

Comparison of ultracentrifugation methods for concentration of recombinant alphaviruses: sucrose and iodixanol cushions

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Abstract

Alphavirus vectors have been successfully used as a gene delivery system in animal models. A prerequisite for the efficient expression of heterologous genes is the application of high doses of recombinant viruses. The currently available methods for viral concentration are laborious, costly and often require specialised equipment. Here, we provide a comparative study of three ultracentrifugation methods used for alphavirus concentration: 20% sucrose cushion, double (20 and 50%) sucrose cushion, and double (20 and 50%) iodixanol cushion. The yield of concentrated recombinant Semliki forest virus from each method was evaluated by determination of the viral infectivity in cell culture and genome copy number as quantified using real-time PCR. The best results were obtained using ultracentrifugation on a double iodixanol cushion, a method that generated a high recovery yield (approximately 40%) and a four-fold increase in virus concentration. The methods of concentration on sucrose appeared less satisfactory. Isosmotic iodixanol-containing virus preparations did not affect alphavirus infectivity and can be used directly for *in vivo* applications.

Key words: concentration of alphaviruses, iodixanol, Semliki forest virus, sucrose cushion, ultracentrifugation.

Abbreviations: BHK-21, Baby Hamster Kidney cell line; EGFP, enhanced green fluorescent protein; GCN, genome copy number; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IU, infectious units; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; SD, standard deviation; SFV, Semliki Forest Virus.

Introduction

Alphaviruses comprise a set of genetically, structurally, and serologically related arthropod-borne enveloped single-stranded positive-sense RNA viruses belonging to the *Togaviridae* family (Strauss et al. 1994). In our research we have worked with the most commonly used Semliki forest virus alphaviral expression system (Liljestrom et al. 1991). Nowadays alphaviral vector systems are widely used for expression of heterologous genes and the development of protective and therapeutic vaccines against a variety of human diseases (Lundstrom et al. 2003; Zajakina et al. 2009).

To improve the biosafety level of the alphaviral expression system, the full Semliki Forest Virus (SFV) genome is split into two vectors. In pSFV1/enhanced green fluorescent protein (EGFP) vector viral structural genes have been substituted by the EGFP marker gene. In order to package the replicon RNA into particles, a helper vector has been used to provide structural proteins (Rheme et al. 2004). This type of expression vector is called a suicide vector because it is unable to propagate due to the fact that it lacks the sequences necessary to synthesise the viral structural proteins (Quetglas et al. 2004).

Much of our current understanding of the alphaviral

replication, propagation and interactions with hosts is derived from studies of viral administration in small laboratory rodents like mice. One of the major obstacles for experiments using animal models is the preparation of highly concentrated viral stocks. Virus injections in animals are limited by their body mass and the total amount of blood in their bloodstream. A safe maximum for a single sample is 1.25% of body weight (1.25 mL per 100 g of body weight) (Schwiebert 2007). This means that, for example, only a maximum volume of 1 to 2 mL in tail vein of adult mice may be injected. Also, primary culture media inevitably contain variable amounts and composition of contaminants derived from packaging cells, as well as various chemical and biological additives such as growth factors, serum and endonucleases. As impurities and contaminants can induce immunological and biological responses, they must be removed by virus purification.

In the present study, we evaluate three methods for alphavirus concentration: (I) sucrose cushion pelleting, (II) double sucrose cushion ultracentrifugation, and (III) double iodixanol cushion ultracentrifugation. Moreover, to simplify the use and accessibility of alphavirus expression systems, we described a real-time PCR-based method for the quantification of genomic material in alphavirus particles. The aim of the study was to compare the effectiveness

of ultracentrifugation through iodixanol cushions with common pelletation in sucrose or ultracentrifugation in sucrose cushions for recombinant alphaviruses.

