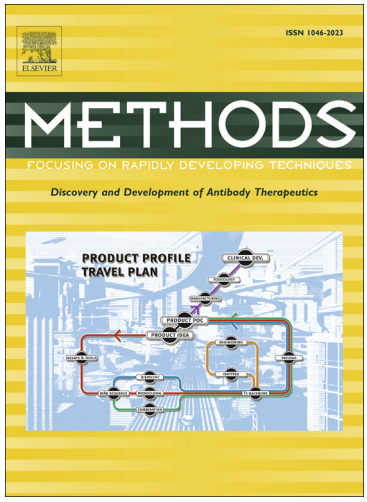
Accepted Manuscript



Characterizing Enterovirus 71 and Coxsackievirus A16 Virus-like Particles Production in Insect Cells

Balaji Somasundaram, Cindy Chang, Yuan Y. Fan, Pei-Yin Lim, Jane Cardosa, Linda Lua

PII: S1046-2023(15)30109-2
DOI: http://dx.doi.org/10.1016/j.ymeth.2015.09.023
Reference: YMETH 3804

To appear in: *Methods*

Received Date: 24 June 2015
Revised Date: 23 September 2015
Accepted Date: 24 September 2015

Please cite this article as: B. Somasundaram, C. Chang, Y.Y. Fan, P-Y. Lim, J. Cardosa, L. Lua, Characterizing Enterovirus 71 and Coxsackievirus A16 Virus-like Particles Production in Insect Cells, *Methods* (2015), doi: http://dx.doi.org/10.1016/j.ymeth.2015.09.023

Characterizing Enterovirus 71 and Coxsackievirus A16 Virus-like Particles Production in Insect Cells

Balaji Somasundaram[a,+], Cindy Chang[a,+], Yuan Y. Fan[b], Pei-Yin Lim[c], Jane Cardosa[c] and Linda Lua[a,*]

[a] The University of Queensland, Protein Expression Facility, Brisbane, QLD 4072, Australia

[b] The University of Queensland, Australian Institute for Bioengineering and Nanotechnology, Brisbane, QLD 4072, Australia

[c] Sentinext Therapeutics Sdn Bhd, Sains@USM, 10050 Penang, Malaysia

[+] Co-first authors

* Corresponding author; Email: l.lua@uq.edu.au, Phone: +61 7 334 63979

Abstract

Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16) are two viruses commonly responsible for hand, foot and mouth disease (HFMD) in children. The lack of prophylactic or therapeutic measures against HFMD is a major public health concern. Insect cell-based EV71 and CVA16 virus-like particles (VLPs) are promising vaccine candidates against HFMD and are currently under development. In this paper, the influence of insect cell line, incubation temperature, and serial passaging effect of and stability of budded virus (BV) stocks on EV71 and CVA16 VLP production was investigated. Enhanced EV71 and CVA16 VLP production was observed in Sf9 cells compared to High Five™ cells. Lowering the incubation temperature from the standard 27°C to 21°C increased the production of both VLPs in Sf9 cells. Serial passaging of CVA16 BV stocks in cell culture had a detrimental effect on the productivity of the structural proteins and the effect was observed with only 5 passages of BV stocks. A 2.7x higher production yield was achieved with EV71 compared to CVA16. High-resolution asymmetric flow field-flow fractionation couple with multi-angle light scattering (AF4-MALS) was used for the first time to characterize EV71 and CVA16 VLPs, displaying an average root mean square radius of 15 ± 1 nm and 15.3 ± 5.8 nm respectively. This study highlights the need for different approaches in the design of production process to develop a bivalent EV71 and CVA16 vaccine.

Keywords: enterovirus; virus-like particle; vaccine; insect cell; field-flow fractionation

1. Introduction

Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16) are structurally similar viruses belonging to Enterovirus genus of the *Picornaviridae* family [1]. EV71 and CVA16 are non-enveloped single-stranded RNA viruses, the causative agents of hand, foot, and mouth disease (HFMD) in children. Enteroviruses could cause severe neurological complications that can potentially be fatal. The large outbreaks of HFMD in Asia-Pacific regions and the lack of therapeutics or preventive vaccines against HFMD is a major public health concern [2, 3]. Moreover, co-circulation and co-infection by both EV71 and CVA16 viruses resulting in elevated numbers of HFMD infections were reported in China [4]. It was also reported that individuals vaccinated with EV71 vaccine candidate could still develop HFMD through CVA16 infection [5], confirming that a monovalent EV71 vaccine is not cross protective against CVA16. Hence, an effective bivalent vaccine against both viruses is needed to prevent HFMD.

Virus-like particle (VLP) vaccines have a proven track record on safety and efficacy, given the success of VLP-based vaccines against human papillomavirus [6], hepatitis B [7] and hepatitis E [8]. VLPs are macromolecular structures of viral proteins that are structurally and immunologically similar to the native virus but they lack the viral genome, thus are non-infectious [9]. Active research to develop VLP-based vaccines against enteroviruses, such as poliovirus, EV71 and CVA16, is being carried out [10].

Unlike the licensed single-protein VLP-based vaccines, VLPs of EV71 and CVA16 comprise multiple proteins and are more complex to produce recombinantly in a heterologous expression host. The native viruses of EV71 and CVA16 are non-enveloped capsids of icosahedral symmetry that are 25–35 nm in diameter [11]. The viral genome consists of three polyprotein regions, where P1 region encodes P1 polyprotein that is proteolytically processed into structural

proteins VP1, VP3 and VP0 by the 3CD protease encoded within the P2 region. These three structural proteins spontaneously assemble into a protomer. The protomers assemble into pentameric intermediates, and 12 pentamers assemble together to form the icosahedral procapsid structures. This knowledge of the native virus structure and assembly has guided enterovirus VLP design and production. Simultaneous expression of P1 polyprotein and 3CD protease in an eukaryotic expression host results in the cleavage of P1 polyprotein into VP1, VP3 and VP0 by 3CD protease [12]. P1 polyprotein is initially cleaved into VP1 and an intermediate product VP0+VP3 (Figure 1). This intermediate product is further cleaved into VP0 and VP3, leading to the spontaneous assembly of structural proteins into protomers, then to pentamers and finally into VLPs.

Following the success of insect cell-based Cervarix® (GSK) HPV VLP vaccine and FluBlok® (Protein Sciences Corporation) influenza vaccine, baculovirus expression system (BEVS) has become a commercially viable platform for vaccine production [13]. BEVS also allows efficient expression of multiple proteins simultaneously making it a powerful and popular platform for multi-protein VLP production [14, 15]. However, the structurally complex multi-protein EV71 and CVA16 VLPs bring in different production challenges [10].

In this work, the influence of insect cell line, expression temperature, serial passaging effect and storage stability of virus stocks on EV71 and CVA16 VLP production were investigated. The potency and safety of VLP-based vaccines are largely dependent on their biophysical properties, hence accurately characterizing the structural integrity of the VLPs is critical [16]. In this study, for the first time, asymmetric flow field-flow fractionation (AF4) with multi-angle light scattering detection was used for biophysical characterization of insect cell-based EV71 and CVA16 VLPs.

2. Materials and methods

2.1 Construction of transfer vectors

Human EV71 (SB12736-SAR-03) and CVA16 (SB3512/SAR/00) VLP cassettes (P1-EMCV IRES-3CD) were synthesized by DNA2.0 and subcloned into the Gateway® destination vector pDEST™ 8 by *att*L/*att*R in vitro recombination using LR Clonase® from Life Technologies (CA, USA).

2.2 Cells and virus

Spodoptera frugiperda (Sf9) and *Trichoplusia ni* (High Five™) insect cell lines were cultured in Sf-900™ II serum-free media (SFM) (Life Technologies). Cells were maintained in suspension at mid-log phase and incubated at 27^{o}C in a shaking incubator set at 120 rpm.

