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Eskelin, Katri

2017-11

Eskelin , K , Lampi , M , Meier , F , Moldenhauer , E , Bamford , D H & Oksanen , H M 2017 ,
' Halophilic viruses with varying biochemical and biophysical properties are amenable to
purification with asymmetrical flow field-flow fractionation ' , Extremophiles , vol. 21 , no. 6 ,
pp. 1119-1132 . <https://doi.org/10.1007/s00792-017-0963-x>

<http://hdl.handle.net/10138/298119>

<https://doi.org/10.1007/s00792-017-0963-x>

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Halophilic viruses with varying biochemical and biophysical properties are amenable to purification with asymmetrical flow field-flow fractionation

Katri Eskelin¹, Mirka Lampi¹, Florian Meier², Evelin Moldenhauer², Dennis H. Bamford¹, and
Hanna M. Oksanen^{1*}

¹Department of Biosciences
Viikinkaari 9
University of Helsinki,
00014 Helsinki, Finland

²Postnova Analytics
Max-Planck-Str. 14
86899 Landsberg, Germany

*Corresponding author: hanna.oksanen@helsinki.fi

Abstract

Viruses come in various shapes and sizes and a number of viruses originate from extremities e.g. high salinity or elevated temperature. One challenge for studying extreme viruses is to find efficient purification conditions where viruses maintain their infectivity. Asymmetrical flow field-flow fractionation (AF4) is a gentle native chromatography-like technique for size-based separation. It does not have solid stationary phase and the mobile phase composition is readily adjustable according to the sample needs. Due to the high separation power of specimens up to 50 μm , AF4 is suitable for virus purification. Here, we applied AF4 for extremophilic viruses representing four morphotypes: lemon-shaped, tailed and tailless icosahedral, as well as pleomorphic enveloped. AF4 was applied to input samples of different purity: crude supernatants of infected cultures, polyethylene glycol precipitated viruses and viruses purified by ultracentrifugation. All four virus morphotypes were successfully purified by AF4. AF4 purification of culture supernatants or polyethylene glycol precipitated viruses yielded high recoveries, and the purities were comparable to those obtained by the multistep ultracentrifugation purification methods. In addition, we also demonstrate that AF4 is a rapid monitoring tool for virus production in slowly growing host cells living in extreme conditions.

Keywords

Asymmetrical flow field-flow fractionation, virus purification, archaeal virus, macromolecular complex, high ionic strength, halophilic virus

Abbreviations

AF4	asymmetrical flow field-flow fractionation
D _{geo}	geometric diameter
HCIV-1	<i>Haloarcula californiae</i> icosahedral virus 1
His1	His1 virus
h.p.i.	hours post infection
HRPV-1	<i>Halorubrum</i> pleomorphic virus 1
HVTV-1	<i>Haloarcula vallismortis</i> tailed virus 1
MALS	multi-angle light scattering
MGM	modified growth medium
MOI	multiplicity of infection
MWCO	molecular weight cut-off
PEG	polyethylene glycol
PFU	plaque forming units
RC	regenerated cellulose
R _g	radius of gyration
SW	salt water
TEM	transmission electron microscopy
VLP	virus-like particle

Introduction

The important roles that viruses play in the environment have become recognized during recent decades. Viruses and their host cells are known to inhabit even the most extreme ecological niches. Halophilic archaea and their viruses dominate in extremely saline environments, whereas high temperature environments are rich sources for crenarchaeal viruses [see reviews by (Atanasova et al. 2016; Luk et al. 2014; Prangishvili 2013)]. However, the biology, virion composition and architecture of relatively few viruses isolated from extreme environments have been studied in molecular detail. In addition, there is only a limited number of high resolution structures of archaeal viruses available (Veesler et al. 2013). One of the reasons is that detailed characterisations require virus specimens of high purity while maintaining biological activity. Obtaining such preparations is often laborious and time consuming, and is restricted to viruses with cultivable hosts. Conditions for maximal virus production are first optimised. For haloarchaeal viruses, this process is lengthy due to the relatively slow growth of the hosts as well as the low adsorption rates and long intracellular virus replication cycles (Atanasova et al. 2016; Luk et al. 2014). Purification steps are then adjusted to remove host and purification process -derived impurities. Usually various purification and concentration techniques are combined. These may include filtration, precipitation, centrifugation, or chromatography where sample components are separated based on the differences in size, charge, hydrophobicity/hydrophilicity, affinity and/or density (Nestola et al. 2015). However, multistep purification processes are often associated with low yields. Typically, haloarchaeal viruses require high ionic strength for maintaining infectivity; this creates additional purification challenges.

Viruses are large and complex macromolecular assemblies. They vary in size and morphology as well as in their biochemical and biophysical properties. Currently known halophilic archaeal viruses infect hosts belonging to the phylum *Euryarchaeota*, the class *Halobacteria* and represent four distinct virus morphotypes (Atanasova et al. 2016). Icosahedral tailed viruses form the majority of haloarchaeal viruses described thus far (Atanasova et al. 2015a), and their morphology resembles

those of the double-stranded DNA bacteriophages of the order *Caudovirales* (Pietilä et al. 2013c). Pleomorphic viruses (pleolipoviruses, the family *Pleolipoviridae*) represent one significant morphotype. Their DNA genome is enclosed by a flexible membrane vesicle that is decorated with randomly arrayed spike complexes (Pietilä et al. 2012; Pietilä et al. 2016). The third morphotype comprises tailless icosahedral viruses with an internal membrane (family *Sphaerolipoviridae*) (Demina et al. 2016; Demina et al. 2017; Porter et al. 2005). Their capsids are decorated with spike complexes located at the five-fold vertices (Gil-Carton et al. 2015; Jäälinoja et al. 2008). The fourth morphotype consists of the lemon-shaped (spindle-shaped) virions. Direct electron microscope (EM) examination of virus-sized particles in environmental samples suggests that this morphology is common (Oren et al. 1997). However, currently only one such virus, His1, has been characterized (Bath and Dyll-Smith 1998; Pietilä et al. 2013a). Its virion is flexible and has short tail structure with six fibres (Hong et al. 2015). The sequence similarity of the major coat protein of His1 suggests that His1 belongs to the *Fuselloviridae* family, where it would form the *Epsilonfusellovirus* genus (Krupovic et al. 2014). The icosahedral tailed and tailless viruses with internal membranes as well as vesicle-like pleomorphic virus morphotypes are also found among bacteriophages, whereas the lemon-shaped viruses are thus far characteristic only for archaea (Pietilä et al. 2014).