Materials and methods

Preparation of recombinant SFV1/EGFP stock and virus ultracentrifugation

A recombinant alphavirus system based on the Semliki Forest virus (SFV) replicon was used. Recombinant SFV particles, namely SFV1/EGFP encoding EGFP, were produced in Baby Hamster Kidney cells (BHK-21) as previously described (Liljestrom et al. 1991; Zajakina et al. 2004). Briefly, RNA transcripts were synthesised *in vitro* by SP6 RNA polymerase using *SpeI* digested pSFV1/EGFP and *NruI* digested pSFV1/Helper plasmids as templates. Both *in vitro* synthesised RNAs were transfected into BHK-21 cells (from American Type Culture Collection) by electroporation as follows. The confluent cell monolayer was trypsinized, washed once with complete Glasgow MEM BHK medium (Gibco) (supplemented with 5% fetal calf serum, 20 mM HEPES (pH 7.3), 2mM glutamine, 10% tryptose broth) once with phosphate-buffered saline (PBS, without MgCl₂ and CaCl₂) and resuspended in 0.8 mL PBS. The cell suspension was mixed with *in vitro* made RNAs (20 µL of the reaction mix) and transferred to a 0.4 cm electroporation cuvette (Bio-Rad). Electroporation was carried out at room temperature by two consecutive pulses at 0.85 kV and 25 mF, using a Bio-Rad Gene Pulser apparatus. These conditions yielded virtually 100% transfection efficiency. Electroporated cells were diluted into complete Glasgow MEM BHK medium, transferred onto tissue culture plates and incubated at 37 °C.

Twenty four hours after electroporation, a volume of 87 mL of virus-containing cell medium (medium from four tissue culture plates after electroporation poured together) was clarified by centrifugation (Optima-L-100XP ultracentrifuge, Beckman, bucket SW28Ti rotor, 150 000 g, 20 min, 4 °C) and equally divided for use in three different concentration protocols (Fig. 1). The first protocol (I) required the direct ultracentrifugation of the virus-containing medium through 5 mL 20 % w/v sucrose solution prepared in TNE buffer [20 mM Tris-HCl (pH 7.), 100 mM NaCl, 2 mM EDTA]. The second (II) and the third (III) methods required the ultracentrifugation of the virus-containing medium through two-step gradients: 3 mL 20% and 3 mL 50% sucrose in TNE or 3 mL 20% and 3 mL 50% iodixanol in TNE, respectively. As an alternative to sucrose, the third method utilized iodixanol, a medical X-ray contrast solution, as previously described for use with other viruses (Peng et al. 2006; Gias et al. 2008).

After ultracentrifugation according to the first protocol, the supernatant was removed and the virus-containing pellet was resuspended by gentle agitation in 1 mL of TNE solution overnight at 4 °C. After ultracentrifugation according to protocols II and III, six 1 mL fractions from the

bottoms of the pierced tubes were collected. Additionally, to remove sucrose (protocol II) from the virus-containing sample, it (3 mL) was dialysed against TNE buffer for 22 h (dialysis membrane Orange Scientific, CelluSep H1, 25 kDa cut-off).

Recombinant alphavirus genome copy number and titer determination

The virus titre, expressed in infectious units (IU) mL⁻¹, in all fractions was quantified using fluorescence microscopy to detect EGFP expression upon BHK-21 cell infection. The infection of BHK-21 cells was carried out in serum-free BHK medium (Gibco) with serial dilutions of each fraction. The BHK-21 cells exhibited bright cytoplasmic EGFP fluorescence after infection with a series of SFV1/EGFP virus dilutions.

Quantification of the SFV1/EGFP virus titre (IU mL⁻¹) was based on the standard assumption, of “one infectious unit is equal to one infected cell.” Briefly, the SFV1/EGFP virus titre was calculated using the following formula: (monolayer socket area / area of one field of view) × (average counted fluorescent cells in 20 fields of view) × (the dilution factor) × (the volume fold, up to 1 mL).

