**Extraction and Purification of Viral Nucleic Acids from Environmental Samples**

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**I. Introduction**

Virus particles (or virions) consist of two or three major components: i) the genetic material made from nucleic acids (i.e., DNA or RNA) arranged in a variety of configurations, that carry the necessary genetic information to “hijack” its host cell for their own replication; ii) a protein coat that protects the more delicate genetic material; and iii) in some cases a virus envelope that surrounds the protein coat made of phospholipids (Fig 1). The shapes of viruses range from simple helical and icosahedral forms to more complex structures. Their capsid structure and arrangement of the genetic material can greatly affect their environmental persistence, extraction efficiency, and detection efficiency of virus particles from the sample matrix.

In the past century, virus detection and identification mainly relied on virus isolation using culture-based methods, through direct observation of virus particles/physical structure using electron microscopy (EM)-based techniques, and/or immunological assays using antibodies. However, without a detailed knowledge of the virus’s genetic material, few inferences could be made about their origin, their infectivity, or occurrence in the environment, especially considering that a routine *in vitro* cell culture method has not been established for some important pathogenic viruses, such as human norovirus. Therefore, molecular methods are currently widely used for virus detection and characterization in clinical, food, environmental samples, etc.

However, virus detection in environmental samples comes with some major challenges – not only because viruses are exceedingly small but because they behave as colloids rather than suspended biological particles in water, which makes them more mobile and transmissible in the environments. For what amounts to small charged protein and lipid packets of genetic material the act of isolating them from the environment is seemingly a daunting task. Contemporary methods typically involve several steps in which each subsequent step is dependent on the performance and quality of the preceding step resulting in often variable results. Figure 2 provides a basic schematic of the various virus extraction and detection steps and control measures. The goal of this chapter is to provide a background on the intricacies of viral nucleic acid extraction from various environmental matrices for use in molecular detection assays such as polymerase chain reaction (PCR).

**II. The Sample Matrix**

One of the first considerations to take into account when trying to isolate viral nucleic acids from any environment is to understand the composition of the matrix. For instance numerous inhibitory compounds may be present that will mostly likely be co-concentrated or co-extracted during sample preparation process. The identification and subsequent mitigation of these compounds will be discussed in more detail in a later section but it is important to evaluate how the samples will be handled during sample preparation in order to identify the most appropriate method for each type of sample matrix. Picking the most appropriate virus sampling method is further complicated by the ever present tradeoff of direct extraction whereby the sample matrix is, in it’s entirety, stripped of nucleic acids and used for downstream applications or in many cases a pre-concentration/isolation step is preferred where virons may be cleaned and isolated from the sample matrix. In this case, just the virus “fraction” of the total sample is used for downstream extraction purposes. Such techniques always leave questions about the inherent biases of concentration and cleanup steps. For instance, what is the virus recovery of the particular virus that is being studied? Were any members of the virus community missed? Best judgment and empirical knowledge should be used when regarding how the sample matrix will be treated prior to nucleic acid extraction.

Another consideration must also be taken into account when choosing sampling methodologies with regards to the estimated concentrations of viruses likely present in the initial sample volume. For detection of a specific plant or animal pathogenic viruses found at low levels in the environment, virus particles must be first concentrated and purified in order to before assay because in most cases, they are highly diluted from the source of contamination. The fewer target viruses found in a sample the larger the sample volume required and subsequent concentration of that sample will be needed before assay. It is also important to consider how much of the concentrate can be economically processed and assayed since this will limit the sensitivity of the assay and number of sample that can be taken.

**1. Sample volumes**

As mentioned above, one of the most troubling aspects regarding efficient viral nucleic acid extraction is the limitation associated with sampling volumes for molecular application. An effective extraction methodology or kit should preferentially be scalable from low to high volume workflows since larger process volumes increase the sensitivity of the assay (Katayama et al., 2002). These methodological limitations occur because initially virus detection methods were developed for use with cell culture assays, where eluate volumes larger volumes can be assayed relative to molecular methods such as PCR. Also, originally virus nucleic acid extraction kits and methodologies were developed for clinical purposes where the concentration of virus particles is considerably higher (e.g. blood, feces, sputum) then those found in the environment. Extraction kits use sample volumes of no more than 200 µl vs 10 – 500 ml for many environmental sample concentrates. Typically providing a higher concentration efficiency to bring sample volumes down below 1 ml is a preferred strategy then trying to extract volumes greater than 1 ml.