In asymmetrical flow field-flow fractionation (AF4), size-based separation takes place in liquid phase using cross-flow as the separation force (Messaud et al. 2009; Wahlund and Giddings 1987). Consequently, the pressure and shear forces are low making AF4 a gentle method for purification of fragile macromolecular complexes such as viruses. Cross-flow pushes sample components against the accumulation wall that consists of an ultrafiltration membrane and a porous frit. Separation is based on exploitation of differences in the hydrodynamic sizes of sample components (Giddings et al. 1976; Wahlund and Giddings 1987). Smaller components have higher tendency to diffuse and they equilibrate farther away from the accumulation wall, whereas larger components stay closer to the accumulation wall. The flow through a narrow channel (nominal thickness typically between

250-500 μm) has a parabolic profile. Thus, the flow rates at the centre of the channel are higher than those close to the channel walls. Consequently, in normal separation mode, small sample components elute before large ones. This applies to sample components in the range of approximately 1 nm to 1 μm (Giddings et al. 1991). Virus diameters typically range between 20 and 300 nm (King et al. 2012) and thus they are expected to elute in normal mode. AF4 has been widely used for separation and characterisation of submicron- and micron-sized bioparticles including viruses [for reviews see (Ratanathanawongs and Williams 2006; Roda et al. 2009)]. It has been exploited to optimise virus-like particle (VLP) production in bacterial, yeast and mammalian cells, to assay VLP assembly and disassembly conditions, to determine size distribution of sample components in virus and VLP specimens, to quantitate particle amounts, and to study the presence of aggregates and fragments in particle preparations (Chen et al. 2015; Chuan et al. 2008; Lang et al. 2009; Somasundaram et al. 2016; Wei et al. 2007). The majority of AF4 applications on viruses and VLPs have used specimens purified to near homogeneity.

Recently, we demonstrated that AF4 is efficient in the purification of the complex icosahedral bacteriophage PRD1 (66 nm in diameter) (Eskelin et al. 2016). PRD1 purification was performed using a neutral buffer with low 2 mM molar ionic strength. To test further the separation power of AF4 in virus purification, we selected four viruses representing different morphotypes requiring high 0.6-1.6 M molar ionic strengths to preserve infectivity (Table 1 and references therein). The viruses studied were the lemon-shaped virus His1 infecting *Haloarcula hispanica*, *Haloarcula vallismortis* tailed virus 1 (HVTV-1), *Halorubrum* pleomorphic virus 1 (HRPV-1), and *Haloarcula californiae* icosahedral virus 1 (HCIV-1). We show that AF4-based purification of viruses was successful in high salinity conditions and yielded high recoveries of infectious viruses.

Material and methods

Virus sample preparation

Viruses and host strains used are shown in Table 1. Host strains were grown at 37°C in modified growth medium (MGM) (Nuttall and Dyll-Smith 1993) containing artificial salt water (SW) (Dyll-Smith 2009; Nuttall and Dyll-Smith 1993). Broth, agar and soft-agar media contained 23, 20 and 18% (w/v) SW, respectively. Virus-specific buffers were the following: His1: 0.5 M NaCl, 35 mM MgCl₂, 10 mM KCl, 1 mM CaCl₂, 50 mM Tris-HCl (pH 7.2); HVTV-1: 1.2 M NaCl, 44 mM MgCl₂, 47 mM MgSO₄, 1.5 mM CaCl₂, 28 mM KCl, 24 mM Tris-HCl (pH 7.2); HRPV-1: 1.5 M NaCl, 100 mM MgCl₂, 2 mM CaCl₂, 20 mM Tris-HCl (pH 7.5); and HCIV-1: 1 M NaCl, 70 mM MgCl₂, 20 mM KCl, 1 mM CaCl₂, 50 mM Tris-HCl (pH 7.2) (Demina et al. 2016; Pietilä et al. 2013a; Pietilä et al. 2013b; Pietilä et al. 2009). Total molar ionic strengths of the buffers were the following: His1: 0.6 M; HVTV-1: 1.6 M; HRPV-1: 1.5 M; and HCIV-1: 1.2 M.

Three different input samples for AF4 purifications were used: (i) agar stocks or culture supernatants, (ii) PEG-precipitated viruses, and (iii) pre-purified viruses from rate-zonal ultracentrifugation in sucrose and differential ultracentrifugation (designated as 1×viruses). They were prepared as previously described using virus specific buffers (Demina et al. 2016; Pietilä et al. 2013a; Pietilä et al. 2013b; Pietilä et al. 2009) (Fig. 1, Table S1). In short, cells were infected at logarithmic growth phase and grown until lysis occurred or with non-lysing hosts up to the previously published time yielding the highest amount of infectious viruses. Cultures of HRPV-1 and HVTV-1 -infected cells were treated with DNase I (50 µg/ml; Sigma) for 30 min at 37°C. Cells and cellular debris were removed by centrifugation to obtain culture supernatants. Virus particles were concentrated from culture supernatants using NaCl-PEG 6000 precipitation.. After resuspension, insoluble particles were removed by short centrifugation to obtain PEG-viruses. Supernatants were subsequently purified through rate-zonal and differential ultracentrifugation to obtain 1×viruses.

CsCl equilibrium and differential centrifugations for His1 were performed as previously described (Pietilä et al. 2013a).

Analysis of His1 virus production in liquid culture

The His1 life cycle was studied by infecting *Har. hispanica* cultures at logarithmic growth phase ($OD_{550} \sim 0.8$) using a multiplicity of infection (MOI) of 10 (time zero) (Pietilä et al. 2013a). The turbidity of infected and uninfected cultures were monitored at OD_{550} . Samples were collected at 0, 6, 24 and 48 hours post infection (h.p.i). Cells and cellular debris were removed by centrifugation (Table S1) and supernatants were analysed by AF4 (see below).

AF4 instrumentation and operation

The AF4 experiments and data collection were performed using an AF2000 MT instrument and Postnova AF2000 software (Postnova Analytics, Landsberg, Germany) as previously described (Eskelin et al. 2016). Teflon spacers of 250 or 350 μm and regenerated cellulose (RC) membranes with a molecular weight cut-off (MWCO) value of 100 kDa (Postnova) were used. For HRPV-1, RC membranes with a 10 kDa MWCO were also used. The injection volume varied from 20 to 5000 μl . The outlet flow was monitored in volts (V) at 260 nm using a UV detector equipped with a preparative flow cell (Shimadzu SPD-20A; Shimadzu, Kyoto, Japan). UV detector sensitivity settings were adjusted according to the input sample absorbance value.

AF4-based virus purifications were performed at 22 °C using the virus-specific buffers as mobile phases and the previously described method (Eskelin et al. 2016). AF4 purification was done in two separate steps: focusing and elution. Sample was injected during the focusing step during of which two opposing lateral flows (each 0.2 ml/min) were applied to enable equilibration of the sample components based on their hydrodynamic properties. This step also concentrates the sample components to a narrow zone close to the injection site. Here the used focusing time varied from 5 to

15 min, depending on the expected polydispersity and protein concentration of the input sample. As good recoveries of infectious viruses were obtained and the intensity of the void peak was low for 1x viruses, where the highest sample loads were used, the focusing time was not further optimised. During elution, a 15-25 min linear cross-flow gradient from 1 ml/min to 0.05 ml/min was applied, followed by a constant cross-flow of 0.05 ml/min. Channel flow was 0.2 ml/min. For the HCIV-1 agar stock, the elution gradient was extended with a linear cross-flow phase of 10 min. Undiluted culture supernatants and diluted PEG and 1xviruses were utilized as input samples. Fractionation of each input sample was repeated at least three times. No day-to-day variation was observed between these technical repetitions. In addition, experiments were duplicated with biological replicates. Fractions (0.5-1.0 ml) were collected from the beginning of elution phase and stored at +4°C.