The recombinant *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) baculovirus for expression of the P1 polyprotein and 3CD protease under the control of polyhedrin promoter was constructed by transformation of pDEST™ 8 constructs into DH10Bac™ competent cells according to the Bac-to-Bac® (Life Technologies) manufacturer's manual. Recombinant bacmids (Bac-P1-3CD) were isolated for insect cell transfection. Sf9 cells in Sf-900™ II SFM were seeded at 2.4 x 10^5 cells/well in a 24-well microtitre plate. For optimal transfection, Cellfectin® II reagent (Life Technologies) was added to 200 ng of recombinant bacmid DNA and incubated at room temperature for 30 min before dispensing onto adherent Sf9 cells. The transfected cells were incubated at 27°C for 5 h before replacing with fresh Sf-900™ II SFM. After 7 days, spent culture medium containing passage 1 recombinant baculovirus (known as budded virus, BV) was harvested. The harvested virus stock was used as inoculum to initiate subsequent virus amplification. Sf9 cells (1 x 10^6 cells/mL) were infected with passage 1 BV stock at an estimated multiplicity of infection (MOI) of 0.1. The culture was incubated at 27^{o}C

and the cell density and viability were monitored using a Countess® automated cell counter (Life Technologies) using the 0.2% trypan blue exclusion method. Passage 2 BV stock was harvested from the culture by centrifugation at 1,500 x *g* for 5 min to remove cells. Virus titers of BV stocks were estimated based on the viable cell size method [17].

2.3 Expression Screen

Passage 4 expression was set up with both Sf9 and High Five™ cells cultured in Sf-900™ II SFM at mid-log phase (3 x 10^6 cells/mL and 1.5 x 10^6 cells/mL, respectively). The cells were infected with passage 3 BV stocks at an estimated MOI of 5. The cultures were incubated at 27°C or 21°C in a shaking incubator set at 120 rpm. Cell densities and viabilities were monitored as described above. Both cells and supernatant were collected every 24 h for Western blot analysis.

2.4 Serial Passaging

Budded virus was continuously serial passaged up to passage 8 to obtain BV stocks for expression at Passage 4 to Passage 9. Sf9 cells in Sf-900™ II SFM were infected with BV stocks from passage 4 to passage 8 at an estimated MOI of 5 and cultured at 27°C. Cell densities and viabilities were monitored daily and samples were collected for Western blot analysis.

2.5 Stability of BV stocks

The stability of Passage 3 BV stocks of EV71 and CVA16 were examined by comparing expression between newly amplified BV stocks, BV stocks stored at 4°C or -80°C for 4 months. Sf9 cells (3 x 10^6 cells/mL) were infected with Passage 3 BV stocks at an estimated MOI of 5 at 21°C and expression was analyzed by Western blot.

2.6 Expression and purification of EV71 and CVA16 VLPs

EV71 and CVA16 VLPs were expressed in Sf9 cells in Sf-900^{TM} II SFM at 21^{o}C for 120 h. Expression culture was harvested by centrifugation at 8,000 x *g* for 20 min and the cells and supernatants were collected separately for purification. The cell pellet was resuspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, pH 7.5) and disrupted using a Sonifer® Cell Disrupter with a 1/2" titanium horn (Branson® Ultrasonics, NC, USA). Cell debris was removed by centrifugation at 15,000 x *g* for 30 min. VLPs in the clarified soluble fraction were recovered by ammonium sulphate ($(NH_4)_2SO_4$) precipitation at 20% saturation for 30 min at room temperature. The precipitated VLPs were pelleted by centrifugation at 10,000 x *g* for 10 min and resuspended in 20 mM Tris, 50 mM NaCl, pH 7.5 before loading onto a Capto™ Core 700 column (CV 4.7 mL) (GE Healthcare Life Sciences, Uppsala, Sweden) at 1 mL/min flow rate in a flow-through mode purification.

For purification of VLPs from the extracellular fraction, culture supernatant was first filtered using a 0.45 μm filter. The filtered supernatant was 10 times concentrated with a tangential flow filtration (TFF) unit with a 300 kDa nominal molecular weight cut-off (MWCO) ultrafiltration cassette (Sartorius AG, Goettingen, Germany) and buffer exchanged into 20 mM Tris, 50 mM NaCl, pH 7.5. The retentate material was centrifuged at 16,500 x *g* for 15 min at 4^{o}C to remove particulates before being loaded onto a Capto™ Core 700 column (CV 4.7 mL) in a flow-through mode purification at 1 mL/min flow rate.

2.7 Western blot analysis

The VLP samples were analyzed on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reduced and denatured conditions. The SDS-PAGE gel was transferred onto PVDF membrane using iBlot® Western blotting system (Life Technologies) and probed with the appropriate primary antibody (rabbit anti-EV71-VP1 polyclonal sera that cross-reacts with

CVA16 VP1, rabbit anti-EV71-VP0 polyclonal sera or rabbit anti-CVA16-VP0 polyclonal sera) at a dilution of 1:4,000 and followed by the secondary antibody (HRP-conjugated goat anti-rabbit IgG) at a dilution of 1:15,000. Chemiluminescent signals were developed using ECL chemiluminescent substrate reagent kit (Life Technologies) and visualized on a Chemi-Doc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., CA, USA).

2.8 Asymmetric flow field-flow fractionation analysis

Asymmetric flow field-flow fractionation (AF4) analysis was performed as per previously described [18], with some modifications. Briefly, the injected samples were focused for 6 min at the marrow injection point. The detector flow through the downstream of detectors was maintained at 1.2 mL/min. At the end of the focus mode, the analytes were eluted under a constant cross flow of 1 mL/min for 30 min, after which the cross flow rate was decreased stepwise to zero for the elution of highly retained analytes. The AF4 analysis was performed using an Eclipse® 3+ system (Wyatt Technology Corporation, CA, USA) coupled with SPD-20A UV/Vis detector (Shimadzu, Kyoto, Japan) and a DAWN Heleos II multi-angle light scattering detector (MALS, Wyatt Technology Corporation). The AF4 system was equilibrated with 50 mM Tris-Glycine, 150 mM NaCl, pH 7.4 prior to analysis. VLP samples were centrifuged (16,500 x *g*, 4°C, 15 min), and 100 µL of the supernatant was injected into AF4 and fractionated. Data acquisition and analysis were formed using the Astra® V software (Version 5.3, Wyatt Technology Corporation). Immunoaffinity chromatography purified EV71 VLPs (supplied by Sentinext Therapeutics) was used as a standard for initial AF4 method development.

2.9 Transmission electron microscopy analysis

Transmission electron microscopy (TEM) was performed using 5 µL of purified VLP sample applied to a Formvar coated, 200-mesh copper grid, rinsed with water, and stained with 1% w/v

uranyl acetate. Sample grids were analyzed with a JEOL 1010 (JEOL, Tokyo, Japan) microscope at 100 kV and the electron micrographs were recorded using a Morada camera (Olympus® Soft Imaging System GmbH).

3. Results and discussion

3.1 Effect of temperature and cell line

The expression of EV71 and CVA16 was tested in two commonly used insect cell lines, Sf9 and High Five™, and at incubation temperatures of either 21°C or 27°C. Both cells and supernatant were collected every 24 h for the detection of viral structural proteins. Figure 2A shows the Western blot analysis of the intracellular and extracellular fractions with anti-EV71-VP1 antibodies. The anti-EV71-VP1 antibody could detect EV71 P1 polyprotein (VP0+VP3+VP1, 96 kDa) and VP1 structural protein (33 kDa). However, in the CVA16 cultures, this antibody detected only CVA16 VP1 structural protein and no CVA16 P1 protein was detected. Non-specific antibody binding was not observed in the uninfected cell samples.

At 27°C, both Sf9 and High Five™ cell lines showed similar levels of EV71 VP1 inside the cells, peaking at 48 hours post infection (hpi). Protein degradation was observed in High Five™ but not in Sf9. At the same temperature, significantly more VP1 was detected in the Sf9 extracellular fraction compared to High Five™. It is unclear if the level of VP1 detected reflects the accumulation of assembled VLPs. The mechanism for trafficking of enterovirus VLPs to the extracellular fraction after intracellular assembly is unknown. CVA16 VP1 production was significantly lower under all conditions when compared to EV71. In the extracellular fractions, CVA16 VP1 degradation was observed in both cell lines, while similar degradation in the intracellular fraction was observed only in High Five™ cells. At 27°C, the optimum cell line for

CVA16 VLP production was Sf9 cells and the optimum time of harvest (TOH) was 48 hpi and 72 hpi for intracellular and extracellular VLPs, respectively (Figure 2A).