**III. Inhibitory Substances**

Molecular methods for virus detection are often seen as not reliable due to the presence of inhibitory compounds and other contaminants that cause false negatives for detection. These issues are caused by naturally occurring inhibitory compounds, such as humic and fulvic acids, RNases and DNases, heavy metals and cations (Ca2+ and Fe3+), as well as a humic acid-like compound in beef extract, which is a traditional eluent for virus concentration from water and biosolids (Gibson et al., 2012; Hata et al., 2011; Rock et al., 2010). The mitigation of inhibitory compounds and other impurities in nucleic acid extracts for the detection of viral genomes in environmental sample matrices is essential for improving detection accuracy and reliability, especially in difficult sample matrices known to contain inhibitory compounds: biosolids, shellfish, water concentrates, various pigmented food items, etc. Achieving the most clean and concentrated nucleic acid possible, regardless of downstream applications, is critical to improving the efficacy of molecular assays or genomic surveys of virus genetic material. Inhibitory substances (organic and inorganic compounds) have been characterized using excitation-emission matrix (EEM) spectrometry, etc. (Rock et al., 2010; Schrader et al., 2012). For some inhibitory compounds that provide measurable excitation and emission spectra significantly different from nucleic acids simple technologies like NanoDrop can easily provide estimates of the presence or absence of inhibitory compounds like humic and fulvic acids in nucleic acid extracts (Wang and Fujii, 2011). However, factors that interfere with nucleic acid extraction and purification have not been fully elucidated.

**1. Mitigation and detection of inhibitory compounds**

Under optimal reaction conditions (i.e., no inhibitors present), quantitative PCR (qPCR) can usually detect as few as 10 gene copies per PCR reaction reliably; however, the presence of PCR inhibitory substances in the sample can greatly reduce the sensitivity and reproducibility of detection. In addition to environmental inhibitors, some virus concentration and extraction methods require the use of elution buffers such as beef extract that also contain inhibitory compounds (Iker et al., 2013a).

There have been several techniques reported to reduce inhibitory effects; e.g., addition of T4 gene 32 protein (Kreader, 1996), bovine serum albumin (BSA) (Kreader, 1996), or polyvinylpyrrolidone (Monpoeho et al., 2000). Removal of inhibitors by using immunomagnetic beads (antigen-antibody reaction) (Schwab and Leon, 1996), cation exchange resin (Abbaszadegan et al., 1993), or gel chromatography (Abbaszadegan et al., 1993; Hata et al., 2011) has also been reported. Early approaches to mitigate such PCR inhibition was to dilute raw nucleic acid extracts by 1:10 or greater, thereby decreasing the concentration of inhibitors significantly, but also nucleic acid concentrations (Gibson et al., 2012). Additionally, the use of pre- or post-nucleic acid extraction procedures to remove or mitigate PCR inhibitors may further degrade viral nucleic acids, especially viral RNA that is highly susceptible to the very active and prevalent RNases. There are several options for post- extraction PCR inhibitor removal, which include aluminum sulfate, protein kinase K, and silica-based spin column to remove inhibitory compounds (Lloyd et al., 2010; Pontiroli et al., 2011; Schriewer et al., 2011). However, such additional procedures may also provide more opportunities for laboratory contamination and increase the overall cost, labor, and time of assays. Therefore mitigation of potential inhibitory compounds should be addressed before or during the nucleic acid extraction step. As an example, (Katayama et al., 2002) developed a virus concentration method using an electronegative filter with an acid rinse procedure followed by alkaline elution to avoid the use of beef extract.

