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Rapid and simplified purification of recombinant adeno-associated virus

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Preclinical gene therapy studies both in vitro and in vivo require high purity preparations of adeno-associated virus (AAV). Current methods for purification of AAV entail the use of centrifugation over either a CsCl or iodixanol gradient, or the use of chromatography. These methods can be cumbersome and expensive, necessitating ultrahigh speed gradient centrifugation or, for chromatography the use of other expensive equipment. In addition, these methods are time consuming, and the viral yield is not high. Currently no commercial purification kits are available for other than AAV serotype 2. A simplified method was used for the purification of AAV, with a viral yield that is able to be used effectively in adult and embryo mice. The method does not require ultrahigh speed gradient centrifugation nor chromatography. Instead, polyethylene glycol (PEG)/aqueous two-phase partitioning is used to remove soluble proteins from the PEG8000 precipitated virus–protein mixture. The procedure obtained rapidly up to 95% recovery of high quality purified AAV. The entire purification process, including HEK293 cell transfection, can be completed readily within one week, with purity seemingly higher than that obtained after one round of CsCl gradient purification.

1. Introduction

Recombinant adeno-associated virus (AAV) is a non-pathogenic gene therapy method that has been used in clinical trials previously. AAV is a good candidate for clinical use as it provides long-term transgene expression in animal models, it is associated with little toxicity, and has good overall safety profiles in both pre-clinical animal studies and in clinical trials. Many investigators have published the need for a highly purified AAV in large quantity in order to perform pre-clinical and clinical trials (Ayuso et al., 2010; Lock et al., 2010).

Currently, the principal methods for purifying AAV include using CsCl ultrahigh speed density gradient centrifugation or chromatography, with the former being adopted by many investigators. This method, however, is time consuming (Ayuso et al., 2010). Iodixanol (OptiPrep) ultrahigh speed density gradient centrifugation is a similar method developed for AAV purification, with a higher viral recovery compared to the CsCl method, but the Iodixanol reagent is expensive and the method still remains relatively time-consuming; the latter drawback prevents its use for assessing quickly gene expression effects of AAV in in vivo studies (Klein et al., 2008; Zolotukhin et al., 1999).

The majority of chromatographic purification methods use either ion exchange or affinity based techniques to purify AAV. The latter method uses an antibody against AAV capsid, which therefore recognizes only assembled (serotype specific) particles, which has limited its popularity among investigators. Heparin-based affinity chromatography has also been used to purify AAV, a technique based on the property that AAV binds heparin sulfate proteoglycan with high efficiency during AAV infection; however, heparin affinity is known to be serotype specific (AAV 2). During heparin-based chromatography, other proteins present in the cell lysate that have affinity for heparin sulfate co-elute with AAV, and in the case of production of AAV in insect cells, baculovirus may also bind to heparin. This contamination would necessitate at least one additional step to purify further AAV from the eluate of heparin-based chromatography. Thus, both the CsCl or iodixanol ultrahigh speed density gradient centrifugation, as well as chromatography are time consuming and serotype-restricted techniques.

AAV has been shown to have more than ten serotypes that show tropism in vivo in a tissue dependent manner (Wu et al., 2006). Commercial purification kits are available only for AAV serotype 2. Specifically, a serotype 2 AAV purification kit is the certain commercially available kit on the market. Current chromatography purification methods are not fit for a variety of AAV serotypes, and ultrahigh speed density gradient centrifugation is time consuming

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and needs expensive equipment (ultrahigh speed centrifuge) that are not always available.

The technique of chemical partitioning in aqueous two-phase systems (ATPs) has been shown to be a powerful method for separating and purifying mixtures of soluble proteins. ATPs can remove undesirable by-products in crude supernatants. These systems are composed of aqueous solutions of either two water-soluble polymers, usually polyethylene glycol (PEG) and dextrin, or else a polymer and a salt, usually PEG and a phosphate or sulfate. Compared with other commonly used separation and purification techniques, ATPs have a number of advantages, such as ease of scale-up, the ability to handle particulate materials, especially for the virus, and the ease of processing. No one has previously reported the use of ATPs to remove soluble bulk proteins from AAV–protein mixtures to further purify AAV.

AAV serotype 8 has been used widely in recent years to transduce multiple organs in animals, but a purification kit is not available commercially. A simple method described in this study can be used in a typical laboratory with only a standard desktop centrifuge, avoiding the need for ultrahigh speed centrifuge or chromatography, to purify AAV serotype 8 and also conventional AAV serotype 2. The method involves PEG8000 precipitation, chloroform treatment, PEG/$(NH_4)_2SO_4$ aqueous two phase extraction and final dialysis. The whole purification process can be achieved in one week, starting with transfecting HEK293 cells in culture, through to harvesting cells to purify the virus for use in in vivo studies. This rapid and simple method should be applicable for other serotype AAV purification and can be used to assess quickly the function of a gene or shRNA carried by the AAV vector. The purity of this method is high enough for use in in vivo studies.

2. Materials and methods

2.1. Chemical reagents and equipment

Polyethylenimine (PEI) was purchased from Polysciences (Warrington, PA). NaCt (sodium citrate), $(NH_4)_2SO_4$, Na_2CO_3, 40% PEG1450, 40% PEG4000 and 40% PEG8000 (w/w) solution were purchased from Sigma–Aldrich (St. Louis, MO). Eppendorf 5810R and 5415R desktop centrifuges with cooling system, 37 °C water bath, sterile cell culture hood and cell culture incubator were used in this study.

