Purification of Adeno-associated Virus (AAV) Vectors Using Norgen's AAV Purification Kit

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INTRODUCTION

Adeno-associated virus is a small single stranded DNA virus and is a member of the Parvoviridae family. AAV is not associated with any known diseases in humans. Recombinant adeno-associated virus (AAV) vectors are highly promising gene delivery tools. They may be used for a variety of gene transfer applications, both in vivo and in vitro. AAV vectors can be used to transduce a wide variety of tissues including lung, liver, muscle, brain, heart, pancreas and spleen, amongst others. A number of clinical trials utilizing AAV have been completed or are currently on-going, targeting a wide range of diseases. Among them are phase 1/2 clinical trials for a number of inherited diseases, such as α1 antitrypsin deficiency, cystic fibrosis, hemophilia B, and phase 1/2 trials for acquired diseases such as Parkinson's disease, severe heart failure, and rheumatoid arthritis. Purification of AAV vectors on both a small and large scale can be time consuming and often requires highly specialized equipment. Norgen's AAV Purification Kits reduce the time required for purifications (2.5-4.5 hours) and the need for specialized equipment such as ultracentrifuges, while still delivering a high yield of purified AAV vector.

MATERIALS AND METHODS

AAV Production

Approximately 1 x 10^7 HEK293 cells were seeded onto 15 cm plates one day prior to transfection, in complete Dulbecco's minimal essential media (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin. The AAV production process is represented graphically by Figure 1. HEK293 cells were transfected according to previously described methods (1), with plasmids containing the rep, AAV9 cap, and helper genes, as well as another plasmid containing the inverted terminal repeat (ITR) flanked AAV genome to be packaged. Cells were co-transfected with a plasmid containing an AAV vector genome encoding heat stable human placental alkaline phosphatase driven by the CAG promoter. The next day, the media was removed and replaced with DMEM + L-glutamine in order to eliminate serum proteins and extracellular plasmids within the media. HEK293 cells were allowed to grow for up to 6 days post transfection, allowing for accumulation of AAV within the media, after which cells and media were harvested into separate fractions.

AAV Purification

Conditioned media containing AAV was purified using the Norgen AAV Purification Kit. Volumes ranging from 1 to 33 mL of media/supernatant containing AAV was purified for the experiment investigating the linearity of elution titer over different input volumes of AAV . For the in vivo and in vitro experiments, 300 mL of AAV9 containing media was split amongst 9 different preps from the Norgen AAV Purification Kit. The total eluate (9.9 mL) was concentrated using a 100 kDa MWCO Amicon® Ultra-4 centrifugal filter unit (Millipore Sigma) down to approximately 0.5 mL for intranasal administration and filtered with a 0.22 µm filter.

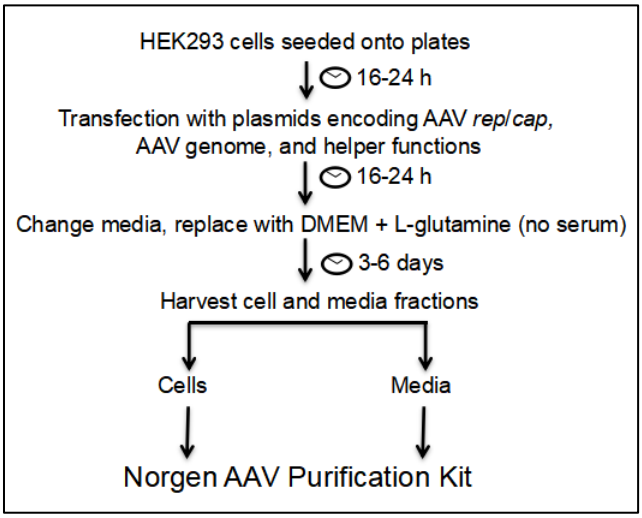

Figure 1. AAV Production and Purification Using Norgen's AAV Purification Kit

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DNA Extraction

Five µL of eluted AAV samples were transferred into PCR tubes. Four µL of Enzyme Incubation Buffer (Norgen's RNase-Free DNase I Kit, Cat# 25710) was added to each sample. Norgen DNAse I (2 U) was also added to each sample to digest contaminating non-viral DNA. Samples were then incubated at 37°C for 30 minutes. EDTA was added to a final concentration of 5 mM before heating the samples at 90°C for 20 minutes in order to inactivate the DNAse.

qPCR Quantification

The extracted AAV DNA was then utilized as template in a real-time PCR reaction. In short, 1 µL of extracted DNA was added to 20 µL of real-time PCR reaction mixture containing TaqMan probe (0.2 mM) and primers (0.5 mM) specific for the AAV2 ITR. The specific probe and primers have been described previously (2). The PCR reactions were amplified under the following conditions: 95°C for 5 minutes for an initial denaturation, 40 cycles of 95°C for 15 second for denaturation and 60°C for 30 seconds for annealing and extension. The reaction was run on a CFX 96 real-time system (Bio-Rad).

