) RNA viruses

(i) Split 1.5 ml of concentrated virus as prepared in Step 9 into two 2 ml tubes.
▲ CRITICAL STEP Use RNase-free solutions and follow handling precautions.
(ii) Using the RNeasy Plus Kit from Qiagen, add an equal volume of lysis buffer containing 10 µl ml^{-1} β-ME to each tube.
! CAUTION Lysis buffer contains guanidinium thiocyanate and β-ME, which are toxic. Use in fume hood and wear personal protection, such as gloves and goggles.
(iii) Vortex the samples and incubate for 5–10 min at room temperature.
(iv) Centrifuge the samples for 3 min at a maximum of 13,000*g* at room temperature.
(v) Add 1 volume of 70% ethanol.
▲ CRITICAL STEP Ensure that the 70% ethanol is made with RNase-free and viral-free water.
(vi) Transfer the supernatant to a gDNA column 750 µl at a time.
(vii) Centrifuge the column for 30 s at >8,000*g* at room temperature.
(viii) Transfer the flow-through into a new labeled RNase-free tube. Repeat Steps B(vi) and B(vii), until all 3 ml of the sample has been passed through the gDNA column.
(ix) Continue with the kit according to the manufacturer's protocol.
(x) Measure the RNA concentration with a Nanodrop or bioanalyzer.
■ PAUSE POINT RNA can be stored for a long term at −80 °C.
? TROUBLESHOOTING
(xi) Validate bacterial and eukaryotic RNA removal using primers for PCR or reverse transcriptase-PCR amplification of the 16S and 18S rDNA genes, respectively[10].

Nucleic acid amplification ● TIMING 3–24 h depending on the amplification type

12| To amplify the DNA using the Genomiphi DNA amplification kit, mix 1–10 ng of template DNA with 9 µl of sample buffer, mix and spin down.

13| Denature the sample by heating for 3 min at 95 °C.

14| Cool on ice for 3 min.

15| For each amplification reaction, combine 9 µl of reaction buffer with 1 µl of enzyme, mix and place on ice, add to the cooled sample from Step 14.
▲ CRITICAL STEP Prepare the reaction mixture immediately before adding to the denatured sample. Preparation of the reaction mixture in advance leads to nonspecific amplification.

16| Incubate the sample for 16–18 h at 30 °C (for a maximum of 18 h, but not shorter than 6 h).

17| Inactivate the enzyme by heating the sample for 10 min at 65 °C.

18| Cool to 4 °C.

19| Clean up the amplified DNA with a modified version of a Qiagen DNeasy Kit.

20| Add 180 µl of buffer ATL, 200 µl of buffer AL and 200 µl of 100% ethanol to the 20 µl of reaction volume. Mix and if the sample is too viscous briefly heat to approximately 55–65 °C.

21| Add to the column provided with the kit, and proceed with the purification steps as specified in the manual.
? TROUBLESHOOTING

● TIMING

Step 1, viral-particle sample preparations: maximum of ~2 d
Steps 2–10, density gradient ultracentrifugation: approximately 2–4 h
Step 11, viral nucleic acid isolation: ~1 or 2 d with optional overnight steps
Steps 12–21, nucleic acid amplification: approximately 3–24 h depending on the amplification type
Box 1, assessing the presence of viral particles using microscopy: 1–2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
Sample loading (Step 10)	Clogged needle	Sample too viscous	If the needle cannot be unclogged, repour and centrifuge the columns
Step 10 (**Box 1**)	No viruses in concentrate	Did not purge retentate from filter (Step 1A(viii))	Remove all pressure from hoses and fully draw retentate in and out of filter
		Pressure too high—viruses destroyed (Step 1A(vi))	Keep pressure below 10 p.s.i.
		Mistakenly collected filtrate (Step 1A(iv–v))	Collect retentate
		Optional postfiltration trapped viruses on membrane (Step 1B(iv))	Eliminate postfiltration step
	No viruses in fraction	CsCl layers wrong densities (Step 2)	Remake densities
		Virions damaged in CsCl (Step 2)	Check virion sensitivity/use different gradient (e.g., sucrose) (see **Table 2**)
		Virions above or below predicted densities (Step 9)	Ensure that loaded sample is lower density and in same buffer
			Readjust gradient densities
	Microbial cells present		Filter collected layer with 0.45–0.22 μm disk filter
			Conduct second ultracentrifugation
			Readjust gradient densities
			Prefilter sample with Nitex mesh
	No viruses or microbes	SYBR Gold too dilute or old (**Box 1**, Step 1)	Make fresh stain
		Not enough sample added (**Box 1**, Step 2)	Add more sample to filter
	High background staining	SYBR Gold too concentrated (**Box 1**, Step 1)	Rinse off excess SYBR Gold or remake SYBR
		Too much sample added	Reduce amount of sample added to filter (dilute sample)
Viral nucleic acid isolation (Steps 11A(xxii) and 11B(x))	Low yield	Viral-particle abundance low (Steps 2–10)	Collect more virions
			Amplify nucleic acids
		Old reagents	Make and purchase fresh reagents
Nucleic acid amplification (Step 21)	No DNA/RNA	Inhibition of amplification (Step 12)	Use less starting material (dilute DNA or RNA)
		Denatured enzyme	Try positive control and/or fresh enzyme

ANTICIPATED RESULTS

The methods presented here should result in the isolation of viral nucleic acids that can be used for PCR, clone library generation and/or direct sequencing of a virome. The total amount of viral nucleic acids derived from this method depends

largely upon the volume or weight of starting material and whether amplification of the DNA or cDNA was conducted. For instance, viruses recovered from 1 g of human feces may yield upward of 50–100 ng of viral DNA without amplification. However, 500 g of feces is required for the same 50–100 ng yield of RNA (see **Table 1**).

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