To quantify the GCN mL⁻¹, viral RNA was isolated from the tested aliquots using TRI Reagent BD (Sigma) dissolved in 30 µL of distilled water, and 11 µL of RNA was reverse transcribed using the First-Strand cDNA Synthesis Kit, (USB-Affymetrix) applying the random hexamer oligonucleotides strategy. The following SFV-specific primers were generated using Beacon Designer software (Premier Biosoft): forward 5'-AGAGTCTCATTCCTGTATGC (SFV genome positions 1061 to 1081 nt), reverse 5'-GTTCTTCCGTTCAACTATCC (SFV genome positions 1182 to 1204 nt). The PCR generated a 143 bp DNA fragment using a MiniOpticon system (Bio-Rad) and the VeriQuest SYBR Green qPCR Master Mix (USB-Affymetrix) with 600 nmol concentrations of each primer and 0.5 µL of cDNA in a 25 µL reaction volume under the following conditions: pre-denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Ten-fold serial dilutions of the plasmid DNA (pSFV1/EGFP) were used to establish a standard curve (Bustin 2000). A linear range was observed when 10² to 10⁷ copies of plasmid DNA were used as templates in the amplification. The assay detection limit was 20 copies per reaction, and the amplification efficiency was 80 to 90%.

The viral samples, negative controls (isolated viral RNA without cDNA synthesis) and serially diluted standards were processed synchronously. The amplification product was confirmed by melting curve analysis (with a melting temperature of 80.2 °C). The results were analysed using OpticonMonitor software (Bio-Rad) and expressed as the gene copy number (GCN) mL⁻¹. The quantitative real-time PCR analysis was performed a minimum of three times for each standard and sample.