In vitro Transduction with AAV

HTX cells were seeded into 10 cm or 6 well dishes so that they would become confluent the next day. Upon reaching confluency, 50 µL of purified vector was pipetted onto cells. Either AAV9 or a bovine AAV isolate (AAV-Ca) was used. Plates were rocked gently back and forth to disperse the vector across the monolayer. Cells were fixed with 2% paraformaldehyde/PBS 48 hours post transduction, and washed 3 times with PBS. Endogenous alkaline phosphatase (AP) was heat inactivated by heating at 68°C for 1 hour. Heat stable AP within the cell monolayer was stained using AP buffer containing 100 mM Tris pH 8.5, 100 mM NaCl, 50 mM $MgCl_2$ and the addition of substrates 5-Bromo-4-chloro-3-indolyl phosphate (Sigma Aldrich) and Nitroblue tetrazolium (Invitrogen).

In vivo administration of AAV

Following concentration with the Amicon Ultra-4 (100 K MWCO), the titer was 4 x 10^{11} ss genomes/mL in a 500 µL volume. This was divided amongst 2 different C57BL/6 mice (~1 x 10^{11} ss genomes per mouse). To target the mouse respiratory tract, AAV was administered using a modified intranasal technique as described previously (3). Mouse tissues were harvested 1 month post transduction. Tissues were processed and prepared as previously described (3).

RESULTS AND DISCUSSION

AAV was purified using Norgen's AAV Purification Kit. Different volumes of input were purified in order to determine AAV recovery across a range of input amounts. For the Norgen AAV Purification Kit, cells and supernatant volumes ranging from 1 mL to 33 mL were purified (Figure 2). A linear increase in vector recovery (R^2=0.99) was observed for these different volumes, demonstrating that the purification process was effective for a range of sample volumes and that the resin did not reach saturation over the range of volumes tested.

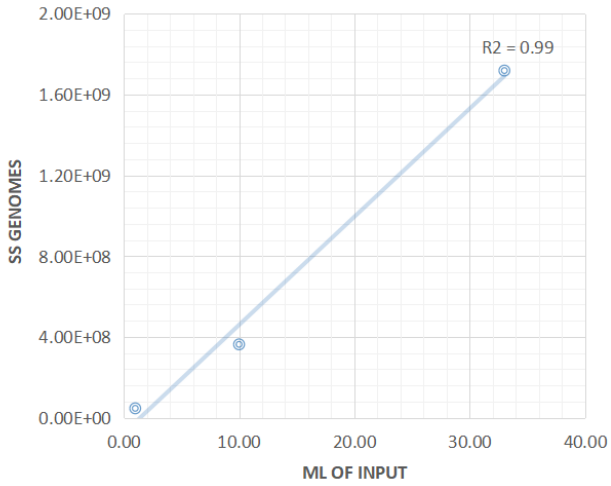
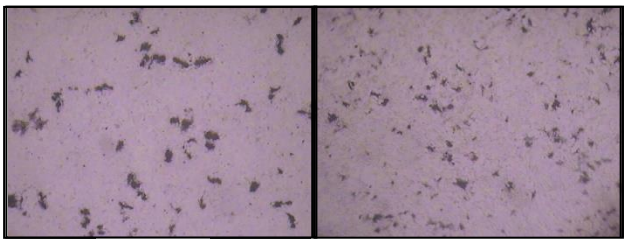


Figure 2. Copy number of single stranded AAV genomes for Norgen AAV Purification Kit elutions purified from 1, 10, or 33 mL of input consisting of supernatant from transiently transfected AAV producer cells. A linear increase in elution copy number across different input volumes demonstrates the utility of the Norgen AAV Purification Kit for small scale AAV purification.



AAV9 **Bovine AAV(AAV-Ca)**

Figure 3. HTX cells transduced with 50 µL eluted vector from the Norgen AAV Purification Kit. Both AAV9 and bovine AAV (isolate AAV-Ca) were tested *in vitro* on HTX cells. Dark/purple staining represents cells that have been transduced by AAV.

