

Purification of Adeno-associated Virus (AAV) Vectors Using Norgen's AAV Mini and Midi Purification Kits

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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 α 1 antitrypsin deficiency, cystic fibrosis, haemophilia 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 (<1.5 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 1×10^7 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*, AAV6 *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 7 days post transfection, allowing for accumulation of AAV within the media, after which cells and media were harvested into separate fractions.

AAV Purification

AAV cell pellet and media fractions were purified separately using Norgen's AAV Purification Mini Kit (Cat# 63200) and Norgen's AAV Purification Midi Kit (Cat# 63300). Volumes ranging from 0.5 to 15 mL of media/cells mixed with media were purified using the Norgen AAV Mini Kit and 2-50 mL of media/mixed cells and media were purified using the Norgen AAV Midi Kit.

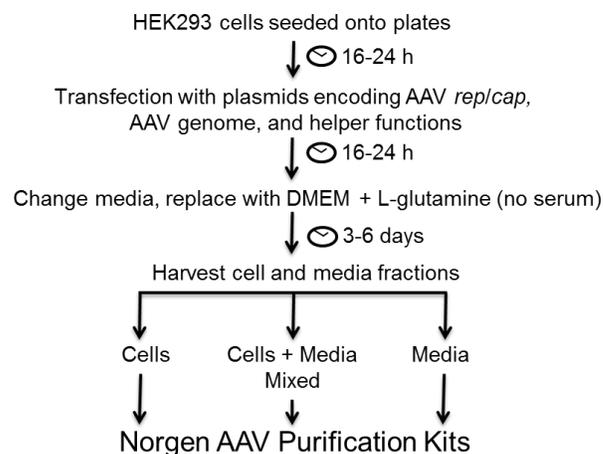


Figure 1. AAV Production and Purification Using Norgen's AAV Purification Kits

DNA Extraction

Fifteen μ L of eluted AAV samples were transferred into PCR tubes. Four μ L 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).

Cell Transduction

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. 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 MgCl₂ and the addition of substrates 5-Bromo-4-chloro-3-indolyl phosphate (Sigma Aldrich) and Nitroblue tetrazolium (Invitrogen).

RESULTS AND DISCUSSION

AAV was purified using Norgen's Mini and Midi kits. Different volumes of input were purified in order to determine AAV recovery across a range of input amounts. For the Norgen AAV Mini Kit, mixed cells and supernatant volumes ranging from 2.5 mL to 15 mL were purified (**Figure 2**). A linear increase in vector recovery ($R^2=0.98$) 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. Similarly, experiments utilizing the Norgen AAV Midi Kit demonstrated a linear increase ($R^2=0.98$) in vector recovery across volumes ranging from 2 to 50 mL (**Figure 3**).

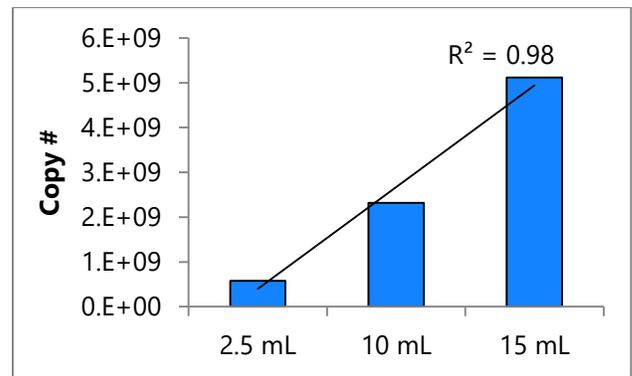


Figure 2. Copy number of single stranded AAV genomes for Norgen AAV Mini Kit elutions purified from 2.5, 10, and 15 mL of input consisting of mixed cells and supernatant from AAV producer cells. A linear increase in elution copy number across different input volumes demonstrates effectiveness of the mini kit for small scale AAV purification.

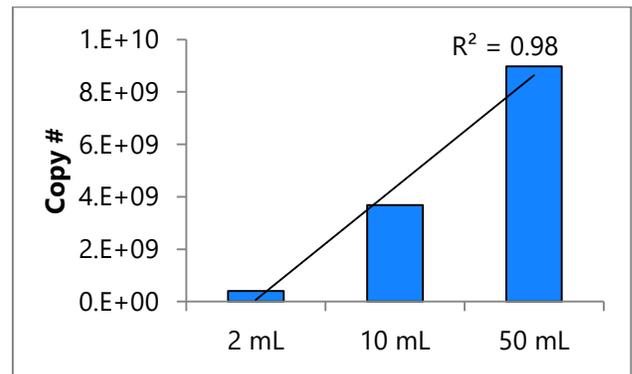


Figure 3. Copy number of single stranded AAV genomes for Norgen AAV Midi Kit elutions purified from 2, 10, and 50 mL of input consisting of mixed cells and supernatant from AAV producer cells. A linear increase in elution copy number across different input volumes demonstrates the utility of the Norgen AAV Midi Kit for small and medium scale AAV purification.

The biological activity of AAV vectors purified using the Norgen AAV Mini Kit and Norgen AAV Midi Kit was investigated by transducing HTX human fibrosarcoma cells with the eluted vector (**Figures 4 and 5** for Mini and Midi kit, respectively). Dark purple staining indicates cell transduction at that location by the AAV vector. An increase in staining was observed for both the Mini and Midi kits with increasing input volumes, demonstrating an increasing yield of biologically active AAV for both kits.

Mock 2.5 mL input 10 mL input 15 mL input

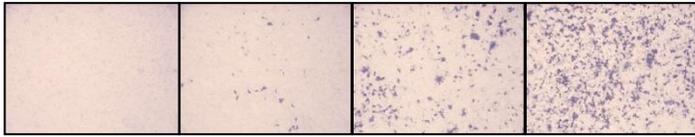


Figure 4. HTX cells transduced with 50 µL eluted vector from 2.5, 10, or 15 mL input using the Norgen AAV Purification Mini Kit. Dark/purple staining represents cells that have been transduced by AAV.

Mock 2 mL input 10 mL 50 mL

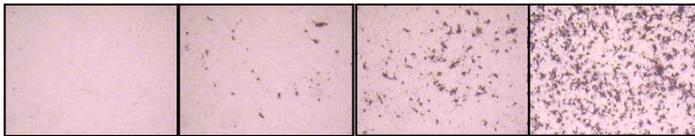


Figure 5. HTX cells transduced with 50 µL eluted vector from 2, 10, or 50 mL input using the Norgen AAV Purification Midi Kit. Dark/purple staining represents cells that have been transduced by AAV.

CONCLUSIONS

1. Norgen AAV Purification Mini Kit and Norgen's AAV Purification Midi Kit enable rapid (<1.5 h) AAV purification from a mixed input of cells and supernatant containing AAV
2. High linearity across a range of volumes at small and medium scale demonstrates efficient binding and release of AAV at different volumes
3. AAV purified using the Mini and Midi Kits is highly active *in vitro*

REFERENCES

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