The incubation temperature was lowered to 21°C to investigate if a lower temperature could prolong cell viability, thus delay cell lysis and allow higher production and/or processing of P1 polyprotein to VP1. EV71 VP1 production (intracellular and extracellular) was enhanced in Sf9 cells, suggesting that the P1 polyprotein cleavage to VP1 was more efficient at 21°C than at 27°C. The reduction in temperature did not have a significant effect on VP1 production in High Five™. The optimum TOH for intracellular EV71 VLPs in Sf9 was 96 hpi and extracellular EV71 VLPs in Sf9 was 120 hpi (Figure 2A). At 21°C, an increase in CVA16 VP1 production was observed in Sf9 but not in High Five^{TM}, similar to EV71. For Sf9 cell line, the optimum TOH for intracellular CVA16 VLPs was 96 hpi, and 144 hpi for extracellular CVA16 VLPs.

To determine CVA16 P1 polyprotein processing, CVA16 samples were also analyzed with anti-CVA16-VP0 antibody Western blot. Figure 2B shows the proteolytic cleavage of P1 polyprotein into intermediates (corresponding to VP0+VP3, 63 kDa) and VP0 (37 kDa). Consistent with the results obtained for anti-EV71-VP1 antibody Western blot analysis, 21°C is the optimal temperature for CVA16 expression and highest level of VP0 was detected at 144 hpi in the extracellular fraction at this temperature (Figure 2B).

EV71 and CVA16 VLPs are structurally similar but significant differences in protein yield and site of protein accumulation were observed when produced in insect cells. Choice of cell line has previously been reported to have an effect on level of expression of foreign genes in the baculovirus-insect cell system [19]. In this study, enhanced production of EV71 and CVA16 VLPs were observed in Sf9 cells compared to High Five^{TM} cells. This result is consistent with

the report by Chung and coauthors describing different approaches to production of EV71 VLPs. They also showed higher yields in Sf9 cells than in High Five™ cells and suggested that this could be due to the cellular and genetic differences of the two cell lines [20].

The baculovirus-insect cell system is a powerful system for recombinant protein expression [21]. However, as a lytic viral expression system, cell lysis-associated proteolysis is a major drawback [22]. Culture temperature and pH are key factors that can influence proteolytic activity and product quality in the baculovirus-insect cell system [23]. Previous studies have shown that lowering the incubation temperatures from 28°C or 30°C to 20°C increased recombinant protein production yield in insect cells [24, 25]. Rapid depletion of oxygen in cultures was observed at elevated temperatures [26]. Such increases in the respiratory and metabolic activity at elevated temperatures often increases the rate of cell death and terminates recombinant protein production [27]. Premature termination of P1 polyprotein and/or 3CD protease production at high temperature would affect P1 polyprotein processing into structural proteins (VP0, VP3 and VP1), a pre-requisite to spontaneous VLP assembly. Production of VLPs using the baculovirus-insect cell system at 21°C may have slowed down cell metabolic activity, thus delaying cell lysis and prolonging the life span of infected cells to allow structural protein processing and maturation of VLPs in the cells.

3.2 Effect of serial passaging on expression

The effect of serial passaging BV stocks on the production of EV71 and CVA16 VLPs was investigated in Sf9 cells at 27°C. Structural protein production analyzed on Western blots probed with anti-EV71-VP1 antibody (Figure 3A) indicated that there was a significant drop in CVA16 VP1 production after 5 passages of BV stocks in cell culture. In contrast, EV71 VP1 production was detected for at least 9 passages, although a gradual decrease in VP1 production was

observed. The cell densities and viabilities post infection of all cultures at each passage were determined and no significant difference was observed (Figure 3B). The titers of BV stocks did not decline with increasing passage number (Table 1) and the observed difference in the effect of serial passaging is not due to the changes in MOI used. The serial passage of recombinant baculoviruses in insect cells can lead to the accumulation of replication-defective deletion mutant viruses known as defective interfering particles (DIPs) [28]. Such accumulated DIPs often lack the foreign gene of interest, and compete with the normal recombinant baculovirus during infection. This in turn results in a decrease in recombinant protein production [29, 30]. Accumulation of DIPs without either or both P1 and 3CD protease genes during the serial passaging of BV stocks for scale-up production could render this VLP production process unviable if it is not carefully monitored.

3.3 Effect of virus stock storage on expression

The effect storage stability at 4°C of passage 3 BV stock on the structural protein production of both EV71 and CVA16 constructs was investigated (Figures 4A and 4B). EV71 and CVA16 structural protein production was analyzed by Western blots, using anti-EV71-VP0 and anti-CVA16-VP0 antibodies, respectively. EV71 passage 3 BV stock retained its ability to infect Sf9 cells leading to comparable structural protein production (Figure 4A) after storage for 4 months at 4°C. However, a significant reduction in the CVA16 structural protein production was observed for BV stock stored at 4°C for 4 months (Figure 4B). Given the sensitivity of CAV16 passage 3 BV virus stock to storage at 4°C, freshly amplified passage 3 BV stock from passage 2 BV stock should be used for each production run. Figure 4C shows that CVA16 passage 2 BV stock maintained better infectivity, thus productivity, when stored at -80°C than at 4°C (Figure

4C). The freeze-thaw effect on BV infectivity could have caused the slight decrease in structural proteins, similar to a previous report [31].

3.4 Purification of EV71 and CVA16 VLPs

EV71 and CVA16 VLPs were expressed in Sf9 cells at 21^{o}C for 120 h. Both VLPs were purified from culture supernatant and cell pellet, separately by Capto™ Core chromatography, and multiple protein bands were detected on the SDS-PAGE gel analysis (Figure 5A). Three protein bands corresponding to P1 polyprotein, VP0+VP3 intermediates and VP0 were detected on the anti-VP0 Western blot analysis (Figure 5B). This confirms that co-expressing P1 polyprotein and 3CD protease resulted in P1 cleavage by 3CD protease and self-assembly of VLPs in insect cells as previously reported [12]. However, the results also suggest that the purified VLP fractions may contain P1 polyprotein and partially processed intermediates VP0+VP3. It is possible that EV71 and CVA16 VLPs produced in insect cells do contain partially processed structural proteins. Another possibility is unprocessed P1 polyprotein and intermediates VP0+VP3 were misforming or aggregating into particles larger than 700 kDa, consequently, being excluded from the Capto™ Core chromatography resin and were co-purified with the assembled VLPs.

Purification of both VLPs from the cells [32, 33] or culture supernatant [20, 34] has been reported previously. Chung et al. have purified EV71 VLPs from cells [32] and culture supernatant [20], and have reported to prefer culture supernatant for ease of purification [20]. Intracellular VLPs are released either mechanically (homogenization/sonication), chemically or enzymatically from the cells [35]. During this lysis process, other host cell proteins are released along with the target VLPs [36], necessitating extra purification processes to purify the VLPs

away from host contaminants thus increasing production costs. Moreover, the buffer composition (salt and pH) can drive the VLPs to either aggregate or dissociate during the lysis process, thereby affecting the structural and colloidal stability of VLPs [10]. In contrast, this extraction step is completely avoided in extracellular VLP purification, thereby decreasing the number of steps, time and cost involved in downstream processing [36].

3.5 Characterization of EV71 and CVA16 VLPs

The first use of AF4-MALS technique for accurately characterizing EV71 and CVA16 VLPs is demonstrated here (Figures 5C and 5D). The AF4-MALS method for enterovirus VLP characterization was developed and optimized using EV71 VLP standard, based on a previously described method [18]. A VLP peak was detected at a retention time of 20 min, and the average root mean square (rms) radius of EV71 VLP standard was calculated as 15.4 ± 1 nm. A peak at a retention time of 12 min (likely comprised of proteins) and an aggregate peak at a retention time greater than 40 min were also observed. This AF4-MALS analytical method was used to characterize the size and distribution of purified VLPs of EV71 (Figure 5C) and CVA16 (Figure 5D). The average rms radius of EV71 and CVA16 VLPs were calculated as 15 ± 1 nm and 15.3 ± 5.8 nm, respectively. As illustrated in Figures 5C and 5D, the heterogeneity of VLP samples could be detected and characterized using AF4-MALS. The VLP yields calculated from the VLP peak areas (Peak B) reveal that EV71 VLP yield is 2.7x higher than CVA16. This method allows quantitative characterization of VLPs in parallel to VLP visualization under TEM (Figure 5E). The size measurement of both EV71 and CVA16 VLPs by AF4-MALS is consistent with reported measurements using dynamic light scattering (30 nm diameter) [37]. Unlike the dynamic light scattering method, AF4-MALS technique allows high resolution of particles based on size, thus enabling highly accurate particle size determination [18].