Multi-angle light scattering (MALS) data was collected as previously described (Eskelin et al. 2016) using a channel flow of 0.5 ml/min for elution. A 25 min linear crossflow gradient was applied starting from 1.0 ml/min to 0.28 ml/min, followed by a 15-min exponential step to a final crossflow rate of 0.05 ml/min. The radius of gyration (R_g) was calculated from the measured MALS signal intensities by applying an intensity distribution function $P(\theta)$ as previously described (Eskelin et al. 2016). Where sphere fit model was applicable, the geometric diameter (D_{geo}) of virus particles was calculated from the obtained R_g -values using the formula $D_{geo}=2 \times R_g / 0.775$. Conversion of R_g - into D_{geo} - values was not performed where MALS data fitting via random coil model gave a better fit than the sphere model.

Analyses of virus infectivity, protein amount, virus purity and yield

Protein and DNA concentrations were estimated from A_{260} values (Eppendorf Photometer, Hamburg, Germany). The number of infectious viruses (plaque forming units, PFU) was determined by plaque assay. Recoveries (%) of protein and infectious viruses were calculated from protein amounts or PFU using the following formulas: $[100\% \times (A_{260, \text{fraction}}) / A_{260, \text{input}}]$ or $[100\% \times (PFU_{\text{fraction}}) / PFU_{\text{input}}]$. Specific

infectivity was calculated as PFU/A₂₆₀. For AF4 purifications, the values were calculated for the fraction that had the highest number of infectious viruses (Tables 2-4). Improvement in purity was determined as the ratio of specific infectivity of the virus-containing AF4 fraction and the corresponding input sample. Obtained values from AF4 purification were also compared to the corresponding values from the ultracentrifugation-based purification methods (Table S2, Table 1 and references therein).

To assess virus purity, collected fractions or dilute input samples (culture supernatants) were precipitated with 10% trichloroacetic acid (TCA, v/v) on ice for 30 min, collected by centrifugation (Eppendorf centrifuge 5415D, 16,000 x g, 30 min, 4°C) and resuspended in 1.5x SDS-PAGE sample buffer. Boiled samples were analysed in 14% or 16% glycine or tricine sodium dodecyl sulphate (SDS) polyacrylamide (SDS-PAGE) gels (Olkkonen and Bamford 1989; Schagger and Von Jagow 1987). Proteins resulting from the precipitation of equal volumes of fractions were analysed in gels, unless otherwise stated. Gels were stained using Coomassie blue and documented using ChemiDoc (Bio Rad, Hercules, USA).

Transmission electron microscopy (TEM)

Samples (2 µl) were adsorbed on copper pioloform-coated grids at room temperature for 1 min. Samples were negatively stained with 1% (w/v) ammonium molybdate (pH 7.0) for 1 min. Input samples (1×HRPV-1 or PEG-HRPV-1) were diluted ten-fold in the virus-specific buffer before staining. Stained samples were visualised by a JEOL 1400 transmission electron microscope (Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki) operating at 80 kV acceleration voltage.

Results

Effect of ionic strength on AF4 separation

In AF4, elevated ionic strength results in a reduced thickness of the electrical double layer and thus a closer contact of the sample components with the membrane and therefore its effect on retention and yield depends on the nature of the sample components as repulsive or attractive interactions may take place (Benincasa and Caldwell 2001; Neubauer et al. 2011; Wagner et al. 2014). The structural components of the studied viruses were expected to be negatively charged at neutral pH (Demina et al. 2016; Pietilä et al. 2009). We first addressed AF4 performance in low (2 mM), medium (0.6 M) and high (1.6 M) ionic strength conditions using standard proteins (150-669 kDa) with acidic pIs (4.4-5.6) (Fig. S1). Two membrane-spacer combinations were used: (i) 100 kDa RC and 350 μ m spacer, and (ii) 10 kDa RC and 250 μ m spacer. We observed that increased ionic strength resulted in longer retention times, irrespectively of the membrane-spacer combination used. In addition, increased salinity in the AF4 mobile phase and the use of the 350 μ m spacer and the 100 kDa RC membrane resulted in peak broadening that was more evident for the 447 and 669 kDa proteins than for the 225 kDa protein (Fig. S1a). For the smallest 150 kDa protein analysed, no UV signal was obtained with the 0.6 M ionic strength mobile phase (Fig. S1b). When the 250 μ m spacer and the 10 kDa RC membrane were used, no band broadening at high ionic strength was observed for the tested proteins (Fig. S1b). Finally, we tested elution of 66 MDa bacteriophage PRD1 using the three different mobile phases, the 10 kDa RC and the 250 μ m spacer (Fig. S1c). The used salinities did not affect virus infectivity (data not shown). The retention time increased at the highest ionic strength mobile phase, but the salinity had no effect on the broadness of virus peaks that eluted as relatively broad zones. Thus, we concluded that AF4 was compatible for virus studies using high ionic strength buffers as a mobile phases. However, depending on the nature of the studied samples, the separation could be compromised due to band broadening and salinity-induced conformational changes.

Lemon-shaped virus His1

AF4 fractograms of the His1 culture supernatant, PEG-precipitated His1 and ultracentrifugation-purified 1×His1 were unique for each sample. The second peak was found in all three input samples (Fig. 2a). These peaks eluting from 28 min onwards contained the majority of infectious viruses (Fig. 2b and c). However, the small discrepancy in the retention times of the virus-containing peaks suggested that the average hydrodynamic sizes of the sample components and the amount of viruses compared to other large-sized macromolecular complexes were slightly different. The fraction with the highest amount of infectious particles was used for calculations of the specific infectivity and yield (Table 2) and for comparisons with the ultracentrifugation purification method (Tables S1 and S2). EM studies have shown that the lemon-shaped virions represent the majority in His1 preparations purified to high homogeneity, but also some larger particles are occasionally observed (Pietilä et al. 2013a). These larger subtypes and the flexible nature of the His1 virion can explain some of the right-side tailing of virus peaks in the fractograms.

The average specific infectivity for AF4-purified His1 culture supernatant was $\sim 4 \times 10^{11}$ PFU/A₂₆₀, which was ~24-fold higher than that of the input sample. The recovery of infectious viruses was ~50% (Table 2). In the case of PEG-His1, the peak fraction contained ~47% of the input virus and the specific infectivity was $\sim 2 \times 10^{11}$ PFU/A₂₆₀ resulting in a ~three-fold improvement. For 1×His1, ~50% of infectious viruses were recovered, but no change in the specific infectivity was observed (Table 2, Fig. 2f). The rate-zonal ultracentrifugation purification of His1 resulted in ~20% yield and specific infectivity of $\sim 4 \times 10^{11}$ PFU/A₂₆₀ (Table S2). SDS-PAGE gel analysis of the AF4-purified His1 culture supernatant showed that most impurities were successfully removed from the virus peak (Fig. 2d and 2e). If needed, the remaining contaminating proteins can be removed by additional

purification steps as demonstrated here for PEG-His1 sample using a tandem purification utilizing AF4 coupled with CsCl equilibrium ultracentrifugation (Fig. 2e).