2.2. Plasmids

Plasmids used to co-transfect in this study are (1) Vector, pAAV-CMV-ZsGreen plasmid, briefly pAAV-GFP carrying the expression cassette flanked by the viral ITRs (provided by Dr. Bing Wang); (2) a packaging plasmid carrying the serotype 8 or serotype 2 AAV rep and cap genes; and (3) a helper plasmid carrying the adenovirus helper functions was purchased from Applied Viromics, LLC. (Fremont, CA). The transgene used in the experiments was ZsGreen (Zoanthus sp. green fluorescence protein (GFP) under the control of a CMV promoter.

2.3. Cell culture and triple plasmid PEI transfection techniques

On day one HEK293 cell are maintained in DMEM (Cellgro, Mediatech. Manassas, VA) with 10% FBS (Cellgro, Mediatech. Manassas, VA). By day 2, at 80% confluent, the culture medium is changed to 2% FBS that morning before transfection in the evening. For PEI triple plasmid co-transfection of HEK293 cells (the plasmids are pAAV-GFP, pHELP and prep-Cap AAV8 or AAV2), plasmids in the ratio of 2:1:1 are PEI mixed by vortexing, incubated for 15 min at room temperature, and then added dropwise to plates. The PEI:DNA ratio is maintained at 2:1 (w/w) (Figs. 1 and 2).

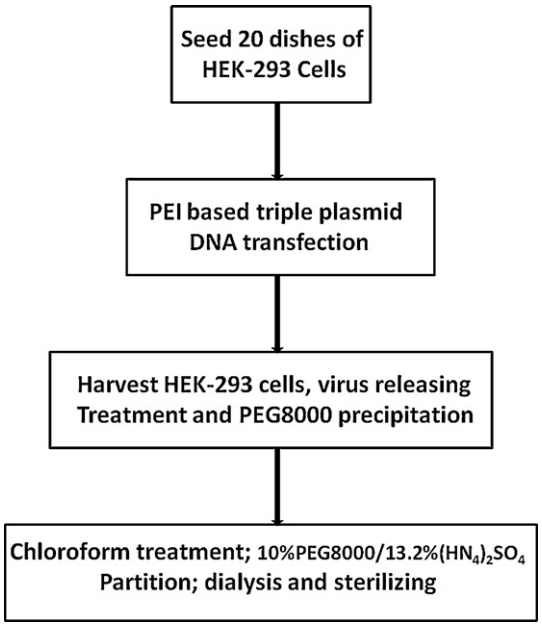

Fig. 1. Flow chart of purification protocol. The diagram illustrates the steps in AAV purification that were investigated in this study.

2.4. Virus harvest and primary treatment

The viral particles were harvested from the culture media of the transfected HEK293 cells. The culture media was centrifuged at 3000 × *g* for 10 min to pellet the cells and cell debris. Around 320 ml of culture medium from twenty 15 cm dishes was taken, clarified and collected.

The cells in culture medium were pooled into one 50 ml conical tube with 5 ml of lysis buffer (50 mM Tris–Cl per 150 mM NaCl per 2 mM $MgCl_2$, pH 8.0), and AAV particles were released from the cells with three freeze/thaw cycles between dry ice-ethanol and 37 °C water bath. 50 U/ml benzonase and 10 U/ml RNase I were added to the virus-released solution and incubated for 30 min at 37 °C water bath. The surfactant, 0.5% sodium deoxycholate, was then added and incubated for an additional 30 min for further treatment of virus released from the DNase and RNase-treated cells broth. To remove the cell debris, the cells were centrifuged at 2500 × *g* for 10 min and the supernatant harvested. For the clarified cell lysate,

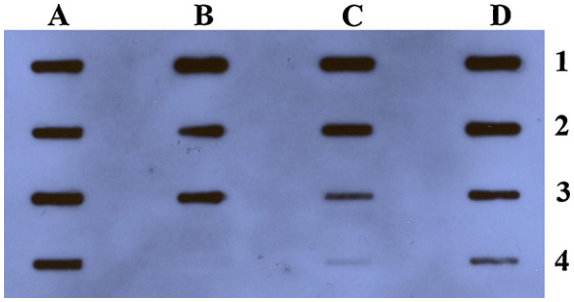

Fig. 2. Titer determination of two-phase partitioning purified AAV-GFP by dot blot. A1–A3 stet repeated 10%PEG8000–13.2%$(NH_4)_2SO_4$ (pH8.0) ATPs purified virus; A4 is chloroform treated rough virus; B1–3 are stet repeated 10% PEG8000–10%$(NH_4)_2SO_4$ (pH8.0) ATPs purified virus; C1–3 are three-times repeated 20%PEG4000–13.2$(NH_4)_2SO_4$ (pH8.0) ATPs purified virus; B4 and C4 are virus in the top PEG fraction; D1–4 are serial dilutions (10 times diluted in PBS) of CsCl purified AAV, starting at $5.2 \times 10^{12} \pm 1.5 \times 10^{11}$.