4. Conclusions

The expression of both EV71 and CVA16 VLPs in insect cells were significantly enhanced by lowering the temperature of standard 27°C to 21°C. The insect cell line of choice for production of both VLPs is Sf9 cell line. The CVA16-expressing baculovirus was more susceptible to serial passage effect in cell culture than EV71. The production of CVA16 structural proteins diminished after 5 passages in cell culture, potentially a challenge to the large-scale production of CVA16 VLPs. Higher EV71 VLP yield was obtained from culture supernatant, whereas higher CVA16 VLP yield was obtained from the cells. These factors will affect the design and development of production processes for a bivalent EV71 and CVA16 VLP. This study also reports the first AF4-MALS characterization of EV71 and CVA16 VLPs. The use of high resolution AF4-MALS, dynamic light scattering and TEM techniques allow biophysical characterization on VLP integrity, which is critical to the development of VLP vaccines.

Acknowledgement

The authors thank Anton Middelberg at the University of Queensland for his valuable suggestions on the AF4 method development.

Reference

[1] T. Solomon, P. Lewthwaite, D. Perera, M.J. Cardosa, P. McMinn, M.H. Ooi, Lancet Infect. Dis., 10 (2010) 778-790.

[2] Q. Mao, Y. Wang, X. Yao, L. Bian, X. Wu, M. Xu, Z. Liang, Hum. Vaccin. Immunother., 10 (2014) 360-367.

[3] C.C.Y. Yip, S.K.P. Lau, P.C.Y. Woo, K.-Y. Yuen, Emerging Health Threats Journal; Vol 6 (2013) incl Supplements, (2013).

[4] W. Liu, S. Wu, Y. Xiong, T. Li, Z. Wen, M. Yan, K. Qin, Y. Liu, J. Wu, PLoS One, 9 (2014) e96051.

[5] W. Xu, C.-f. Liu, L. Yan, J.-j. Li, L.-j. Wang, Y. Qi, R.-b. Cheng, X.-y. Xiong, Virol J., 9 (2012) 8.

[6] R. Kirnbauer, F. Booy, N. Cheng, D.R. Lowy, J.T. Schiller, Proc. Natl. Acad. Sci. U. S. A., 89 (1992) 12180-12184.

[7] P. Valenzuela, A. Medina, W.J. Rutter, G. Ammerer, B.D. Hall, Nature, 298 (1982) 347-350.

[8] F.C. Zhu, J. Zhang, X.F. Zhang, C. Zhou, Z.Z. Wang, S.J. Huang, H. Wang, C.L. Yang, H.M. Jiang, J.P. Cai, Y.J. Wang, X. Ai, Y.M. Hu, Q. Tang, X. Yao, Q. Yan, Y.L. Xian, T. Wu, Y.M. Li, J. Miao, M.H. Ng, J.W. Shih, N.S. Xia, Lancet, 376 (2010) 895-902.

[9] Q. Zhao, S. Li, H. Yu, N. Xia, Y. Modis, Trends Biotechnol., 31 (2013) 654-663.

[10] B. Somasundaram, L.H.L. Lua, Pharmaceutical Bioprocessing, 3 (2015) 45-59.

[11] G. Stanway, J. Gen. Virol., 71 (Pt 11) (1990) 2483-2501.

[12] Y.C. Hu, J.T.A. Hsu, J.H. Huang, M.S. Ho, Y.C. Ho, Biotechnol. Lett., 25 (2003) 919-925.

[13] M.M. Cox, Vaccine, 30 (2012) 1759-1766.

[14] L.A. Palomares, J.A. Mena, O.T. Ramírez, Methods, 56 (2012) 389-395.

354 [15] L.A. Palomares, O.T. Ramírez, Biochem. Eng. J., 45 (2009) 158-167.

355 [16] L.H.L. Lua, N.K. Connors, F. Sainsbury, Y.P. Chuan, N. Wibowo, A.P.J. Middelberg,
356 Biotechnol. Bioeng., 111 (2014) 425-440.

357 [17] V. Janakiraman, W.F. Forrest, S. Seshagiri, Nat. Protoc., 1 (2006) 2271-2276.

358 [18] Y.P. Chuan, Y.Y. Fan, L. Lua, A.P.J. Middelberg, Biotechnol. Bioeng., 99 (2008) 1425-
359 1433.

360 [19] W.F. Hink, D.R. Thomsen, D.J. Davidson, A.L. Meyer, F.J. Castellino, Biotechnol. Prog., 7
361 (1991) 9-14.

362 [20] C.Y. Chung, C.Y. Chen, S.Y. Lin, Y.C. Chung, H.Y. Chiu, W.K. Chi, Y.L. Lin, B.L.
363 Chiang, W.J. Chen, Y.C. Hu, Vaccine, 28 (2010) 6951-6957.

364 [21] R.D. Possee, Curr. Opin. Biotechnol., 8 (1997) 569-572.

365 [22] S. Naggie, W.E. Bentley, Biotechnol. Prog., 14 (1998) 227-232.

366 [23] P.E. Cruz, P.C. Martins, P.M. Alves, C.C. Peixoto, H. Santos, J.L. Moreira, M.J.T.
367 Carrondo, Biotechnol. Bioeng., 65 (1999) 133-143.

368 [24] M. Donaldson, H.A. Wood, P.C. Kulakosky, M.L. Shuler, Biotechnol. Bioeng., 63 (1999)
369 255-262.

370 [25] K.D. Cain, K.M. Byrne, A.L. Brassfield, S.E. LaPatra, S.S. Ristow, Dis. Aquat. Organ., 36
371 (1999) 1-10.

372 [26] T. Gotoh, K. Chiba, K.-I. Kikuchi, Biochem. Eng. J., 17 (2004) 71-78.

373 [27] J.N. Andersen, P.G. Sriram, N. Kalogerakis, L.A. Behie, The Canadian Journal of Chemical
374 Engineering, 74 (1996) 511-517.

375 [28] M. Kool, J.W. Voncken, F.L.J. Van Lier, J. Tramper, J.M. Vlak, Virol, 183 (1991) 739-746.

376 [29] P. Krell, Cytotechnology, 20 (1996) 125-137.

377 [30] S. Kumar, L.K. Miller, Virus Res., 7 (1987) 335-349.

378 [31] H. Jorio, R. Tran, A. Kamen, Biotechnol. Prog., 22 (2006) 319-325.

379 [32] Y.C. Chung, M.S. Ho, J.C. Wu, W.J. Chen, J.H. Huang, S.T. Chou, Y.C. Hu, Vaccine, 26
380 (2008) 1855-1862.

381 [33] Q.W. Liu, K.X. Yan, Y.F. Feng, X.L. Huang, Z.Q. Ku, Y.C. Cai, F. Liu, J.P. Shi, Z. Huang,
382 Vaccine, 30 (2012) 6642-6648.

383 [34] M. Gong, H. Zhu, J. Zhou, C. Yang, J. Feng, X. Huang, G. Ji, H. Xu, P. Zhu, J. Virol.,
384 (2014).

385 [35] A.P.J. Middelberg, Biopharmaceutical Production Technology, Wiley-VCH Verlag GmbH
386 & Co. KGaA2012, pp. 79-105.

387 [36] A. Roldao, M.C.M. Mellado, L.R. Castilho, M.J.T. Carrondo, P.M. Alves, Expert Rev
388 Vaccines, 9 (2010) 1149-1176.

389 [37] S.Y. Lin, Y.C. Chung, H.Y. Chiu, W.K. Chi, B.L. Chiang, Y.C. Hu, Journal of bioscience
390 and bioengineering, 117 (2014) 366-371.

391

392

Figure captions

Figure 1: Diagrammatic representation of P1 polyprotein processing and assembly of structural proteins. P1 polyprotein is proteolytically cleaved into intermediate VP0+VP3 and VP1 proteins. The intermediate VP0+VP3 is further cleaved into VP0 and VP3 proteins. Structural proteins VP0, VP3 and VP1 first assemble to form a protomer. A pentamer is made of five protomers and twelve copies of pentamer come together to form a VLP.