Icosahedral tailed virus HVTV-1

The AF4 fractograms of HVTV-1 culture supernatant, PEG-HVTV-1 and 1×HVTV-1 showed two major peaks with different intensities (Fig. 3a). For 1×HVTV-1, the highest signal intensity was observed for the second peak (eluting from 25 min), which contained the infectious viruses (Fig. 3b). Culture supernatant and PEG-HVTV-1 had peaks at similar positions (Fig. 3a). From the peak fractions, ~40-50% of the input virus was recovered from the culture supernatant and PEG-HVTV-1, whereas ~70% recovery was observed for 1×HVTV-1 (Fig. 3b, Table 3). The best ~60-fold improvement in the specific infectivity was observed for the AF4-purified culture supernatant (Table 3). For PEG and 1×input samples, the specific infectivities ($\sim 7 \times 10^{10}$ PFU/A₂₆₀ and $\sim 2 \times 10^{11}$ PFU/A₂₆₀) were improved approximately eight-fold and five-fold, respectively. Comparison of protein patterns of the input samples and AF4 -purified virus verified the observed improvement in purity (Fig. 3c and Fig. S2). The rate zonal ultracentrifugation method resulted in ~3% recovery of the infectious virus with specific infectivity of $\sim 3 \times 10^{10}$ PFU/A₂₆₀ (Table S2).

Pleomorphic vesicle-like HRPV-1 virus

All three HRPV-1 input samples separated as two major peaks that differed in their intensity and shape (broadness) (Fig. 4a). The peak that started to elute at ~25 min was the most intense in 1× HRPV-1 sample and contained the highest number of infectious viruses (Fig. 4b). Approximately ~30% of infectious viruses was recovered in the peak fraction. Approximately 40% and 90% recoveries were obtained for the AF4-purified culture supernatant and PEG-HRPV-1, respectively (Fig. 4b, Table 4). The specific infectivity values for the AF4-purified culture supernatant, PEG-HRPV-1 and 1×HRPV-1 were $\sim 4 \times 10^{11}$, $\sim 7 \times 10^{11}$ and $\sim 1 \times 10^{12}$ PFU/A₂₆₀, respectively. The best ~60-fold improvement in purity was obtained for the culture supernatant. For the AF4-purified PEG-

HRPV-1, the specific infectivity was improved ~3-fold, whereas for 1×HRPV-1 no significant improvement was observed (Table 4). The average recovery of infectious viruses purified by rate-zonal ultracentrifugation was ~11% and specific infectivity $\sim 5 \times 10^{11}$ PFU/A₂₆₀ (Table S2).

SDS-PAGE gel analysis verified the presence of two major HRPV-1 structural proteins VP4 and VP3 (Pietilä et al. 2009) in the fractions enriched with infectious viruses, whereas the majority of host-derived proteins were found in the first fractions (Fig. 4c, Fig. S3). TEM analysis of the AF4 virus fractions showed that in addition to viruses of ~40-50 nm in diameter, some filamentous material was also present (Fig. 4d).

The effectiveness of the fractionation process of polydisperse specimens can be easily assayed by reinjecting collected fractions into the AF4 for a second run (Giddings and Yang 1985) or by using dilute samples (Fig. S4a). Re-injection of the AF4-purified PEG-HRPV-1 fractions (Nos 5-8; Fig. 4a) resulted in two separate peaks showing that it contained two different infectious virus particle populations with different hydrodynamic properties (Fig. S4a-b). Treatment of the sample by proteinase K resulted in a fractogram, where a single peak with a shorter retention time (indicating a smaller average size) was observed (Fig. S4c). Such a treatment produces HRPV-1 particles that lack the VP4 spike protein regions that protrude from the membrane vesicles (Pietilä et al. 2010). Our results indicate that the two virus populations observed in the non-treated sample had different spike protein compositions. The spikes are known to be organized randomly on the membrane vesicle (Pietilä et al. 2012). Proteinase K eluted along with the first peak immediately after the focus flow was switched off (Fig. S4c).

The size distribution of the components in the PEG-HRPV-1 and 1×HRPV-1 samples was analysed by AF4 instrument coupled with MALS detection (Fig. 5). For both samples, the first peak (~24 to 32 min) showed the same R_g estimate of ~15 nm and indicated that the particles were relatively homogenous in size compared to each other (Fig. 5b and c). The corresponding intensity distribution

profile showed good agreement with the applied spherical model (Fig. S5). Conversion of the obtained R_g into geometric diameter yielded estimates of ~39 nm. In contrast, the second PEG-HRPV-1 peak eluting from ~32 min onwards contained sample components that were on average larger than those of 1×HRPV-1. The obtained estimates for R_g using random coil model were 89 and 55 nm, respectively.

Icosahedral inner-membrane containing virus HCIV-1

To expedite virus production, we tested the applicability of virus agar stocks (specific infectivity $\sim 4 \times 10^9$ PFU/A₂₆₀) as input samples for AF4. We utilised HCIV-1 as a model (Table 1). AF4 fractograms for HCIV-1 agar stock were spiky and no base line separation was obtained, but two major peaks were observed (Fig. 6a). The second peak eluting from ~27 min onwards contained the majority of infectious viruses (Fig. 6b). For the peak fraction, the yield of infectious viruses was ~35% and the corresponding specific infectivity was $\sim 3 \times 10^{10}$ PFU/A₂₆₀ resulting in ~8-fold improvement in HCIV-1 purity. Comparison of protein patterns of fractions to the input sample and 1x purified HCIV-1 verified the observed improvement in specific infectivity for AF4 fractionated virus, but revealed that several impurities were still present (Fig. 6c).

His1 virus production

Optimisation of virus production and purification as well as analysis of the outcome in slowly growing halophilic microbes is laborious and time-consuming. Here, we studied whether AF4 could be used to analyse progeny virus production in growing cells using His1 virus and *Har. hispanica* host strain as a model (Fig. S6). The fractogram for uninoculated MGM growth medium had a single high intensity peak that eluted immediately after the focus flow was switched off (Fig. S6a).

Uninfected host culture (control) had the same void peak that was followed by an additional lower intensity peak (elution 8-12 min; Fig. 6a). The fractograms for the samples taken immediately after infection (0 h.p.i.) or at 6 h.p.i. (Fig. S6b) did not differ from those of uninfected controls (Fig. S6a).

Consequently, the measured virus concentrations of $\sim 2 \times 10^9$ PFU/ml (0 h.p.i.) and $\sim 7 \times 10^8$ PFU/ml (6 h.p.i.) were below the AF4 detection limit. The 24 and 48 h.p.i. samples from His1-infected culture had an additional peak (elution 12-18 min; Fig. S6b). Quantitation of infectious particles showed that the majority of viruses ($\sim 2\text{-}3 \times 10^{11}$ PFU/ml) were in this peak (Fig. S6c).