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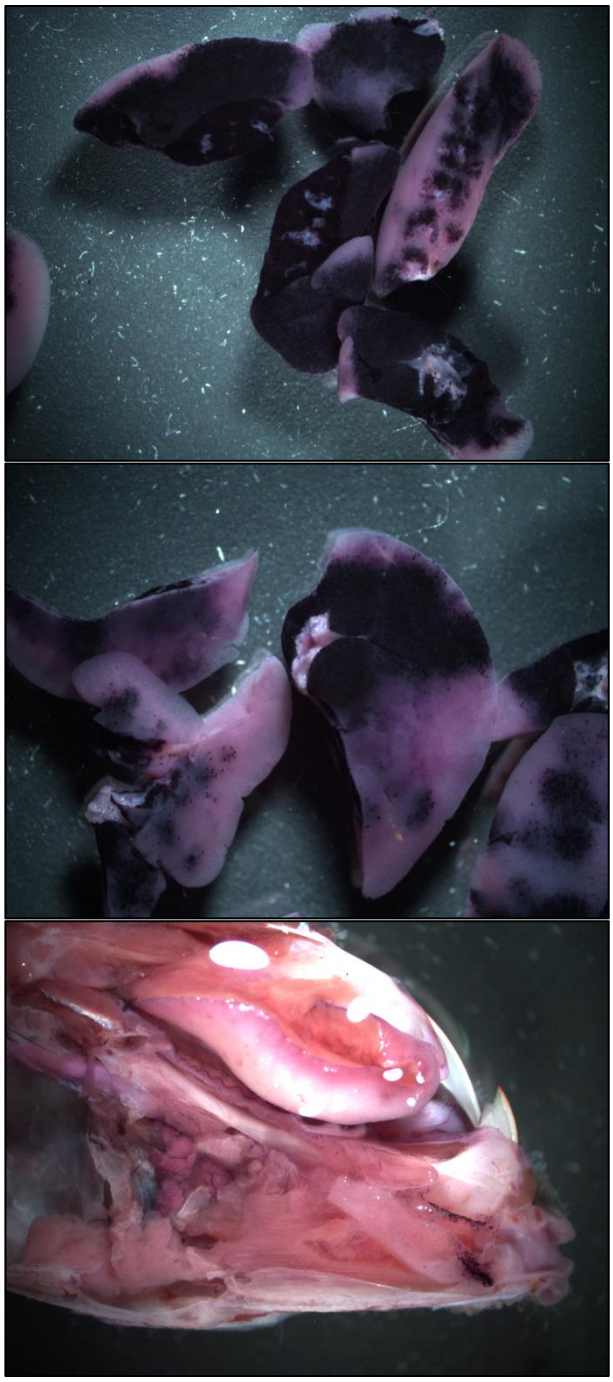

Figure 4. In vivo testing of AAV vector purified using the Norgen AAV Purification Kit. **Top and middle panels:** areas of transduction in the mouse lung from AAV9 purified using the Norgen AAV Purification Kit following intranasal administration. **Bottom panel:** mouse nose transduced by AAV9 purified using the Norgen AAV Purification Kit following intranasal administration. Dark/purple staining represents cells that have been transduced by AAV and are expressing the alkaline phosphatase reporter gene.

The biological activity of AAV vectors purified using the Norgen AAV Purification Kit was investigated by transducing HTX human fibrosarcoma cells with the eluted vector (Figure 3). Dark purple staining indicates cell transduction at that location by the AAV vector.

To investigate the utility of the Norgen AAV Purification Kit for in vivo use, AAV9 containing cell culture media/supernatant was used as input. Approximately 300 mL of AAV9 media/supernatant was purified by pooling eluate from 9 preps of the Norgen AAV Purification Kit. Purified eluate was concentrated with an Amicon 100 K centrifugal filtration device and then filtered with a 0.22 µm syringe filter yielding a 500 µL preparation containing 4 x 10^{11} ss genomes/mL. Approximately 250 µL of AAV9 vector was delivered via the intranasal route to mice and one month post transduction, tissues were harvested. Robust dark purple staining (indicating expression of the alkaline phosphatase reporter gene) was observed in the tissues of the mouse respiratory tract, including the various lobes of the lung as well as the nose (Figure 4). These results demonstrate the utility of the Norgen AAV Purification Kit for in vivo studies utilizing AAV.

CONCLUSIONS

1. The Norgen AAV Purification Kit enables rapid (2.5-4.5 h) AAV purification from cells or supernatant containing AAV. High linearity across a range of volumes (1 to 33 mL) demonstrates efficient binding and release of AAV. Multiple preps can be combined into a single prep and concentrated (via Amicon or other methods) for in vivo use
2. AAV purified using the kit is highly active in vitro and in vivo

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