Figure 2: Effect of temperature and cell line on EV71 and CVA16 VLP expression. EV71 and CVA16 VLPs were expressed in Sf9 and High Five^{TM} insect cell lines at 27°C and 21°C. A) EV71 and CVA16 expression profiles were examined on Western blot analysis using anti-VP1 antibody. EV71 and CVA16 samples harvested at 24, 48, 72 and 96 hours post infection (hpi) from 27^{o}C were analyzed. At 21^{o}C, EV71 samples were harvested at 48, 72, 96 and 120 hpi and CVA16 samples were harvested at 72, 96, 120 and 144 hpi, for analysis. B) CVA16 P1 polyprotein processing in Sf9 insect cells were analyzed by Western blot using anti-CVA16-VP0 antibody. The P1 polyprotein, intermediate VP0+VP3 and VP0 were observed on anti-CVA16-VP0 Western blot. Lanes: M, molecular weight marker; C, uninfected cell control.

Figure 3: Effect of serial passaging on EV71 and CVA16 VLP expression. A) Analysis of VP1 expression at different passage number by anti-VP1 Western blot. B) Plots of viable cell densities of Sf9 cultures at different passage number.

Figure 4: Effect of storage stability of virus stocks on EV71 and CVA16 VLP expression. Anti-VP0 western blot analysis on the expression profile of A) EV71, B) CVA16 using passage 3 virus stocks stored at 4°C for 4 months, and C) CA16 VLPs using passage 2 virus stocks stored at 4°C and -80°C for 4 months.

Figure 5: Purification and characterization of EV71 and CVA16 VLPs. VLPs from both intracellular and extracellular fractions were purified. Intracellular VLPs were purified by ammonium sulphate precipitation followed by Capto^{TM} Core 700 chromatography. Extracellular VLPs were purified using Capto^{TM} Core 700 chromatography. Purified VLPs were analyzed on SDS-PAGE (A) and anti-VP0 Western blot (B). C & D) Fractograms from asymmetric flow field-flow fractionation coupled with multi-angle light scattering of EV71 VLP standard in comparison with purified VLPs of EV71 (C) and CVA16 (D). Proteins (Peak A), VLPs (Peak B) and aggregates (Peak C) were separated and detected in the VLP samples. E) Characterization of VLPs with transmission electron microscopy.

430 Table 1: Virus titers of EV71 and CVA16 budded virus stocks.

Passage number	EV71 virus titer (10^8 IU/mL)	CVA16 virus titer (10^8 IU/mL)
3	2.77	2.77
4	3.35	2.16
5	3.65	2.24
6	3.05	6.25
7	4.76	6.79

431

Figure 1

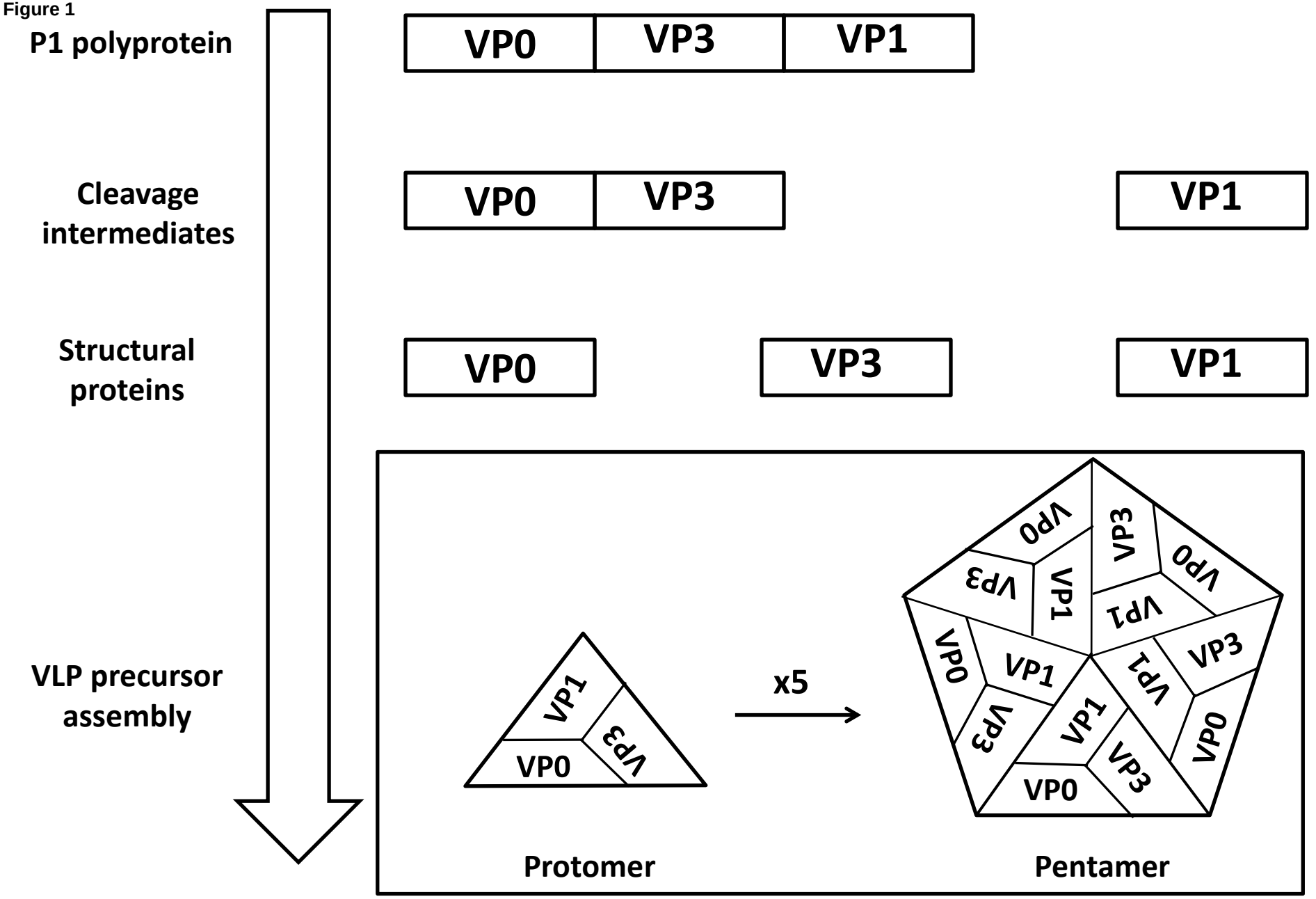
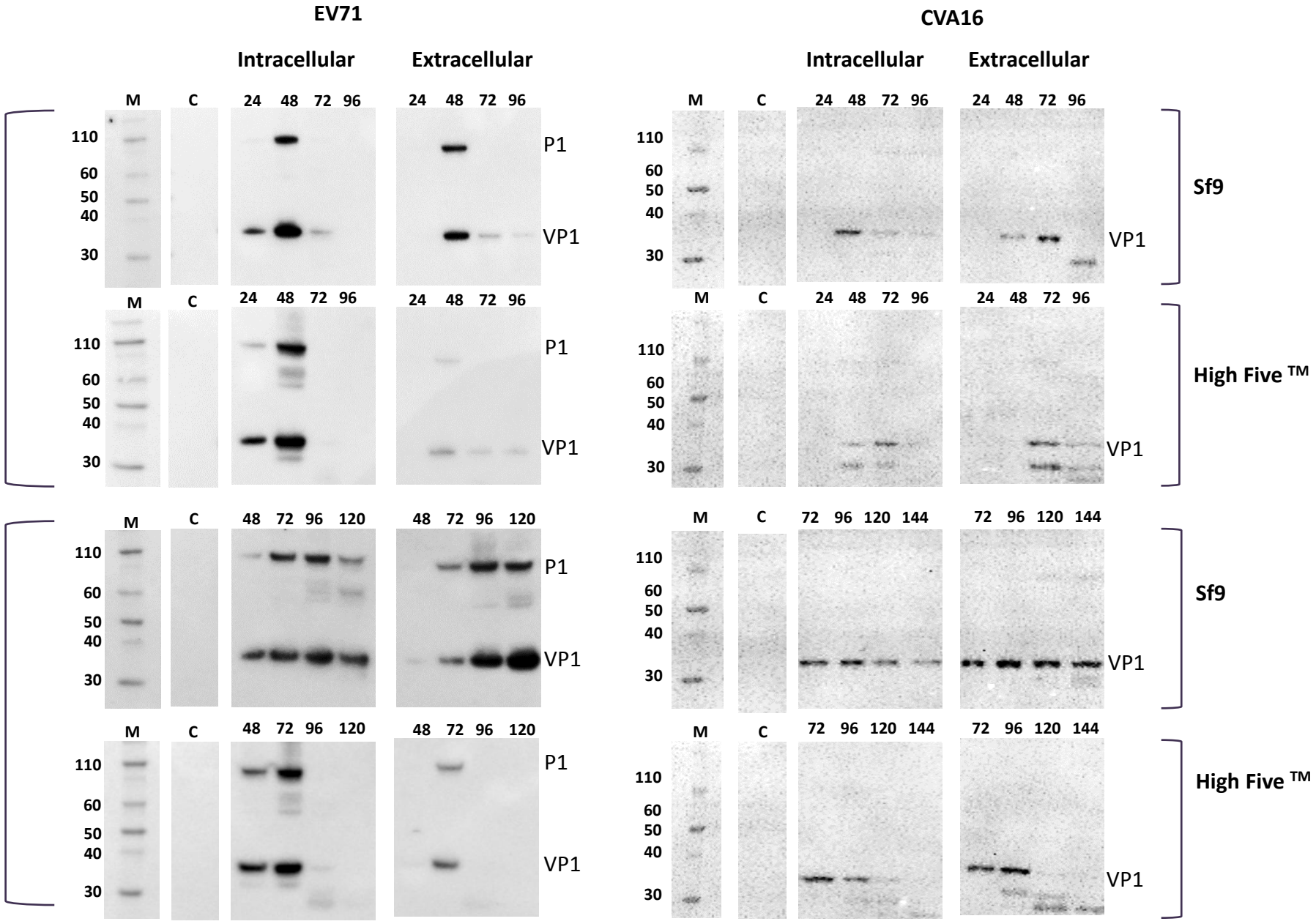