Discussion

Systematic searches for halophilic viruses and the available methods to cultivate halophilic microbes have rapidly increased the number of described haloarchaeal viruses (Atanasova et al. 2015a; Atanasova et al. 2016; Atanasova et al. 2015b; Atanasova et al. 2012; Luk et al. 2014). Detailed genetic, biochemical and structural characterisations are nevertheless lacking for most isolates. Consequently, rapid native purification and characterisation methods would enhance the knowledge on haloarchaeal viruses. High salinity requirements bring additional challenges to such attempts. For instance, traditional chromatography typically utilizes low ionic strength buffers for sample binding and high ionic strength buffer for elution. AF4 has been successfully used for assaying the quality and quantity of viruses and VLPs (Chen et al. 2015; Chuan et al. 2008; Eskelin et al. 2016; Lang et al. 2009; Somasundaram et al. 2016; Wei et al. 2007). However, in most cases purified specimens have been used.

We applied AF4 for purification of four haloarchaeal viruses that required high total molar ionic strengths (0.6 and 1.6 M) to maintain biological activity (Demina et al. 2016; Pietilä et al. 2013a; Pietilä et al. 2010; Pietilä et al. 2013b). Previous studies on the effects of salt in AF4 mobile phases are limited to relatively low ionic strength (75-150 mM) (Benincasa and Caldwell 2001; Neubauer et al. 2011; Wagner et al. 2014). By comparing protein and virus elution in AF4 in low (2 mM), medium (0.6 M) and high (1.6 M) ionic strength mobile phases, we concluded that AF4 is applicable to high ionic strength buffers. However, high salinity increased retention times. In addition, the separation was affected by the used spacer thickness, membrane pore size and properties of the sample components.

AF4 was utilized to purify His1, HVTV-1 and HRPV-1 using culture supernatants, PEG precipitated viruses and ultracentrifugation purified 1×viruses as input samples. The best increase in purity was observed for culture supernatants, where the specific infectivity values of the virus-containing AF4

fractions were improved ~24, ~60, ~64-fold for His1, HVTV-1 and HRPV-1, respectively. The AF4 purified HCIV-1 agar stock gave ~8-fold higher specific infectivity. The obtained specific infectivity values of the AF4 purified His1, HVTV-1 and HRPV-1 were high: $\sim 1 - 4 \times 10^{11}$ PFU/A₂₆₀. They were comparable to the reported values for His1, HVTV-1, and HRPV-1 ($\sim 4 \times 10^{11}$, $\sim 6 \times 10^{10}$, and $\sim 1 \times 10^{12}$ PFU/A₂₆₀) that were purified to near homogeneity using rate-zonal, equilibrium and differential ultracentrifugations (Pietilä et al. 2013a; Pietilä et al. 2010; Pietilä et al. 2013b). In these previous reports, the purities were assayed using denaturing protein gel analysis and cryo-EM or TEM coupled with negative staining showing that samples with such specific infectivities can be considered as high purity. For HCIV-1 agar stocks, the obtained purity after AF4, $\sim 3 \times 10^{10}$ PFU/A₂₆₀, deviated most from that reported for specimens purified to near homogeneity using ultracentrifugation-based methods ($\sim 1 \times 10^{12}$ PFU/A₂₆₀) (Demina et al. 2016). In general, various pre-treatments prior to AF4 could further improve separation and purity. For example, removal of host DNA and RNA by nuclease treatment improved separation of sample components of bacteriophage PRD1 lysate and PEG precipitate (Eskelin et al. 2016). Also further optimisation of the cross-flow and channel flow rates, their ratio and cross-flow gradient type could improve separation. For instance, elution of virus particles under the influence of higher cross flow force would prevent the potential mixing of the infectious viruses with the well-retained compounds that elute when the cross-flow force is low. In addition, minimisation of the time for focusing and elution would result in shorter running times.

PEG in the presence of NaCl selectively precipitates high molecular weight complexes (Yamamoto et al. 1970). The specific infectivity values for PEG-precipitated His1, HVTV-1 and HRPV-1 samples were ~5-32 -fold higher than those of the corresponding culture supernatants (Tables 2-4) showing that PEG precipitation results in significant removal of impurities from virus preparations. By AF4, 3 to 8 -fold increments in specific infectivities were obtained for the PEG precipitated viruses. However, the obtained specific infectivity values for the AF4-purified PEG-viruses were

comparable with those of culture supernatants. For 1×viruses, AF4 purification of HVTV-1 resulted in a significant ~5-fold improvement, whereas the purities of 1×His1 and 1×HRPV-1 were not affected. For AF4-purified bacteriophage PRD1, the best increment in purity was also obtained for culture supernatants and PEG-PRD1, whereas 1×PRD1 was not further purified by AF4 (Eskelin et al. 2016). To our knowledge no such data exists for other virus systems. Interestingly, PEG- and 1x HRPV-1 eluted as two distinct particle populations that were of different sizes but were nevertheless infectious. AF4 analysis of the proteinase K –treated HRPV-1 subpopulations suggested that they differed in their spike compositions. The obtained R_g estimate for HRPV-1 particles in the first peak was ~39 nm (Fig. 5b-c). The average diameter based on cryo-electron tomography of HRPV-1 is 41 nm (Pietilä et al. 2012).

Multistep ultracentrifugation purifications are often associated with reduced yields. For the four studied haloarchaeal viruses, ~13-17% yields have been reported (Demina et al. 2016; Pietilä et al. 2013a; Pietilä et al. 2010; Pietilä et al. 2013b). Here, the obtained yields from the rate-zonal and differential centrifugation purifications ranged from ~3-20% (Table S2), whereas those from AF4 purifications were significantly higher (~30-90%, Tables 2-4). Increased ionic strength did not affect AF4 separation, as the yields were comparable to those reported for PRD1 virus (~50-100%), which was purified by AF4 using low 2 mM ionic strength (Eskelin et al. 2016). Notably, protein contents (Figs. 3-4, 6) and specific infectivity values of several consecutive virus-containing fractions of HVTV-1, HRPV-1 and HCIV-1 were similar (Tables 3, 4) and the total yields could be further improved by pooling fractions. In contrast, careful fractionation for His1 was required to achieve the best purity (Fig. 2d). In practice total yields of infectious viruses from a single AF4 fractionation experiment of 1-5 ml of culture supernatant were low. However, higher input sample volumes or repeated fractionations can be utilized. Injection volumes up to 1 L have been reported for dilute samples (Lee et al. 1998). Alternative methods towards preparative AF4-purifications include increased channel dimensions (thicker or wider spacers) (Bria et al. 2017). Sample dilution during

AF4 is a known drawback of the method. In our study, the highest dilution was observed for the AF4 fractionated 1xviruses and PEG-viruses, whereas little (~2-fold) or no dilution was observed for the culture supernatants. If needed, dilution can be reduced by using thinner spacers, miniaturized channel design or a slot flow option, where the sample-free buffer zone and the sample-containing zone are separately collected (Müller et al. 2015; Prestel et al. 2006).

