A novel method for purification of recombinant adeno-associated virus vectors on a large scale

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Abstract **A novel method for recombinant adeno-associated virus (rAAV) purification on large scale is described. The method involves three steps, including chloroform treatment, PEG/NaCl precipitation and chloroform extraction. The whole procedure can be performed in four hours. Using this purification method, we can reproducibly obtain, from 4×10^9 of proviral cells cultured in roller bottles, purified rAAV-GFP stocks with titers of around 5×10^{13} particles/mL and purity greater than 95%. The infectious titers of the vector stocks were up to 2×10^{12} TU/mL, thus particle-to-infectivity rate was about 25. Under an electronic microscope, most rAAV particles appeared full and a few were in intermediate form. Empty particles were rarely seen. The purified rAAV-GFP stocks have been successfully used in *in vitro* and *in vivo* transfection experiments. Therefore, this new method offers a simple, rapid and cost-effective way for large-scale rAAV purification.**

Keywords: recombinant adeno-associated virus, chloroform, purification.

Recombinant adeno-associated virus (rAAV) has proven to be a promising gene delivery vector and has been tested in ever-widening array of human diseases. However, its application has been limited due to difficulties in obtaining enough quantity of purified rAAV at high efficiency and low expense. Many efforts have been made to improve rAAV yielding. Recently, our laboratory[1] and Conway et al.[2] independently constructed a recombinant herpes simplex virus type 1 (rHSV-1) vector possessing packaging functions for rAAV. Large-scale production of rAAV can easily be realized by infecting an rAAV proviral cell line with this rHSV helper virus.

However, purification and concentration of rAAV from crude cell lysate remains to be improved. The conventional rAAV purification method involves stepwise precipitation by using ammonium sulfate to isolate and concentrate rAAV from cell debris, followed by 2 or 3 rounds of CsCl density gradient centrifugation[3]. This method is elaborate and time-consuming and often results in poor recovery. Column chromatographic procedures have proved to be simple and efficient in rAAV purification, especially with heparin affinity chromatography[4,5] and immuno-affinity chomatography[6], based on the identification of heparin sulfate proteoglycan as a cellular receptor for AAV-2[7] and the identification of specific antibodies that recognize only assembled AAV-2 virons[8]. However, the cost is relatively high for large-scale rAAV preparation.

To simplify the purification process and decrease the expenses, we have developed a three-step procedure for large-scale rAAV purification, which involves chloroform treatment, PEG/NaCl precipitation, and chloroform extraction. The novel purification procedure described in this study proved to be simple, rapid, inexpensive, high recovery, practical and reproducible for large-scale rAAV preparation.

1 Materials and methods

(ⅰ) Materials. The plasmid pSNAV-GFP is an rAAV-GFP proviral construct that was constructed by inserting GFP gene derived from pGREEN LANTERN-1 (GIBCO BRL) into the cloning site of an rAAV vector pSNAV[9]. HeLa cell line and BHK-21 cell line were purchased from American Type Culture Collection. BHK/SG1 cell line established by our group is an rAAV proviral cell line harboring GFP expression cassette. The cells were grown in RPMI 1640 containing 10% foetal bovine serum (Hyclone) at 37℃. Generation of HSV1-rc/ΔUL2 expressing AAV-2 Rep and Cap, which possess packaging functions for rAAV, has previously been described[1]. The virus was propagated on BHK-21 cells. Glass roller bottles (ϕ110 mm×480 mm, Wheaton products) were employed for large-scale cultivation. Cell number is about 8×10^8 per bottle when the cells are confluent.