P1 polyprotein
VP0
VP3
VP1
Cleavage intermediates
VP0
VP3
VP1
Structural proteins
VP0
VP3
VP1
VLP precursor assembly
VP1
VP3
VP0
x5
Protomer
Pentamer

Figure 2

2A


EV71
CVA16
Intracellular
Extracellular
Intracellular
Extracellular
M
C
24 48 72 96
24 48 72 96
24 48 72 96
24 48 72 96
48 72 96 120
48 72 96 120
72 96 120 144
72 96 120 144
110
60
50
40
30
P1
VP1
Sf9
High Five ™
°C
21°C

2B

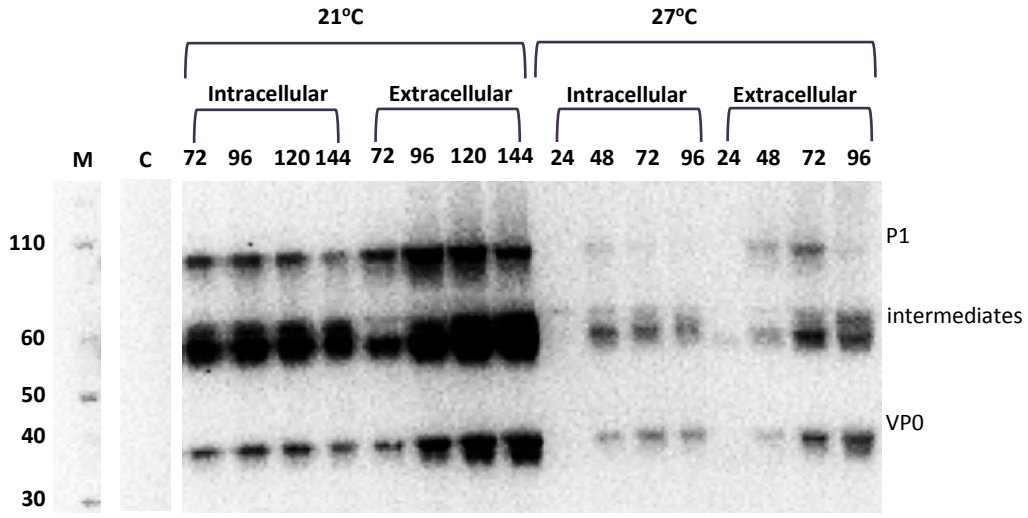
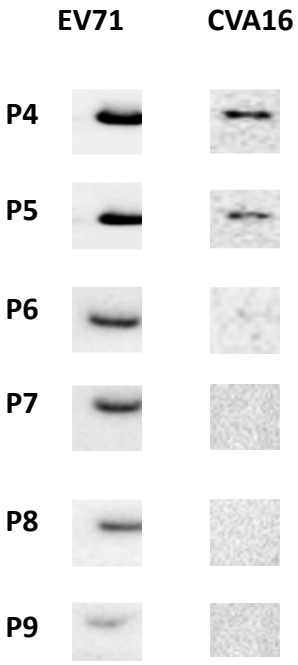
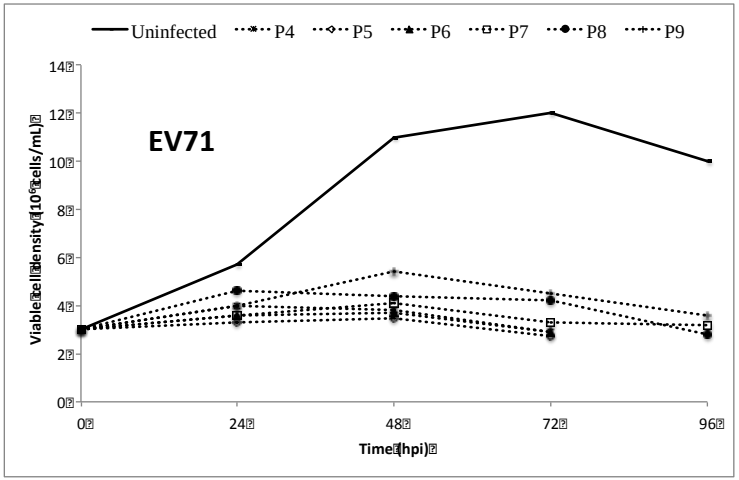

21°C
27°C
Intracellular
Extracellular
Intracellular
Extracellular
M
C
72 96 120 144 72 96 120 144 24 48 72 96 24 48 72 96
110
60
50
40
30
P1
intermediates
VP0

