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AAV2 production with optimized N/P ratio and PEI-mediated transfection results in low toxicity and high titer for *in vitro* and *in vivo* applications

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A B S T R A C T

The adeno-associated virus (AAV) is one of the most useful viral vectors for gene delivery for both *in vivo* and *in vitro* applications. A variety of methods have been established to produce and characterize recombinant AAV (rAAV) vectors; however most methods are quite cumbersome and obtaining consistently high titer can be problematic. This protocol describes a triple-plasmid co-transfection approach with 25 kDa linear polyethylenimine (PEI) in 293T cells for the production of AAV serotype 2. Seventy-two hours post-transfection, supernatant and cells were harvested and purified by a discontinuous iodixanol density gradient ultracentrifugation, then dialyzed and concentrated with an Amicon 15 100,000 MWCO concentration unit. To optimize the protocol for AAV2 production using PEI, various N/P ratios and DNA amounts were compared. We found that an N/P ratio of 40 coupled with 1.05 μg DNA per ml of media (21 μg DNA/15 cm dish) was found to produce the highest yields for viral replication and assembly measured multiple ways. The infectious units, as determined by serial dilution, were between 1×10^8 and 2×10^9 IU/ml. The genomic titer of the viral stock was determined by qPCR and ranged from 2×10^{12} to 6×10^{13} VG/ml. These viral vectors showed high expression both *in vivo* within the brain and *in vitro* in cell culture. The use of linear 25 kDa polyethylenamine PEI as a transfection reagent is a simple, more cost-effective, and stable means of high-throughput production of high-titer AAV serotype 2. The use of PEI also eliminates the need to change cell medium post-transfection, lowering cost and workload, while producing high-titer, efficacious AAV2 vectors for routine gene transfer.

1. Introduction

Recombinant adeno-associated viruses (rAAV), a 22 nm naked, single-stranded DNA member of parvoviridae family (Hoggan et al., 1966), has emerged as one of the preferred gene-delivery viral vectors in the past decade. Use of rAAV has lead to promising leaps in gene therapy (Bainbridge et al., 2008; Bowles et al., 2012; Jayandharan et al., 2011; Kaplitt et al., 2007; Niemeyer et al., 2009). It has become such a powerful tool in the laboratory and clinic because of its ability to infect a large range of tissues. It exhibits long-term gene transduction, offers a wide variety of cell and tissue tropism due to availability of multiple serotypes, exhibits low immunogenicity, and has an overall good safety profile (Lock et al., 2010; Wright, 2009). High-throughput production of rAAV serotype 2 (rAAV2) is the goal of our viral vector core, and it requires the subsequent implementation and optimization of a production protocol that is adaptable to multiple inserted transgenes, in order to obtain a pure and high titer virus without extensive workload and cost.

In this protocol, rAAV2 was produced by a three-plasmid co-transfection system in HEK293 T cells, wherein (1) a helper plasmid (pHelper) contained the adenovirus genes E2a, E4 and viral associated RNAs necessary for AAV reproduction, (2) a vector plasmid carries the transgene flanked by inverted terminal repeats (ITRs), and (3) the packaging plasmid provides the AAV serotype 2 replication (rep2) and encapsidation (cap2) genes. The HEK293 T cell line provides trans-acting adenovirus E1 genes. This protocol reports an optimization of rAAV2 production by reducing the amount of DNA used for transfection (to 1.05 μg/ml), which decreased the amount of work and cost in DNA purification while observing no decrease in viral titers.

Established calcium-phosphate transfection (Ca–P) has been found to be more laborious, expensive, and yield inconsistent and unstable results because of narrow optimal conditions, especially

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temperature and pH (Wright, 2009). Comparatively, using linear 25 kDa polyethylenimine (PEI) as a transfection reagent is cost-effective, has improved stability, and has the ability to function at a wider pH range (Lock et al., 2010; Reed et al., 2006). The use of PEI also eliminates the need to change cell medium post-transfection (Durocher et al., 2007), drastically lowering cost and workload.

PEI is a polymer in which a secondary amine occupies the third atom per monomer unit. It is the amino group of PEI that causes the polymer to display a high cationic charge density potential and buffer capacity (Boussif et al., 1995b; Clamme et al., 2003a,b). This allows the PEI to form ionic interactions with the phosphate backbones of DNA to form a condensed PEI-DNA complex that can be transported into the cell *via* endocytosis (Godbey et al., 1999). Behr (Behr, 1997) observed that DNA-PEI complexes escape endosomes *via* a proton sponge effect that promotes osmotic swelling and disrupting of the endosomal membrane. However, how DNA later enters the nucleus is still unknown. Within the complex, the particular ratio between molar units of PEI nitrogen atoms to units of DNA phosphate atoms, known as the N/P ratio, has been found to correlate with enhanced transfection efficiencies (Guo et al., 2012; Ogris et al., 1998; Reed et al., 2006). However, the N/P ratio can differ between protocols, and most ratios utilize an excess of PEI for transfection. One reason that these ratios sometimes include excessive PEI is centered on PEI's ability to enhance the proton sponge effect and disrupt endosomes (Boeckle et al., 2004)

In the described experiments, it was observed that using a linear 25 kDa PEI to transfect HEK293T cells with a three plasmid system using N/P ratio of 40, with an excess of PEI, yields high titer rAAV2, without observable cell death after 72 h transfection. In addition to harvesting viral particles in lysed whole cell homogenate, the supernatant was also harvested using a 40% PEG8000/2.5 N NaCl precipitation. While some consider this to be trivial to final viral titers (Reed et al., 2006; Zolotukhin et al., 1999), others have found supernatant to contain up to 50% of total viral particles (Ayuso et al., 2010) and suggest that it is a pure (no cellular debris) source of rAAV vectors (Lock et al., 2010). To further isolate virions, a discontinuous iodixanol gradient centrifugation followed by dialysis in an Amicon 15 100,000 MWCO concentration unit was employed. Optimization of this PEI protocol resulted in the use of less DNA, decreased workload, and lower cost for reagents. A simple, effective, and adaptable approach for producing high titer recombinant AAV2 using 25 kDa linear PEI is described. This further demonstrates the utility of AAV2 *in vitro* in cell culture and *in vivo* in the rodent brain.