(ⅱ) Production of rAAV-GFP on large scale. BHK/SG1 cells were proliferated in 5 roller bottles by incubating at 37℃ at 1 roll/min. Confluent cells were infected by helper virus HSV1-rc/ΔUL2 at MOI of 0.1 in a volume of about 10 mL. 2 h later, 200 mL serum free RPMI 1640 media were added to each roller bottle followed by incubation at 37℃. To eliminate the necessity of handling large volumes of the start material, 100 mL culture medium was removed from each bottle after 24—36 h. The cells were incubated for additional 24—48 h until all the cells exhibited cytopathic effect (CPE) and were easy to fall into medium by shaking vigorously. Cultures, including cells and media, from 5 roller bottles were collected into 500-mL flasks (250 mL per flask), and referred to as start material.

(ⅲ) Purification of rAAV-GFP. See fig. 1 for the steps. 10% (*v*/*v*) chloroform was added to the start material, which was then incubated at 37℃ with vigorously

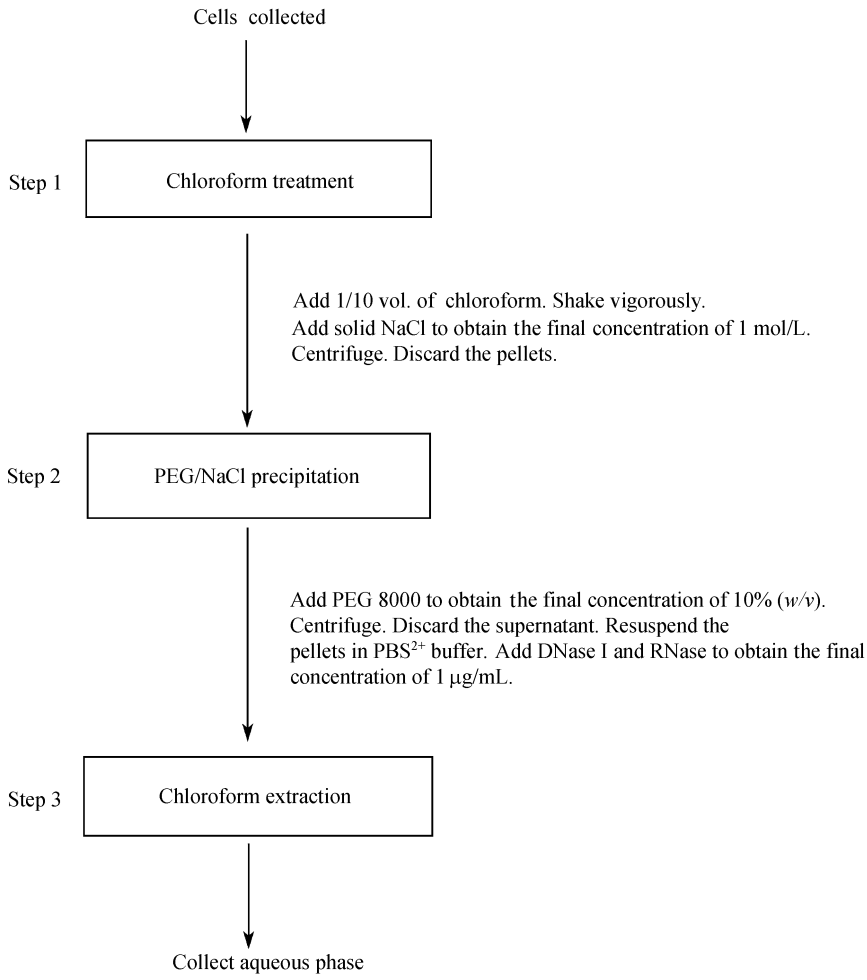

Fig. 1. The diagram illustrating the three steps in rAAV purification and concentration.