Figure 3

3A



3B



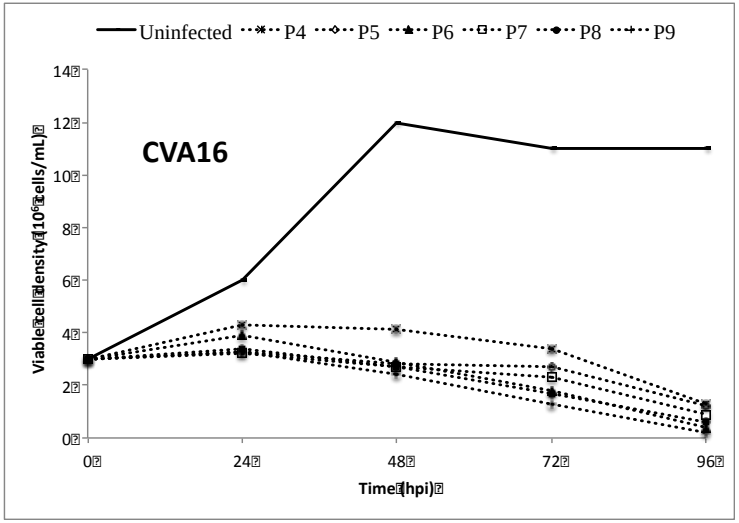


Figure 4

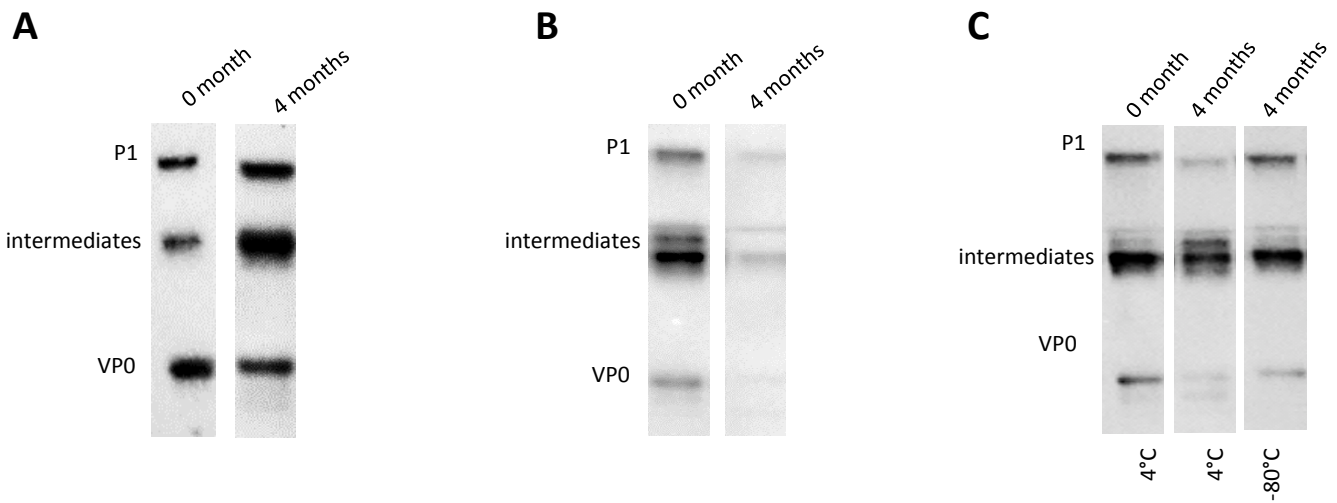
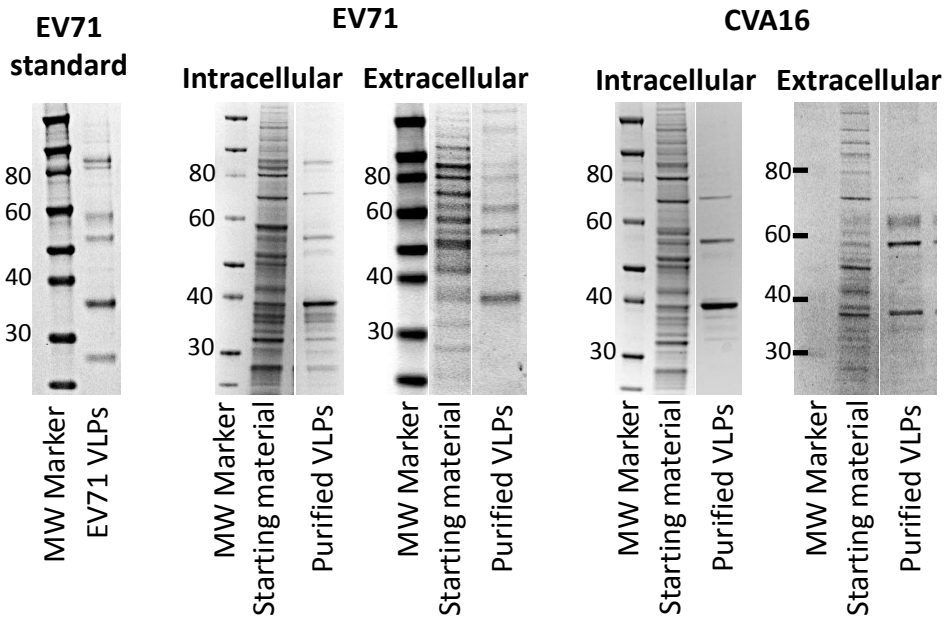
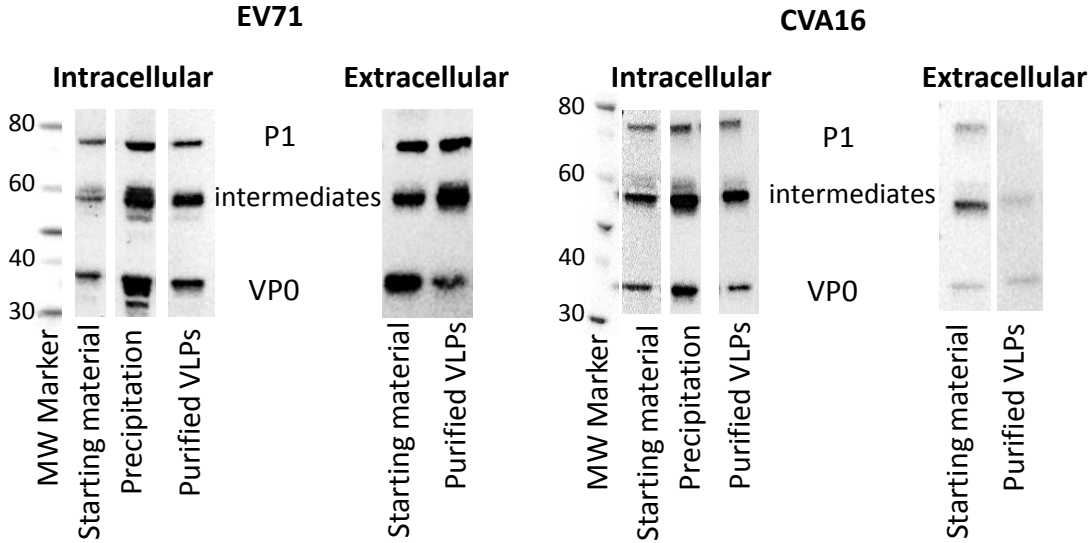

A
0 month
4 months
P1
intermediates
VP0
B
0 month
4 months
P1
intermediates
VP0
C
0 month
4 months
4 months
P1
intermediates
VP0
4°C
4°C
-80°C