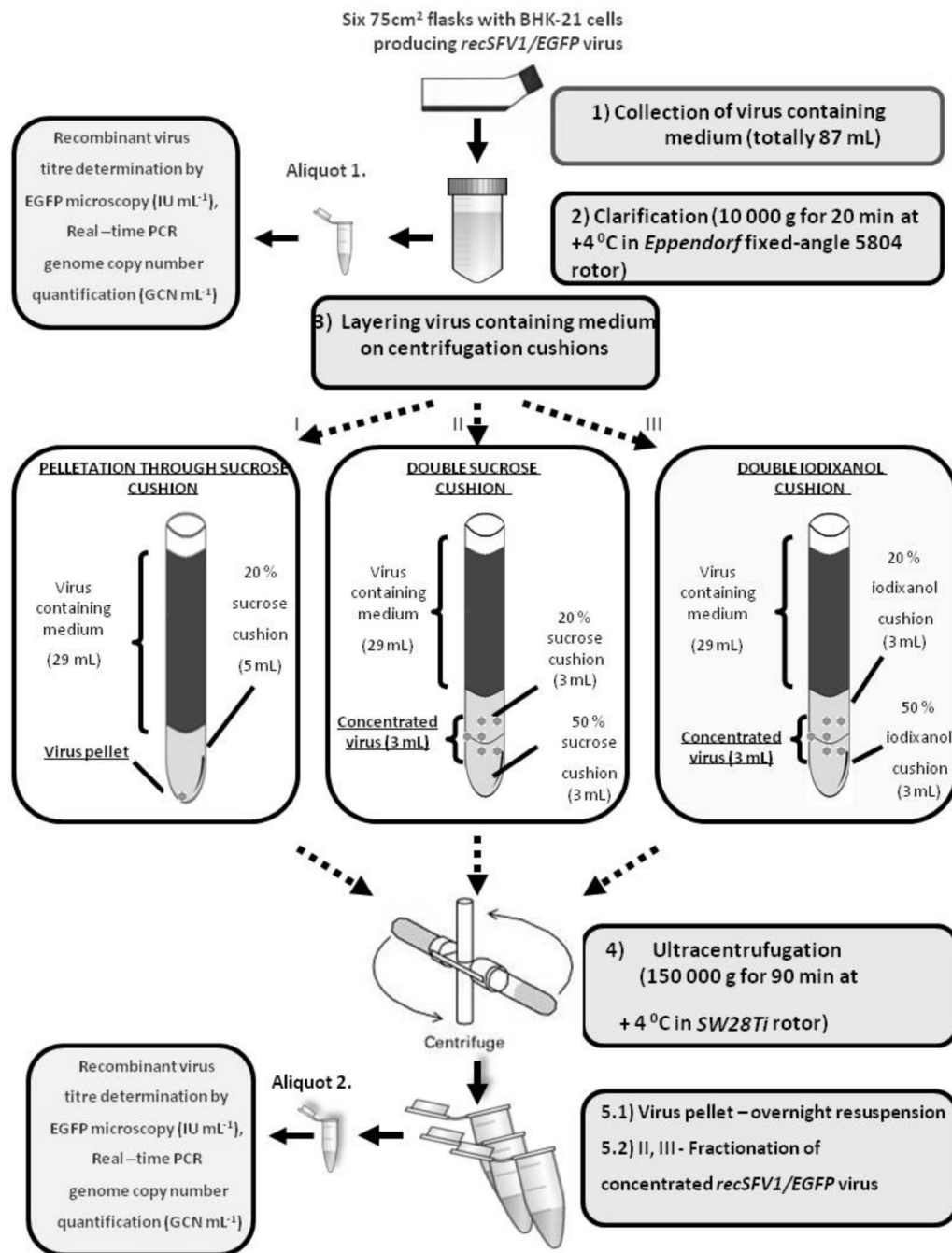


Fig. 1. Schematic representation of the recombinant SFV1/EGFP virus concentration methods studied: pelletation through a sucrose cushion (I), double sucrose cushion ultracentrifugation (II), and double iodixanol cushion ultracentrifugation (III). The BHK-21 cells were co-electroporated with in vitro transcribed SFV1/EGFP and Helper1 RNAs to elicit the secretion of recombinant SFV1/EGFP particles into the cell medium. (1) Two days post-electroporation, the virus-containing medium was collected and (2) clarified by centrifugation. (3) The virus-containing supernatant was layered onto sucrose or iodixanol cushions according to the indicated protocol (I, II, or III). (4) After ultracentrifugation, the virus-containing samples were collected. 5.1) The virus pellet was resuspended after pelletation. 5.2) The double cushion preparations were fractionated (bottom to top). The virus-containing aliquots from each method before (aliquot 1) and after centrifugation (aliquot 2) were subjected to EGFP fluorescence microscopy (IU mL⁻¹) and real-time PCR (GCN mL⁻¹).

Statistical analysis

Both virus GCN mL⁻¹ and titer data in each experiment were analysed using Statistica software (StatSoft Inc.). Results were presented as the mean ± standard deviation. Statistical calculations showed that the results are distributed in accordance with normal distribution, and are found to be statistically significant ($P \leq 0.05$; one-way ANOVA test), suggesting that our assays were prone to error.

Results

The ultracentrifugation on double sucrose cushion revealed the largest quantity of infection-competent virus particles in the second to fourth fractions from the bottom of the vial. However, in the double iodixanol cushion ultracentrifugation, the largest virus quantity was observed in the third to fifth fractions. These fractions were combined (3 mL) and used for the quantification of virus titre expressed in IU mL⁻¹ and in GCN mL⁻¹ real-time PCR quantification. Remarkably, the traces of infectious virus were detected in almost all fractions.

Therefore, three different SFV1/EGFP virus preparations (by three protocols) were obtained and then subjected to virus titre determination. The viral infectivity (IU mL⁻¹) was measured in four samples: in the unconcentrated supernatant and in the virus preparations obtained using pelleting (protocol I), double sucrose (protocol II) and double iodixanol (protocol III) cushions (Fig. 2A). The highest yield ($4.88 \times 10^8 \pm 15.59\%$ IU mL⁻¹) was obtained using protocol III, which generated a 3.64-fold higher virus concentration per milliliter compared with the initial unconcentrated viral stock ($1.34 \times 10^8 \pm 6.57\%$ IU mL⁻¹). Pelletation (protocol I) generated a low amount of infectious virus particles (recovery rate of 6.9%), and the concentration of these particles was only increased by 2.02-fold per milliliter. Moreover, the reproducibility of

this method was insufficient, as in sucrose pelletation the viral particles during settling on a bottom were too strongly deformed due to sticking to the bottom in such degree, that in consequence it was impossible to resuspend the pellet, resulting in a high standard deviation ($2.70 \times 10^8 \pm 34.46\%$). Finally, among three independent experiments, Protocol II generated a 2.95-fold increase in viral concentration.