shaking for 1 h until all cells were lysed, which was determined by checking a drop of the culture under a microscope. Then solid NaCl was added till the final concentration was 1 mol/L by constant shaking for 30 min at room temperature. The supernatant was harvested by centrifugation at 12 000 r/m for 15 min at 4℃ and precipitates were discarded. An appropriate amount of solid PEG8000 was added to the supernatant to obtain a final concentration of 10% (*w*/*v*) by intermittent shaking at room temperature. The supernatant was cooled in ice water and let to stand still for 1 h. The precipitated rAAV particles were recovered by centrifugation at 11 000 r/m for 15 min at 4 ℃. The pellets were re-suspended in PBS^{2+} buffer with the final volume of 5 mL, and this suspension was referred to as crudely purified rAAV-GFP stock. DNase and RNase, both at the final concentration of 1 μg/mL, were added to the culture and incubated for 30 min. An equal volume of chloroform was added to the suspension which was then shaken vigorously for 2 min. The organic and aqueous phases were separated by centrifugation at 12000 r/m for 5 min at 4℃. The aqueous phases containing the rAAV-GFP were collected and referred to as the purified rAAV-GFP stock.

(ⅳ) Purity assay by SDS-polyacrylamide gel. The purity of rAAV-GFP was evaluated by electrophoresis in 10% SDS-PAGE. Each viral stock was mixed with an equal volume of 2×loading buffer and incubated in boiling water for 3 min. 10 μL samples were loaded for each lane and run at 200 V. The gel was stained with 0.25% (*w*/*v*) Coomassie Brilliant Blue R250 dissolved in a solution containing 45% methanol (*v*/*v*) and 10% glacial acid (*v*/*v*), and destained until clear bands with low background were shown.

(ⅴ) Electron microscopy for rAAV. An aliquot of purified rAAV-GFP stock was subjected to an electron microscope after negative staining with 2% uranyl acetate and then loading on 150 mesh nickel grids.

(ⅵ) Determination of genome containing particles of rAAV-GFP. Dot blot hybridization was used to determine the rAAV-GFP particles in purified rAAV-GFP

stock. An aliquot of the stock was incubated in boiling water for 5 min, serial 10-fold dilutions were applied to positively charged nylon membrane. The probe used for hybridization was a digoxigenin-labeled PCR fragment of GFP gene. To establish a standard curve, serial dilutions of pSNAV-GFP DNA were applied in parallel.

(vii) Determination of rAAV-GFP infectious titer. rAAV-GFP infectious titer was determined by assay for transgenic expression. HeLa cells were seeded on 24-well tissue culture plates with 5×10^5 cells per well and incubated at 37℃ overnight. Serial 10-fold dilutions of purified rAAV-GFP stock were added to each well and the adenovirus was also added at multiplicity of infection (MOI) of 5 to each well. 36 h later, the infectious titer of rAAV-GFP was determined by counting the green fluorescent cells.

2 Results

(i) Isolation, concentration and purification of rAAV-GFP. The flow chart of purification process is shown in fig. 1. Three steps, including chloroform treatment, PEG/NaCl precipitation, and chloroform extraction, were involved. The procedure can be performed within four hours. More than 100 folds of concentrating could be achieved from the start material. The recovery of rAAV infectious from the start material was estimated to be more than 90%.

(ii) Purity of rAAV-GFP. Capsid of AAV-2 is composed of three kinds of proteins: VP1, VP2 and VP3, with molecular weight of 87, 72 and 62 ku, respectively. The ratio of VP1, VP2 and VP3 in AAV-2 viron is 1∶1∶10. Therefore, three bands with specific patterns could be seen when AAV-2 virons were analyzed by SDS-PAGE. Analysis of rAAV-GFP purity at different stages of purification by Coomassie brilliant blue stained SDS acrylamide gel electrophoresis is shown in fig. 2. In this experiment, 5 mL final purified stock of rAAV-GFP was obtained from 500 mL start material harvested from 5 roller bottles (ϕ110 mm×480 mm) containing about 4×10^9 Cgfp cells. 5 μL aliquots of the purified rAAV-GFP stock (lane 2) and the crudely purified rAAV-GFP stock (lane 1) were subjected to 10% SDS acrylamide gel electrophoresis. Three clear bands representing AAV capsid proteins VP1, VP2 and VP3 could be seen on both lanes 1 and 2. However, many undesired proteins with various molecular weights could also be visualized on lane 1, while very low background was seen on lane 2, demonstrating that chloroform extracting could efficiently remove contaminants from rAAV stock with no significant loss of rAAV. The purity of rAAV-GFP was estimated to be more than 95% in the final rAAV-GFP stock by computer analysis. No significant capsid proteins could be visualized in the same volume of the start material of rAAV-GFP (lane 3). It is notable that the Commasse brilliant blue staining used in this study is a method less sensitive than silver staining or Western blot used more often by other reseachers, indicating that the concentration of rAAV virons is rather high.