Figure 5

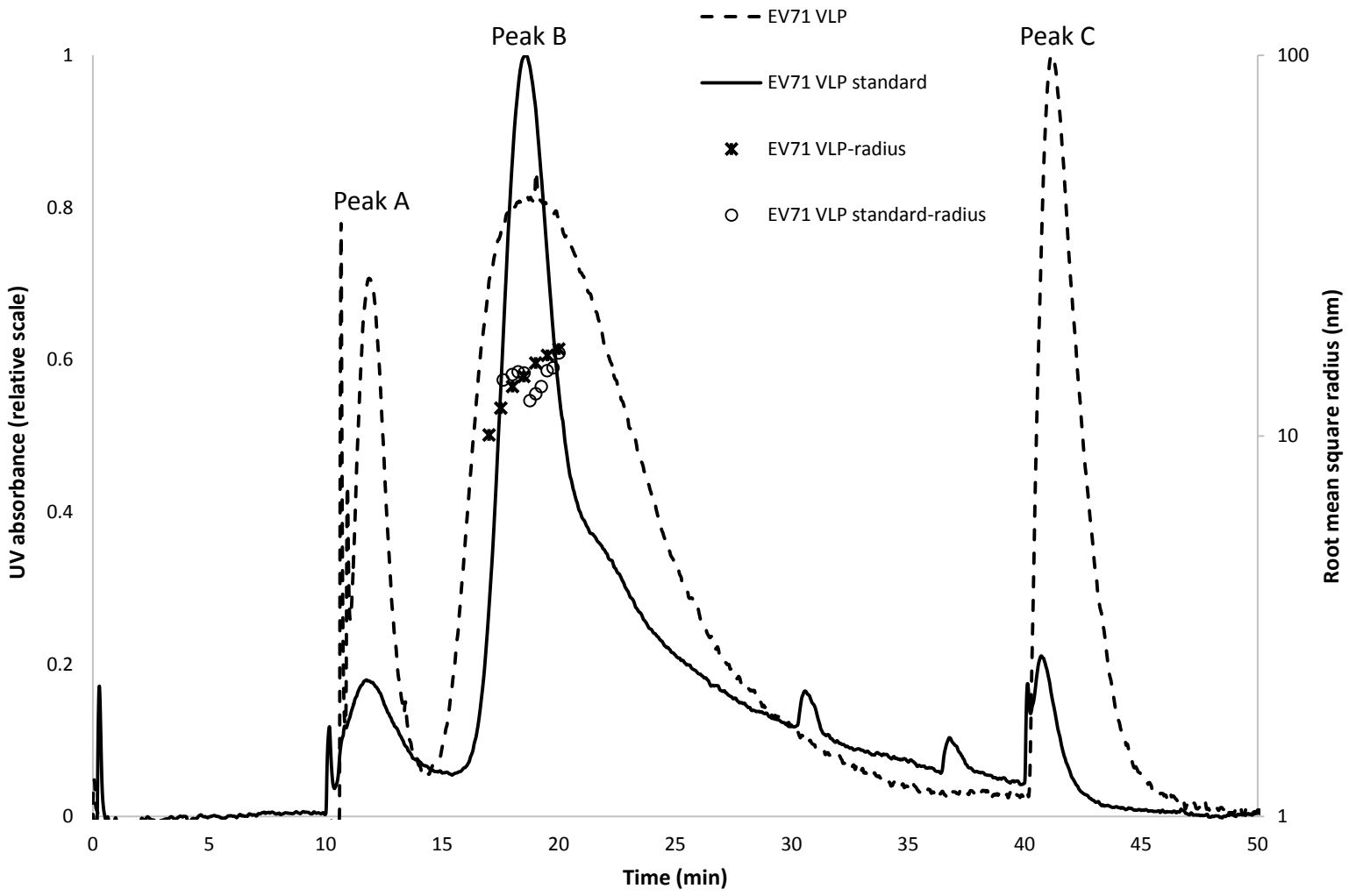
5A



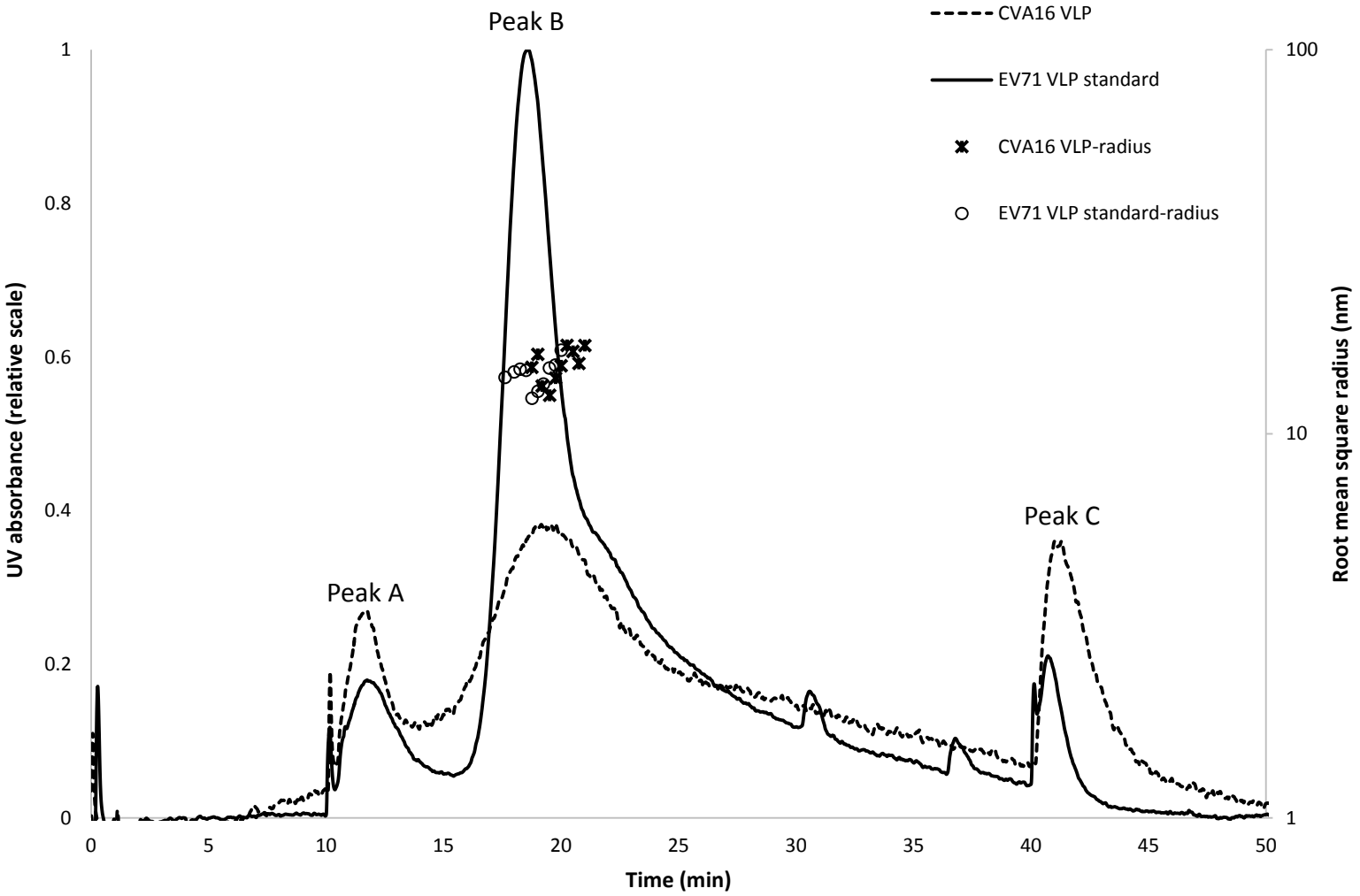
5B



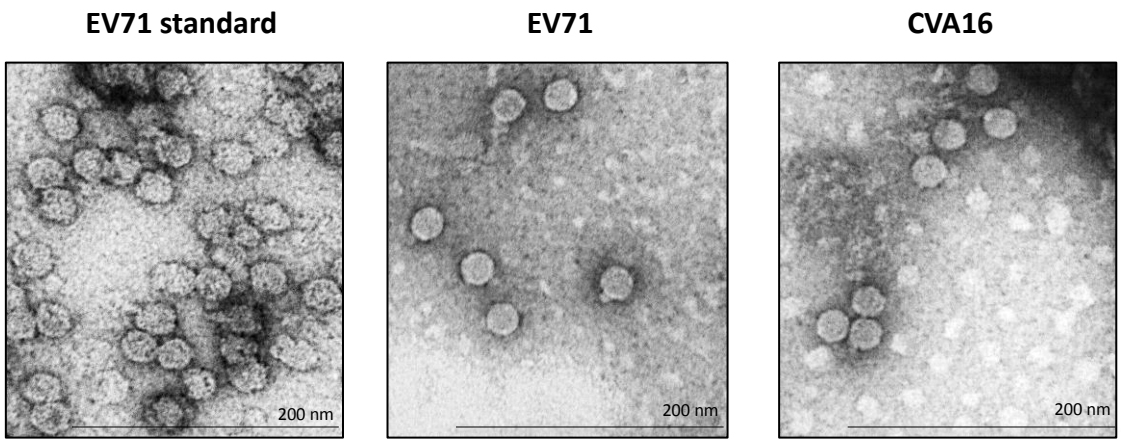
5C


EV71 VLP
EV71 VLP standard
EV71 VLP-radius
EV71 VLP standard-radius
Peak A
Peak B
Peak C
UV absorbance (relative scale)
Root mean square radius (nm)
Time (min)
0
0.2
0.4
0.6
0.8
1
1
10
100
0
5
10
15
20
25
30
35
40
45
50

5D


Peak B
CVA16 VLP
EV71 VLP standard
CVA16 VLP-radius
EV71 VLP standard-radius
Peak A
Peak C
UV absorbance (relative scale)
Root mean square radius (nm)
Time (min)
0
0.2
0.4
0.6
0.8
1
1
10
100
0
5
10
15
20
25
30
35
40
45
50

5E



Highlights

- Expression at 21°C enhanced the production of EV71 and CVA16 VLPs in Sf9 cells
- CVA16 expression yield is adversely affected by serial passaging of virus
- Higher VLP yield is obtained with EV71 compared to CVA16
- VLP size distributions of EV71 and CVA16 VLPs are characterized by AF4-MALS