Next, the real-time PCR results (GCN mL⁻¹) revealed virus concentration efficiencies and recovery ranges (Fig. 2B) similar to those measured using EGFP expression (IU mL⁻¹) (Fig. 2A). However, the obtained absolute values for GCN mL⁻¹ were two-fold higher than those calculated for IU mL⁻¹. For example, viruses concentrated using protocol III yielded the following values after centrifugation: $8.62 \times 10^8 \pm 12.78\%$ GCN mL⁻¹ and $4.88 \times 10^8 \pm 15.59\%$ IU mL⁻¹ for the same sample. The presence of defective (non-infectious) particles carrying genomes can introduce errors into RT-PCR-based quantifications. Moreover, for productive cell infection, more than one virus particle is necessary. Therefore, the IU mL⁻¹ virus titre can be significantly lower in comparison to the GCN mL⁻¹ titre.

Discussion

Recombinant alphaviruses can be produced efficiently *in vitro* with a virus titer of approximately 10^7 infectious units per mL (IU mL⁻¹) of cell culture medium. However, experiments with *in vivo* applications require an increased concentration of virus: approximately 10^8 to 10^9 IU mL⁻¹ for viral stocks. There are several criteria important in selecting appropriate methods for virus concentration and purification: (i) capacity for processing large volumes of viral preparations with high yield, (ii) preservation of viral stability, (iii) easy scale-up and (iv) low cost of operation (Morenweiser 2005). The nature of contaminating substances reflecting the choice of virus production method

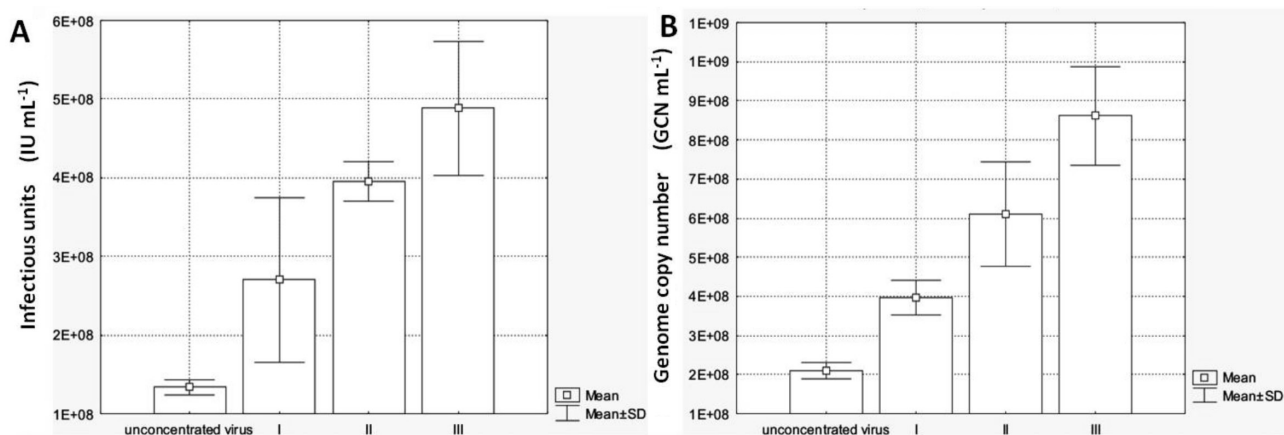


Fig. 2. Comparison of the SFV1/EGFP virus titre obtained by each respective virus concentration protocol: sucrose cushion pelleting (I), double sucrose cushion ultracentrifugation (II), and double iodixanol cushion ultracentrifugation (III). (A) The virus titre was determined by the expression of the EGFP gene (IU mL⁻¹). (B) The virus titre was evaluated using real-time PCR quantification of the viral GCN mL⁻¹.