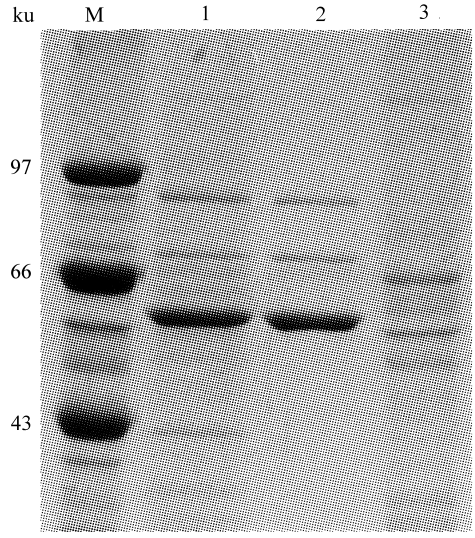

Fig. 2. Coomassie Brilliant Blue stained SDS-polyacrylamide gel electrophoresis of rAAV-GFP at various stages of purification. Each stock was mixed with an equal volume of 2×loading buffer and incubated in boiling water for 3 min before loading. 10 μL sample was loaded in each lane. The start material was shown in lane 3. The crudely purified stock of rAAV-GFP is shown in lane 1, and the purified rAAV-GFP is shown in lane 2. M, Standard proteins whose molecular weights are indicated on the left.

(iii) Electron microscopy. An aliquot of the purified rAAV-GFP stock after negative staining was taken to visualize rAAV-GFP particles by electron microscopy. Large amounts of AAV particles with clean background could be seen clearly. Most AAV particles appear full with a few intermediates (fig. 3). Empty particles were rarely seen. The result indicates that the purified rAAV-GFP stock contains rAAV particles of high concentration and purity.

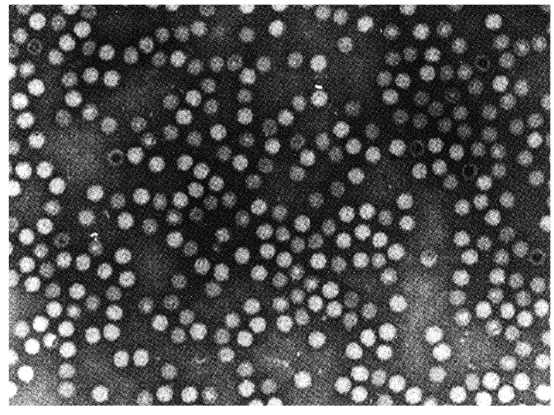

Fig. 3. Purified and concentrated recombinant adeno-associated virus particles visualized by electron microscopy after negative staining with uranyl acetate (× 35620).

(iv) Titers of the purified rAAV-GFP. rAAV genome containing particles was determined by the dot-blot method using digoxigenin-labeled GFP gene as probe. The physical titer of the purified rAAV-GFP is estimated to be about 5×10^{13} particles/mL. Infectious titer of the purified rAAV-GFP is about 2×10^{12} TU/mL determined by transgenic expression. The particle-to-infectivity rate is about 25, indicating that the infectivity of rAAV was not damaged in the purification process.