We recently showed that AF4 provided a rapid method for monitoring the progress of PRD1 infection in gram-negative bacterium *Salmonella* (Eskelin et al. 2016). AF4-MALS has also been applied for the quantitation of influenza virus in mammalian cell culture supernatants (Bousse et al. 2013). Here we showed that the AF4 can be utilised for real-time monitoring of archaeal His1 virus production at late stages of infection. A rapid one-hour assay is a benefit when compared to traditional plaque assays that take several days for His1. Furthermore, for viruses, which exit the cell by non-lysing manner and the progress of infection does not manifest as significantly decreased culture turbidity, the visualisation and quantitation of virus production from the AF4 fractograms is a clear methodological advancement. The early stages of His1 infection could not be studied by AF4 with the current instrumental set up having a preparative UV cell. For PRD1 the detection limit was $\sim 5 \times 10^9$ PFUs (Eskelin et al. 2016). However, such detection limitations can be overcome by injecting more sample or by utilising an analytical UV cell offering higher sensitivity. For influenza virus, the detection limit of $\sim 8 \times 10^8$ PFU/ml ($\sim 2 \times 10^6$ particles) with AF4 equipped with an analytical UV cell has been reported (Bousse et al. 2013).

In conclusion, we showed that AF4 purification enables rapid one-step purification of infectious viruses from crude samples and can be applied for broad range of viruses with different morphology, size and outer surface chemistry. Furthermore, we showed that AF4 is amenable for purification of specimens in high ionic strength conditions. The obtained purity for AF4 purified samples was

comparable to that obtained by multistep purification method including PEG-precipitation rate-zonal and differential ultracentrifugations. However, the obtained yields for infectious viruses were higher in AF4. We also showed that AF4 is not only suitable for virus purification in challenging buffer conditions, but also applicable for studies on their composition as shown here for the protease K treated HRPV-1. As AF4 separation channel allows fractionation at +5 to +90 °C, it can potentially be utilized on studies of psychrophilic and hyperthermophilic macromolecular complexes such as viruses of thermophilic archaea or bacteria.

Acknowledgements

The authors acknowledge the University of Helsinki and Academy of Finland (grant 306833) for support provided to Centre for Virus and Macromolecular Complex Production (ICVIR, University of Helsinki), part of Instruct-FI, used in this study. This study was supported by Academy Professor (Academy of Finland) funding grants 283072 and 255342 (D.H.B.). We also acknowledge Academy of Finland grant 272507. The authors thank Päivi Suorsa, Helin Veskiväli and Sari Korhonen for excellent technical assistance, and Salla Välipakka for assistance in the His1 work.

FIGURE LEGENDS

Fig. 1

Virus purification workflow. Purity was evaluated by measuring absorbance values (A_{260}/ml), the number of infectious viruses (PFUs), specific infectivity (PFU/ A_{260}) and by Coomassie-stained glycine or tricine SDS-PAGE gels. For technical details, see materials and methods and Table S1.

Fig. 2

AF4 purification of lemon-shaped His1 virus. **(a)** Representative AF4 fractograms of His1-infected *Har. hispanica* culture supernatant (blue), PEG-His1 (red) and 1×His1 (green). Cross-flow gradient is shown with the dashed black line. UV range setting for culture supernatant was 0.001 and 0.01 for PEG-His1 and 1×His1, respectively. The 350 μm spacer and RC membrane with MWCO of 100 kDa was used. **(b-c)** Absorbance measurements (lines) and analysis of infectious viruses (columns) of input samples and AF4-fractionated **(b)** culture supernatant and PEG-His1 as well as **(c)** 1×His1. Averages and bars representing standard deviation are shown. **(d)** Protein content of the culture supernatant (input), AF4 fractions and 1×His1. **(e)** Protein content of PEG-His1 (input), AF4-purified His1 (fractions 4-7 pooled) and tandem purification of PEG-His1 by AF4 and CsCl equilibration centrifugation. **(f)** Protein content of AF4-fractionated 1×His1. Proteins in **d-f** were analysed in tricine SDS-PAGE gels. Positions of His1 major structural proteins VP21, its lipid-modified form (asterisks) as well as minor protein VP27 are shown. Molecular mass marker (M1) sizes are indicated in kilodaltons (kDa).

Fig. 3

AF4 purification of icosahedral tailed virus HVTV-1. **(a)** Representative fractograms of purification of culture supernatant (blue), PEG-HVTV-1 (red) and 1×HVTV-1 (green). Cross-flow gradient is shown with the dashed black line. The 250 μ m spacer and RC membrane with 100 kDa MWCO was used. **(b)** Absorbance measurements (lines) and number of infectious viruses (columns) of fractions. Averages and bars representing standard deviation are shown. **(c)** Protein content of AF4-fractionated PEG-HVTV-1, input sample and 1×HVTV-1 in tricine SDS-PAGE gels. Positions of HVTV-1 major structural proteins are shown with asterisks; gp refers to gene product. Molecular mass markers (M) are indicated in kilodaltons (kDa).

Fig. 4

AF4 purification of pleomorphic HRPV-1 virus. **(a)** Representative fractograms of the culture supernatant (blue), PEG-HRPV-1 (red) and 1×HRPV-1 (green). Cross-flow gradient is shown with the dashed black line. **(b)** Absorbance measurements (lines) and virus content (columns) of fractions. Averages and bars representing standard deviation are shown. **(c)** Analysis of protein content of AF4-fractionated culture supernatant by tricine SDS-PAGE. For fractions 1 and 2, half of the sample volume was loaded. Positions of HRPV-1 major structural proteins (VPs) are indicated. Molecular mass markers (M) are indicated in kilodaltons (kDa). **(d-e)** TEM pictures from the AF4-purified **(d)** culture supernatant and **(e)** PEG-HRPV-1.

Fig. 5

Size distribution of PEG-HRPV-1 and 1×HRPV-1 sample components. **(a)** Representative fractograms of the AF4 analyses of PEG-HRPV-1 (red, right y-axis) and 1×HRPV-1 (green, left y-axis). The elution program included a 25-min linear elution gradient that was followed by exponential decrease to 0.05 ml/min; V_{out} was 0.5 ml/min (dashed black line). **(b-c)** Light scattering signal (LS90°, left y-axis) and size distribution of sample components (R_g ; right y-axis) of **(b)** PEG-HRPV-1 and **(c)** 1×HRPV-1. R_g values were determined using a sphere model for the particles eluting between 26-30 min and a random coil model for molecules eluting between 33 and 47 min.

Fig. 6

AF4 purification of icosahedral internal membrane-containing HCIV-1. **(a)** Representative fractogram of HCIV-1 agar stock. Cross-flow gradient is shown with the dashed black line. Note the extension of the elution gradient with a linear cross-flow phase of 10 min. The 250 μ m spacer and RC membrane with 100 kDa MWCO was used. **(b)** Absorbance measurements (lines) and virus content (bars) of fractions. **(c)** Analysis of protein content of fractions (F) in a tricine SDS-PAGE gel. Positions of HCIV-1 major structural proteins (VPs) are indicated. Molecular mass markers (M) are indicated in kilodaltons (kDa).

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Table 1. Characteristics of the studied haloarchaeal viruses.