Table 1. The concentration factor (fold increase) for each method used. SD of IU mL⁻¹ (A) or GCN mL⁻¹ (B) were measured in three independent experiments. The recovery yield characterises the percentage of the virus recovered after concentration (in 1 mL for protocol I and in 3 mL for protocols II and III) compared with the unconcentrated virus stock in a total of 29 mL of cell supernatant (see in the text)

	EGFP expression				Real-time PCR			
	Unconcentrated virus	I (pelletation)	II (double sucrose cushion)	II (double iodixanol cushion)	Unconcentrated virus	I (pelletation)	II (double sucrose cushion)	II (double iodixanol cushion)
Concentration factor (×)	1	2.02	2.95	3.64	1	1.88	2.91	4.10
SD of IU (%)	6.57	34.46	5.86	15.59	8.43	9.19	17.83	12.78
Recovery yield (%)	–	6.9	30.5	37.7	–	6.5	30.1	42.5

must also be considered while choosing the purification strategy.

It was difficult to choose the most appropriate method for concentrating recombinant alphaviruses because no studies comparing the efficiencies of the different methods have been published. Therefore purification of enveloped viruses remains problematic and still is dependent on empirical approaches. Several methods have been developed for the concentration of enveloped viruses (Killington et al. 1996). Purification strategies such as chromatography, ultrafiltration, precipitation and density - gradient ultracentrifugation are based on viral size and their antigenic properties.

For example, chromatographic methods have many limitations. In affinity chromatography they are associated with the high costs of antibodies. Second, they are serotype specific, which precludes application of the same process to all alphaviral serotypes. Purification of viral particles by ion-exchange chromatography cannot discriminate between full and empty viral capsids (Potter et al. 2002). In other chromatography modifications salt concentration and pH adjustments are necessary for optimisation of binding of the viral particles to chromatographic resins. The relative fragility of the viral particles must also be taken into account while choosing a chromatographic purification protocol. Enveloped viruses are difficult to purify due to the fragility of the virion envelope, which may be disrupted by rapid changes in osmotic pressure (Vaney et al. 2011). High salt concentrations in buffers, rapid changes in ionic strength, as well as exposure to a pH greater than 8.0, can result in a conformational change associated with loss of activity, aggregation or disruption of viral particles. In some purification schemes, degradation of the virus due to osmotic shock was also observed. Chromatography is more expensive than other methods, and furthermore, the cleaning-in-place of the resin is very restricted. Additionally, it is difficult to separate virus particles from high molecular weight contaminants that coelute with the virus in the excluding volume of the column. Because many chromatographic elution buffers

used for viral purification procedures are not suitable for *in vivo* manipulations, additional purification steps such as dialysis or concentration may be necessary.

Membrane filtration has also been used for concentration and partial purification of viral particles (Morenweiser 2005). While membrane filtration is an attractive procedure, there are problems with membrane fouling and, at high pressures, loss of viral infectivity, possibly due to shear forces that may act on the viral envelope. In addition, since separation is based on size differences, large molecular weight transduction inhibitors are coconcentrated with the viral particles, resulting in a reduction in viral transduction efficiencies.

For some viruses, precipitation with chemicals, such as polyethylene glycol (PEG), ammonium sulfate or calcium phosphate, has been useful in promoting viral aggregation with subsequent removal by low-speed centrifugation (Segura et al. 2011). PEG precipitation techniques are simple and render it easy to concentrate viruses, but the excess amount of extracted PEG hampers the PCR reaction. Polymers may co-precipitate along with viral particles, and unfortunately some viruses may lose infectivity (Andreadis et al. 1999). To overcome such difficulties purification methods frequently are used in combination (two or more purification methods may be performed sequentially). However, usually each of them requires either specialised, expensive equipment or exclusively sophisticated personnel and complex sample preparation procedures, which are time consuming and labour intensive. Thus, all these difficulties overshadow the benefits of these methods.