3 Discussion

Much progress has been made toward new strategies for rAAV production on a large scale. However, the efficient and cost-effctive method for rAAV purification is not available at present. Conventional rAAV purification method[3] involves stepwise precipitation by ammonium sulfate to isolate rAAV from cell debrises and concentration of rAAV, followed by 2 or 3 rounds of CsCl density gradient centrifugation. Then the residual CsCl is removed by dialysis. This method is elaborate and time-consuming and often results in poor recovery and reduced infectivity of rAAV. In addition, CsCl is a potential toxic reagent. Therefore, new strategies for rAAV purification are being developed. Using a monoclonal antibody that recognizes only assembled AAV virons[8], Grimm et al.[6] have described a procedure for rAAV purification by immuno-affinity column chromatography. The procedure can be performed in one day, and it results in an approximately 70% yield of rAAV that is at least 80% in purity. Two independent laboratories[4, 5] have developed the protocols in which heparin, an analog of the natural receptor for AAV[7], is used as the affinity matrix for rAAV purification. A potential problem with the use of this affinity approach is that many cellular proteins are also known to associate physically with heparin. Thus, it requires the incorporation of a specific strategy to remove contaminating heparin-binding proteins. To solve this problem, Zolotukhin et al.[5] semi-purified virus from a "free/thaw" cell lysate by centrifugation in a density step-gradient of an iodixanol non-ionic medium. The semi-pure preparation was then applied to an HPLC heparin column for further purification. The procedure takes less than 1 day and results in 50%—70% recovery of the virus that is more than 99% in purity. In contrast to the protocol described by Zolotukhin et al., Clark et al.[4] lysed vector producing cells by exposing them to 56℃ for 45 min. The combination of detergent lysis with heat treatment denatured undesired proteins and resulted in flocculent precipitate, which was removed before heparin chromatography. This approach results in a recovery greater than 70% of virus that is more than 99% in purity. Although these approaches greatly simplify the purification procedure and improve the recovery, the expense is relatively high for large-scale rAAV preparation.

We have focused our attention on developing a novel method for rAAV purification on a large scale. The resistance of AAV-2 particles to chloroform makes it an ideal reagent for rAAV isolation and purification. Noting that AAV has similar physical properties with some bacteriophage, for example, they all show resistance to chloroform, we tried for the first time to develop a novel method for rAAV purification by modifying a classic method for isolation and purification for bacteriophage λ[10]. Three purposes were achieved by treating the start material with chloroform: i) the helper virus HSV1-rc/ΔUL2 was inactivated completely, avoiding inactivating helper virus by incubation at 56℃ for 30—60 min; ii) the cells were lysed easily and rAAVs released efficiently and rapidly compared with the conventional approach by several rounds of freezing and thawing which is less efficient in rAAV releasing; iii) large amounts of cellular proteins were denatured and precipitated, and could be removed easily by centrifugation. As an alternative to ammonium sulfate, we have investigated the use of 1 mol/L NaCl/10% PEG for precipitating rAAV virons from large bulk of rAAV-containing stock. More than 100-fold concentration could be achieved. The crudely purified rAAV was extracted with chloroform and the aqueous phase was recovered by centrifugation. This step is very efficient in removing undesired proteins and residual PEG with almost no loss of rAAV. The purified rAAV stock could be stored at 4℃ for more than 1 month without significant decrease of infectious titer. These stocks have been successfully used in *in vitro* and *in vivo* transfection experiments. Further steps should be taken to remove residual chloroform before the stocks are used in clinical trials. Compared with conventional methods for rAAV purification, this protocol is time-saving and results in high recovery. The infectivity of rAAV is not damaged. Compared with the methods using column chromatography, this protocol is cost-effective and has a priority in large-scale rAAV preparation. rAAV obtained by this protocol have been used in *in vitro* or animal experiments for gene therapy. Clinical grade reagents can also be obtained by further purification. The protocol described in this note can be applied not only in the purification of rAAV in which HSV is used as a helper, but also in the purification of rAAV from helper-free or *in vitro* packaging system.

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