Virus and its family	Host strain	Mode of infection	Virion morphotype**	Virion characteristics	Specific infectivity***	Genome	References
His1 virus <i>Fuselloviridae</i> *	<i>Haloarcula hispanica</i> ATCC 33960	Persistent infection without host lysis.		Lemon-shaped particles (~44×77 nm) with short tails (~7 nm). Elastic proteinaceous virions with lipid-modified major coat protein. Particle density 1.39 g/ml (CsCl).	~4×10 ¹¹ PFU/A ₂₆₀	Linear dsDNA ~17 kb.	Bath and Dyall-Smith, 1998, Bath et al., 2006, Pietilä et al., 2013, Hong et al., 2015
<i>Haloarcula vallismortis</i> tailed virus 1 (HVTV-1)	<i>Haloarcula vallismortis</i> ATCC 29715	Host cell lysis.		Icosahedral proteinaceous head (~76×96 nm) and non-contractile tail (~73 nm). Particle density 1.47 g/ml (CsCl).	~3×10 ¹² PFU/mg of protein or 6×10 ¹⁰ PFU/A ₂₆₀	Linear dsDNA genome ~102 kb.	Atanasova et al., 2012, Pietilä et al., 2013
<i>Halorubrum</i> pleomorphic virus 1 (HRPV-1), <i>Pleolipoviridae</i>	<i>Halorubrum</i> sp. PV6	Persistent infection without host lysis.		Pleomorphic enveloped virus (~44×55 nm) with embedded protein spikes organised in random fashion. Glycosylated spike proteins. Particle density 1.29 g/ml (CsCl).	~3×10 ¹³ PFU/mg of protein or ~1×10 ¹² PFU/A ₂₆₀	Circular ssDNA ~7 kb.	Pietilä et al., 2009, Pietilä et al., 2012.
<i>Haloarcula californiæa</i> icosahedral virus 1 (HCIV-1), <i>Sphaerolipoviridae</i>	<i>Haloarcula californiæa</i> ATCC 33799	Host cell lysis		Icosahedral proteinaceous virions (~70 nm) and inner membrane. Particle density 1.3 g/ml (CsCl).	~1×10 ¹² PFU/mg of protein or ~1×10 ¹² PFU/A ₂₆₀	Linear dsDNA ~31 kb.	Demina et al., 2016.

* Not officially approved by the ICTV.

** Not to scale

*** Specific infectivity values are given to specimens purified to high homogeneity through rate-zonal and equilibration centrifugation referred to as 2×purified virus.

Table 2. AF4 purification of culture supernatant of His1-infected *Har. hispanica*, PEG-His1 and sucrose gradient-purified 1×His1. Input samples and AF4 fractions having the highest number of infectious viruses were compared. Averages ± standard deviation from three technical repetitions are shown.

	Culture supernatant		PEG-His1		1× His1	
	Input	AF4: fraction 5	Input	AF4: fraction 5	Input	AF4: fraction 8
Volume (ml)	1.0	0.8	1.0**	0.8	0.1**	0.5
PFU/ml	9.8x10 ¹⁰	7.9x10 ¹⁰	7.1x10 ¹¹	4.2x10 ¹¹ ±1.1x10 ¹¹	4.3x10 ¹³	4.0x10 ¹² ±5.0x10 ¹¹
PFU	9.8x10 ¹⁰	6.3x10 ¹⁰ ±1.9x10 ¹⁰	7.1x10 ¹¹	3.3x10 ¹¹ ±8.5x10 ¹¹	4.3x10 ¹²	2.0x10 ¹² ±2.5x10 ¹¹
PFU yield (%)	100	51±10	100	47±12	100	47±5.8
A ₂₆₀ /ml	6.7*	0.16±0.02	9.8	2.2±0.3	61.1	6.4±0.5
A ₂₆₀ units	6.7*	0.13±0.01	9.8	1.7±0.2	6.1	3.2±0.3
A ₂₆₀ yield (%)	100	2.0±0.2	100	18±6.4	100	53±4.4
PFU/A ₂₆₀	1.5x10 ¹⁰	3.6x10 ¹¹ ±5.4x10 ¹⁰	7.3x10 ¹⁰	1.9x10 ¹¹ ±7.4x10 ¹⁰	7.0x10 ¹¹	6.1x10 ¹¹ ±2.6x10 ¹⁰

* The average A₂₆₀/ml value for MGM growth medium was 5.9 when measured against buffer.

** Input sample volume was adjusted to 1 ml or 0.1 ml by adding virus-specific buffer. PEG-His1: 200 µl sample + 800 µl buffer; 1×His1: 50 µl sample + 50 µl buffer.

Table 3. AF4 purification of HVTV-1 culture supernatant, PEG-HVTV-1 and sucrose gradient-purified 1×HVTV-1. Input samples and AF4 fractions having the highest number of infectious viruses were compared. Averages ± standard deviation from three technical repetitions are shown.

	Culture supernatant		PEG-HVTV-1		1×HVTV-1	
	Input	AF4: fraction 5	Input	AF4: fraction 5	Input	AF4: fraction 6
Volume (ml)	1.0	0.8	0.1**	0.8	0.1	0.8
PFU/ml	3.9x10 ¹⁰	2.0x10 ¹⁰ ±3.5x10 ⁹	9.2x10 ¹¹	2.8x10 ¹⁰ ±9.8x10 ⁹	3.2x10 ¹²	2.8x10 ¹¹ ±7.1x10 ¹⁰
PFU	3.9x10 ¹⁰	1.6x10 ¹⁰ ±2.8x10 ⁹	4.6x10 ¹⁰	2.2x10 ¹⁰ ±7.9x10 ⁹	3.2x10 ¹¹	2.3x10 ¹¹ ±5.7x10 ¹⁰
PFU yield (%)	100	42±7.2	100	48±17	100	71±18
A ₂₆₀ /ml	19.6*	0.17±0.03	48.4	0.38±0.04	36.6	1.96±0.21
A ₂₆₀ units	19.6	0.14±0.03	4.8	0.31±0.03	3.7	1.57±0.17
A ₂₆₀ yield (%)	100	1.0±0.2	100	6.3±0.6	100	43±4.6
PFU/A ₂₈₀	2.0x10 ⁹	1.2x10 ¹¹ ±1.7x10 ¹⁰	9.5x10 ⁹	7.2x10 ¹⁰ ±2.0x10 ¹⁰	4.4x10 ¹⁰	2.1x10 ¹¹ ±3.4x10 ¹⁰

* The average A₂₆₀/ml value for MGM growth medium was 16.6 when measured against buffer.

**Input sample volume was adjusted to 0.1 ml by adding virus-specific buffer. PEG-HVTV-1: 50µl sample + 50 µl buffer; 1×HVTV-1: 50 µl sample + 50 µl buffer.

Table 4. AF4 purification of culture supernatant of HRPV-1-infected *Halorubrum* sp. PV6, PEG-HRPV-1 and sucrose gradient-purified 1×HRPV-1. Input samples and AF4 fractions having the highest number of infectious viruses were compared. Averages $\pm$ standard deviation from three technical repetitions are shown.