However, viruses are quite large when compared with biomolecules such as proteins, peptides, glycoproteins, sugars and nucleic acids that are present in lysates harvested from virus-infected cells. While proteins typically range from 0.005 to 0.08 × 10⁶ Da, viruses generally exceed 5 × 10⁶ Da and can have a size of 20 to 200 nm. This allows to concentrate viral particles using ultracentrifugation in dense solutes such as Dextran, colloidal silica, Percoll, CsCl or sucrose (Morenweiser 2005). Density-gradient centrifugation allows achieving a greater degree of

purification, in which viruses are separated by sedimentation through a gradient of a dense substance.

Unlike CsCl gradients, mild conditions of sucrose gradients do not inactivate the infectivity of viruses, do not cause aggregation of viral particles upon storage, are not toxic, are cheap and before *in vivo* use it is enough to perform a simple dialysis against saline to fully eliminate sucrose traces in concentrated virus preparations (Burova et al. 2005). In general, sucrose gradient centrifugation is considered to be chemically and physically appropriate for even labile viral particles.

The direct pelletation of viral particles through ultracentrifugation on sucrose remains a commonly used technique for the concentration of different enveloped viruses (Pyke et al. 2004; Tan et al. 2005; Sjoberg et al. 2006). However, our experimental results showed that this method did not achieve significantly concentrated recombinant alphavirus particles due to their extreme loss of virus infectivity. Using this method we lost more than 90 % of viral particles. (Fig. 2). This, probably, occurred due to squeezing force causing damages to the viral particles. Ultracentrifugation in double sucrose cushion gave better results. Using this technique recombinant alphaviral particles during ultracentrifugation were concentrated on the border between layers of 20 and 50% sucrose. This method appeared more appropriate for sensitive SFV1/EGFP particles, and resulted in lesser loss of concentrated viral particles. Using this method, 70% of the initial amount of viral particles were lost.

Alternatively, we used double iodixanol cushion for ultracentrifugation of viral particles. Iodixanol is less viscous than sucrose. It also has a low osmolarity and therefore could be subsequently used in viral infectivity assays directly without the need for prior removal. This unique feature of iodixanol made our work without application of dialysis less time-consuming. Iodixanol also was commercially available in isosmotic buffer. This allowed concentrating the virus under isosmotic conditions and better preserving the integrity and biological activity of the viral particles (Gias et al. 2008). In iodixanol centrifugation we lost only approximately 60% of viral particles. Our results agree with results in purification techniques previously reported in resembling studies (Lawrence, Steward 2010), as it was reported that usually during concentration procedures the recovery of viral particles varied from 15 to 60%, depending upon the technique used. Therefore we consider the double iodixanol cushion to be the most optimal method for concentration of alphaviral particles with standard losses.

Before use *in vitro* or *in vivo*, it was essential to determine the viability and titer after ultracentrifugation of the collected alphaviral particles. Since alphaviral replication defective SFV1/EGFP alphavirus do not produce plaques, its titres can not be determined by commonly used methods. Thus, for recombinant viruses encoding nonfluorescent proteins, the titers were measured using laborious indirect

immunofluorescence procedure. However, SFV1/EGFP alphavirus expresses EGFP marker gene, which can be readily detected using standard fluorescence microscopy (Lundstrom 2007).

Additionally, quantitative real-time PCR was used to determine the number of viral genomes in GCN mL⁻¹. The GCN mL⁻¹ also reflected the concentration of the SFV1/EGFP particles without considering their viral infectivity. Therefore, the proposed PCR-based genome quantification method was used as an alternative to immunofluorescence analysis.

In summary, we compared three similar methods for concentrating recombinant alphaviruses. All methods described here do not require sophisticated equipment and can be carried out with equipment that is present in routine microbiology laboratories. Double iodixanol cushion ultracentrifugation generated the highest recovery yield (40%), and the viral concentration was increased four-fold compared with the initial amount of the virus. This ultracentrifugation method is convenient, saves time and is an inexpensive for use with small-scale lab samples. It does not require any additional steps (such as dialysis or desalting) to remove the concentrating solution and can be used directly for *in vivo* applications. This method can be optimised for the concentration of other viruses as well.

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