**2. Controls to monitor inhibitory effects and detection efficiency**

Since the above-mentioned techniques to mitigate inhibition cannot reduce the inhibitory effects entirely and may cause loss of viruses during the process, it is essential to evaluate the magnitude of inhibition for each sample and even each detection process (i.e., nucleic acid extraction, RT, PCR, etc.) (Demeke and Jenkins, 2010; Hata et al., 2011; Opel et al., 2010). More specifically, viral nucleic acid preparation is considered a critical step in accurate quantification of viral genomes by qPCR and digital PCR (dPCR), and thus MIQE guidelines recommend reporting the details of process controls included in the assay (Bustin et al., 2009; Huggett et al., 2013). Exogenously added control virus particles and/or control nucleic acids outlined in Figure 2 have been used for this purpose (Gregory et al., 2006; Hata et al., 2013, 2011; Iker et al., 2013a) and are strongly suggested as part of proper QA/QC protocols when evaluating viral nucleic acid preparation.

The following controls are used to monitor the efficiency of viral nucleic acid extraction and molecular detection:

1. Process control – virus particles added to original sample matrices prior to concentration or virus extract to monitor overall recovery from original sample matrices. Viruses that are different from viruses added to the extraction control provide an overall evaluation of the recovery efficiency should attempts to recover viruses from sample matrix fail.
2. Extraction control – virus particles added to the sample prior to viral nucleic acid extraction to monitor extraction efficiency (Bosch et al., 2010). Various types of native and artificial viral particles, such as mengovirus (Costafreda et al., 2006)(da Silva et al., 2007), murine norovirus (Hata et al., 2011)(Hata et al., 2013), armored RNA (artificial viral RNA coated with coliphage MS2 capsid protein) (Pasloske et al., 1998), etc. have been used for this purpose. This control has been often used to monitor the efficiency of extraction-RT-(q)PCR.
3. Internal control RNA (RT-PCR control) – RNA released from virus particles or artificially synthesized RNA to assess the efficiency of RT-PCR as well as to estimate the viral nucleic acid extraction efficiency together with the use of a process control virus (extraction control) (Costafreda et al., 2006). The development of primer sharing controls (PSCs), which are artificial RNAs that are amplified by with the same primer pairs and result in the same amplicon sizes as the targets to accurately assess RT-PCR inhibition, has been reported (Hata et al., 2011).
4. Internal control DNA (PCR control) – exogenously introduced non-target control DNA that is used to monitor PCR efficiency. The PSC-DNA (Hata et al., 2011) or plasmid DNA (Iker et al., 2013a) have been used for this purpose.

In reality few choose to not go through such extensive measures of evaluating every step the process citing concerns over cost, time, and necessity. For most laboratories that work extensively with viral nucleic acid preparations, especially for molecular detection, the use of all of these proposed controls is mainly limited to internal controls and sometime extraction controls to evaluate the level of inhibition (if any) during the extraction or amplification process. If the work requires truly quantitative detection then these controls are almost always required to show that the quantitative values have at least some merit. In practice, especially for protocols and sample matrices that are well known to an established laboratory, the addition of virus particles as a process and extraction control requires little effort and cost. Their addition(s) make it easier to track down an explanation for any unusual results should something unexpected occur.

**IV. Nucleic Acid Preparation and Purification**

**1. Traditional viral nucleic acid extraction/purification techniques**

Early or “in-house” methods of viral nucleic acid extraction/purification techniques, such as heat release (Nishimura et al., 2010; Richardson et al., 1988) phenol-chloroform extraction (Ma et al., 1995), and guanidium extraction (Schwab et al., 1996; Shieh et al., 1995) have been argued as a “cost effective” and in some cases “quick” technique. However, such techniques are not considered to be effective in removing inhibitors, have low extraction efficiency, use potentially harmful chemicals (chloroforms, phenols, etc.), end up being more time-consuming and labor intensive when problems are realized downstream, and potentially further damage nucleic acids. Additionally, these in-house methods lack reproducibility and quality assurance from laboratory to laboratory and even sample to sample. With the more recent development of the commercially produced extraction kits these traditional methods have begun to go by the wayside in favor of more standardized technologies.