	Culture supernatant		PEG-HRPV-1		1×HRPV-1	
	Input	AF4: fraction 6	Input	AF4: fraction 6	Input	AF4: fraction 6
Volume (ml)	5.0	0.8	1.0 ^{**}	0.8	1.0 ^{**}	0.8
PFU/ml	2.5×10^{10}	$5.7 \times 10^{10} \pm 1.7 \times 10^{10}$	3.5×10^{11}	$3.8 \times 10^{11} \pm 1.3 \times 10^{11}$	1.9×10^{12}	$7.7 \times 10^{11} \pm 2.0 \times 10^{11}$
PFU	1.3×10^{11}	$4.6 \times 10^{10} \pm 1.4 \times 10^{10}$	3.5×10^{11}	$3.0 \times 10^{11} \pm 1.0 \times 10^{11}$	1.9×10^{12}	$6.2 \times 10^{11} \pm 1.6 \times 10^{11}$
PFU yield (%)	100	37 $\pm$ 11	100	90 $\pm$ 46	100	32 $\pm$ 9
A ₂₆₀ /ml	4.0 [*]	0.16 $\pm$ 0.02	1.76	0.56 $\pm$ 0.004	1.1	0.68 $\pm$ 0.02
A ₂₆₀ units	19.9	0.12 $\pm$ 0.01	1.76	0.44 $\pm$ 0.004	1.1	0.54 $\pm$ 0.01
A ₂₆₀ yield (%)	100	0.6 $\pm$ 0.1	100	27 $\pm$ 2	100	54 $\pm$ 6
PFU/A ₂₆₀	6.3×10^9	$4.0 \times 10^{11} \pm 1.0 \times 10^{11}$	2.2×10^{11}	$6.7 \times 10^{11} \pm 2.3 \times 10^{11}$	1.8×10^{12}	$1.1 \times 10^{12} \pm 2.7 \times 10^{11}$

* The average A₂₆₀/ml value for non-inoculated MGM was 3.4 measured against virus-specific buffer as a blank.

**Input sample volume was adjusted to 1 ml by adding virus-specific buffer. PEG-HRPV-1: 200 μ l sample + 900 μ l buffer; 1× HRPV-1: 50 μ l sample + 1200 μ l buffer.

d) F6: culture supernatant

E) F6: PEG-HRPV-1

Supplementary figure S1. Effect of ionic strength on the elution of standard proteins and bacteriophage PRD1. **(a)** Protein elution in mobile phases of low (2 mM) and medium (0.6 M) molar ionic strength. The 350 μ m spacer and RC membrane with 100 kDa MWCO was used. UV signal intensity was measured in volts (V) at 260 nm (left y-axis, alcohol dehydrogenase on the right y-axis). Cross-flow gradient is shown with the dashed black line (right y-axis in **b**). **(b)** Protein elution in low, medium and high (1.6 M) molar ionic strength. The 250 μ m spacer and 10 kDa RC membrane was used. **(c)** Elution of the 66 MDa PRD1 particle in different ionic strength mobile phases. Experiments performed with the 250 μ m spacer and the 10 kDa RC membrane are shown in blue. Fractogram for the 350 μ m spacer and 100 kDa RC membrane is shown in black. **(d-e)** Sizes (kDa), pIs and retention times (t_r) of the proteins in different ionic strength: the 350 μ m spacer and 100 kDa RC in **d** and the 250 μ m spacer and 10 kDa RC membrane in **e**.

Supplementary figure S2. AF4 purification of HVTV-1 virus. Protein content analysis of AF4-fractionated HVTV-1 culture supernatant by tricine SDS-PAGE. See Fig. 3A for the fractogram. HVTV-1 major structural proteins are marked with asterisks. Gp refers to gene product. Molecular weight markers (M) are indicated in kilodaltons (kDa).

Supplementary figure S3. Protein content of HRPV-1 input samples and AF4 fractions. Fractions from AF4 purification of **(a)** PEG-HRPV-1 and **(b)** 1xHRPV-1 were analysed by tricine SDS-PAGE. For PEG-HRPV-1 fractions, equal volumes of resuspended protein precipitates were loaded into gel. For 1xHRPV-1 fractions 5-7, half the volume of the resuspended AF4 fractions were loaded. Positions of HRPV-1 major structural proteins (viral proteins; VPs) are shown. Sizes of molecular mass marker proteins (M) are indicated in kilodaltons (kDa). See Fig. 4A for the corresponding fractograms.

Supplementary figure S4. Refractionation and proteinase K treatment of PEG-HRPV-1. **(a)** Virus-containing fractions 5-8 collected from AF4 analysis of PEG-HRPV-1 were re-injected (100 μ l) to AF4 (see Fig. 4A for the original fractogram) and compared to the ten-fold diluted input sample. Channel flow was 0.2 ml/min. **(b)** Tricine SDS-PAGE analysis of the protein content of refractionated fractions. 1xHRPV-1 is shown as a control and its major structural proteins (VP4: 44 kDa and VP3: 11 kDa) are indicated. **(c)** AF4 purified PEG-HRPV-1 was treated with proteinase K prior to refractionation (blue line). Injection volume was 100 μ l. Untreated sample was analysed as a control (red line). AF4 operation conditions were as in **a**.

Supplementary figure S5. Obtained spherical (a, c) and random coil fits (b, d) for PEG-HRPV-1 (a-b) and 1xHRPV-1 (c-d). Spherical fit showed good agreement with the model at 29 min. Random coil fit at 40 min is shown.

c)

	Time (min)	Volume (ml)	A_{260}	A_{280}	A_{260}/A_{280}	PFU/ml	PFU/ A_{260}	PFU	Yield (%)
fr1	6-8.5	0.5	0.319	0.262	1.23	1.0E+09	3.2E+09	5.1E+08	0.7
fr2	8.5-11	0.5	0.711	0.355	2.00	5.9E+08	8.3E+08	3.0E+08	0.4
fr3	11-13.5	0.5	0.265	0.140	1.90	4.7E+09	1.8E+10	2.4E+09	3.2
fr4	13.5-16	0.5	0.155	0.100	1.56	4.9E+10	3.1E+11	2.4E+10	33.2
fr5	16-18.5	0.5	0.075	0.052	1.43	1.6E+10	2.1E+11	7.8E+09	10.6
fr6	18.5-21	0.5	0.042	0.031	1.38	1.5E+09	3.6E+10	7.5E+08	1.0
input		1.0				7.3E+10		7.3E+10	

Supplementary figure S6. AF4 analysis of His1 infection in liquid culture. **(a)** Fractograms of MGM growth medium and uninfected *Har. hispanica* culture supernatant from cells grown for 24 hours. Channel flow was 0.2 ml/min. The 250 μ m spacer and RC with a 100 kDa MWCO were used. **(b)** Fractograms of His1-infected culture supernatants at the point of infection and at 6, 24 and 48 h.p.i.. Virus titers were 2.0×10^9 , 7.3×10^8 , 2.4×10^{10} and 1.3×10^{10} PFU/ml, respectively. **(c)** Number of infectious viruses and specific infectivity of fractions from the analysis of culture supernatant from 48 h.p.i. sample.