2. Materials and methods

2.1. AAV vectors

Three vectors were used in each experiment: (1) An AAV vector containing GFP or GOI (gene of interest) flanked by the ITRs; (2) a packaging vector containing the AAV serotype rep2 and cap2 genes; (3) a helper vector containing the adenovirus helper functions. (All plasmids purchased from Cell biolabs Inc.).

2.2. PEI preparation and calculation

PEI (Polyethylenimine, linear, MW 25,000 from Polysciences, cat # 23966) was prepared to a final concentration of 7.5 mM (based on monomer units). pH was adjusted to 8.0 and filtered through 0.22 μm filter. 20 ml-aliquots were frozen and thawed three times, and stored at −20 °C. The amount (μl) of PEI was calculated based on the following equation (Reed et al., 2006): PEI (μl) = $3 \times D \times R/S$, where D = total amount of plasmid DNA used (μg), R = N/P ratio (ratio of nitrogen content in PEI to phosphorous content in DNA), S = concentration of the PEI stock (mM, monomer unit).

2.3. Preparation of AAV-293T cells for transfection

HEK 293 T cells (gift from Dr. Thomas Kukar; also available from ATTC) were maintained in complete medium (4.5 g/L-Glucose and L-Glutamine containing DMEM supplemented with 10% FBS and 1% Pen-Strep) and incubated at 37 °C, 5% CO_2. One day before transfection, HEK 293 T cells were seeded onto 20 150 mm plates at a density of 1×10^7 cells per plate in 18 ml of complete medium. The cells were approximately 70% confluent on the day of transfection.

2.4. Co-transfection of three plasmids

All plasmid concentrations were adjusted to 1 μg/ul in TE buffer before mixing the following: 105 μg of pAAV vector, 105 μg of pAAV-R_2C_2 and 210 μg of pHelper (total of 420 μg DNA for 2015 cm plates; equal to 1.05 μg per ml of media).

The transfection mixture, which included a total of 420 μg DNA, 4 ml of 1.5 M NaCl, 6.72 ml of PEI and 29.28 ml of sterile dd H2O in a 50 ml sterile tube, was vortexed a few seconds, and incubated at room temperature for 20 min. 2 ml of the mixture was then added to each plate in a dropwise manner. The plates were returned to the incubator for 72 h at 37 °C, 5%CO_2.

2.5. Virus harvest

Cell culture media and transfected cells were harvested separately. 40% PEG (cat# P2139, Sigma) in 2.5 N NaCl was added to the supernatant to a final concentration of 8%, and incubated on ice for 2 h. The cell pellet was suspended in 14 ml of lysis buffer (50 mM Tris–Cl, 150 mM NaCl and 2 mM $MgCl_2$) and stored at 4 °C. Following the 2 h incubation, the supernatant was centrifuged at 2500 g for 30 min at 4 °C to pellet the PEG-precipitated virus. The cell lysate and pelleted supernatant precipitate were combined and then treated with 750 μl of 10% sodium deoxycholate (cat# BP349-100, Fisher) to final concentration 0.5%, and benzonase (cat# E8263-25KU, Sigma) to a final concentration of 50 μg/ml. After 30 min incubation at 37 °C, 3 ml of 5 M NaCl were added to the sample to decrease aggregation of the virus followed by a 30 min 50 °C incubation, and three freeze–thaw cycles between −80 °C and 37 °C. Debris was pelleted by spinning at 12,000 × *g* for 30mins at 4 °C, and separated from the lysate with the use of a popper pipette needle attached to a 20 ml syringe. The lysate was then pipetted into a Beckman 25 × 89 quick seal tube and immediately underlaid with 9 ml of 15%, 6 ml of 25%, 5 ml of 40% and 5 ml of 60% iodixanol (cat# D1556-250, sigma) using a pipetting needle attached to a 10 ml syringe. If necessary, 1× PBS was added to fill the tube completely.

2.6. Centrifugation and virus retrieval

The quick seal tube containing the prepared iodixanol gradient and lysate was centrifuged at 500,000 × *g* using rotor 70 Ti for 1.5 h at 18 °C. To retrieve the virus, the tube was then secured in a clamp stand set to eye-level, after which an 18 G needle was inserted into the top of the tube to allow air to enter. Another 18 G needle attached to a 10 ml syringe was inserted just below the interface of the 40% and 60% iodixanol layers with the bevel of the needle up. Approximately 4 ml of sample were extracted containing the AAV in the 40% iodixanol layer.

2.7. Dialysis and concentration

The extracted virus was then added to 7 ml of PBS previously dispensed into the top portion of an Amicon 15 100,000 MWCO (cat# UFC910024, Fisher) concentration unit followed by a 10 min

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