**2. Viral nucleic acid extraction kits**

There are a number of commercially available viral nucleic acid extraction kits on the market today. Many extraction kits currently in use today were developed originally for use in clinical specimens and provide excellent and rapid virus recovery for samples low in inhibitory compounds at a low cost to the user. However, only recently have kits become available to deal with water and food sample processing that contain exceedingly high levels of inhibitors. Silica membrane-based nucleic acid extraction/purification method, a commonly used method for environmental water samples, cannot always effectively remove humic acid (Hata et al., 2011; Iker et al., 2013b; Rock et al., 2010). The principle of this method is based on attachment and detachment of nucleic acids to silica by altering pH and ionic strength (Mio et al., 2006) - nucleic acids tend to bind silica at acidic condition in the presence of chaotropic salts but do not bind tightly near neutral pH. This accounts for the presence of humic acid, which causes major PCR inhibition, in virus concentrates. In addition, plant pigments behave chemically similar to nucleic acids and are typically co-extracted causing problems similarly to humic acid-like substances. Proteinacious materials like meat and dairy interfere with the concentration process by binding tightly to virions making them difficult to extract and purify (Postollec et al., 2011).

Most recently magnetic bead based extraction and purification methods (e.g. MagNA pure Roche, PowerMag MO BIO, Dynabeads Invitrogen) have become a popular way to both disrupt sample material through mechanical homogenizations as well as reversibly bind the nucleic acids to the surface of the bead. The beads can then be magnetically separated from the disrupted sample for further purification making it an ideal approach for robotic high throughput applications. Although this approach is typically very effective at disrupting and retrieving nucleic acid material from the sample matrix, the magnetic beads will also bind inhibitory compounds. The nucleic acid material can then be eluted from capture beads and further purified.

**V. Quality Assurance and Quality Control**

Part an effective nucleic acid proper quality assurance and quality control (QA/QC) plan. For example extraction room/space needs to be physically and/or temporally separated from sample processing, reagent preparation, and post-PCR handling spaces to prevent cross contamination of nucleic acid extracts. A PCR/molecular-laboratory should ideally be divided into three areas: a sample preparation area (the extraction area); a reagent mixing preparation area (the clean area); and detection area or sequencing area (the dirty or post PCR area). Additionally these areas must be cleaned of potential RNases and DNases or other compounds that may cause loss or contamination of raw nucleic acid extracts. Prevention methods include but are not limited to cleaning of pipets and work surfaces with enzymatic disrupting products and/or the use of UV light sterilization will be effective at prevent nucleic acid loss post extraction. Consumables such as tubes and tips that come into contact with samples and extractions should also be taken into account. For example, loss of virus particles spiked into molecular grade water (i.e., under non-buffering conditions) might also be attributed to virus adsorption to the microcentrifuge tubes during the extraction process (Patel et al., 2007). Thus low binding DNA/protein tubes and tips are required to mitigate the loss of virus particles or nucleic acids post extraction. Proper storage of nucleic acid extracts is also of concern as degradative enzymes are more active at warmer temperatures contributing to nucleic acid degradation. Also the simple act of freezing and thawing nucleic acids will act against DNA/RNA integrity, therefore it is suggested that if raw nucleic acid extracts are subjugated to numerous freeze though cycles that aliquots be made and stored at a constant temperatures should sample be need to be repeatedly accessed. The temperature at which nucleic acids are stored should also be taken into consideration. For short term storage of less than 1-3 months nucleic acids may be held in a -20**°**C non-cycling freezer, should samples need to be stored for longer periods they should be placed in a -80**°**C with power backups and alarmed monitoring systems.

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**Fig 1.** Overview of virus structure. The presence or absence of outer membranes can greatly alter concentration and extraction efficiency of various viruses. The type of genetic material will also effect the stability of the genomes post extraction.

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**Fig 2.** Overview of virus extraction workflow. Nucleic acid extraction and purification begins with evaluating the sample matrix to optimize downstream workflows for the specific sample matrix and detection method.