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Production of Adeno-Associated Virus Vectors in Cell Stacks for Preclinical Studies in Large Animal Models

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TITLE:

Production of Adeno-Associated Virus Vectors in Cell Stacks for Preclinical Studies in Large Animal Models

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KEYWORDS:

Adeno-associated virus vectors, gene therapy, cell stacks, affinity purification, preclinical studies, large animal models, transgene expression, AAV6, AAV6.2FF

SUMMARY:

Here we provide a detailed procedure for large-scale production of research-grade AAV vectors using adherent HEK 293 cells grown in cell stacks and affinity chromatography purification. This protocol consistently yields >1 x 10^{13} vector genomes/mL, providing vector quantities appropriate for large animal studies.

ABSTRACT:

Adeno-associated virus (AAV) vectors are among the most clinically advanced gene therapy vectors, with three AAV gene therapies approved for humans. Clinical advancement of novel applications for AAV involves transitioning from small animal models, such as mice, to larger animal models, including dogs, sheep, and nonhuman primates. One of the limitations of administering AAV to larger animals is the requirement for large quantities of high-titer virus. While suspension cell culture is a scalable method for AAV vector production, few research labs have the equipment (e.g., bioreactors) or know how to produce AAV in this manner. Moreover,

AAV titers are often significantly lower when produced in suspension HEK 293 cells as compared to adherent HEK293 cells. Described here is a method for producing large quantities of high-titer AAV using cell stacks. A detailed protocol for titering AAV as well as methods for validating vector purity are also described. Finally, representative results of AAV-mediated transgene expression in a sheep model are presented. This optimized protocol for large-scale production of AAV vectors in adherent cells will enable molecular biology laboratories to advance the testing of their novel AAV therapies in larger animal models.

INTRODUCTION:

Gene therapy utilizing adeno-associated virus (AAV) vectors has made huge strides over the past three decades[1,2]. Demonstrated improvements in a diverse range of genetic diseases, including congenital blindness, hemophilia, and diseases of the musculoskeletal and central nervous system, have brought AAV gene therapy to the forefront of clinical research[3,4]. In 2012, the European Medicines Agency (EMA) approved Glybera, an AAV1 vector expressing lipoprotein lipase (LPL) for the treatment of LPL deficiency, making it the first marketing authorization for a gene therapy treatment in either Europe or the United States[5]. Since then, two additional AAV gene therapies, Luxturna[6] and Zolgensma[7], have received FDA approval, and the market is expected to expand quickly over the next 5 years with as many as 10–20 gene therapies expected by 2025[8]. Available clinical data indicate that AAV gene therapy is a safe, well-tolerated, and efficacious modality making it one of the most promising viral vectors, with above 244 clinical trials involving AAV registered with ClinicalTrials.gov. The increasing interest in clinical applications involving AAV vectors requires robust and scalable production methods to facilitate the evaluation of AAV therapies in large animal models, as this is a critical step in the translational pipeline[9].

For AAV vector production, the two main requirements are the AAV genome and the capsid. The genome of wild-type (wt)-AAV is single-stranded DNA that is approximately 4.7 kb in length[10]. The wt-AAV genome comprises inverted terminal repeats (ITRs) found at both ends of the genome, which are important for packaging, and the *rep* and *cap* genes[11]. The *rep* and *cap* genes, necessary for genome replication, assembly of the viral capsid, and encapsulation of the genome into the viral capsid, are removed from the viral genome and provided in trans for AAV vector production[12]. The removal of these genes from the viral genome provides room for therapeutic transgenes and all the necessary regulatory elements, including the promoter and polyA signal. The ITRs remain in the vector genome to ensure proper genome replication and viral encapsulation[13,14]. To improve the kinetics of transgene expression, AAV vector genomes can be engineered to be self-complementary, which mitigates the need for conversion from single-stranded to double-stranded DNA conversion during AAV genome replication, but reduces the coding capacity to ~2.4 kb[15].

Beyond AAV genome design, capsid serotype selection determines the tissue and cell tropism of the AAV vector *in vivo*[2]. In addition to tissue tropism, different AAV serotypes have been shown to display different gene expression kinetics[16]. For example, Zincarelli et al.[17] classified different AAV serotypes into low expression serotypes (AAV2, 3, 4, 5), moderate expression serotypes (AAV1, 6, 8), and high expression serotypes (AAV7 and 9). They also categorized AAV serotypes

into slow-onset expression (AAV2, 3, 4, 5) or rapid-onset expression (AAV1, 6, 7, 8, and 9). These divergent tropisms and gene expression kinetics are due to amino acid variations in the capsid proteins, capsid protein formations, and interactions with host cell receptors/co-receptors[18]. Some AAV capsids have additional beneficial characteristics such as the ability to cross the blood-brain barrier following intravascular administration (AAV9) or reside in long-living muscle cells for durable transgene expression (AAV6, 6.2FF, 8, and 9)[19–20].

This paper aims to detail a cost-effective method for producing high-purity, high-titer, research-grade AAV vectors for use in preclinical large animal models. Production of AAV using this protocol is achieved using dual-plasmid transfection into adherent human embryonic kidney (HEK)293 cells grown in cell stacks. Furthermore, the study describes a protocol for heparin sulfate affinity chromatography purification, which can be used for AAV serotypes that contain heparin-binding domains, including AAV2, 3, 6, 6.2FF, 13, and DJ[21–22].

A number of packaging systems are available for the production of AAV vectors. Among these, the use of a two-plasmid co-transfection systems, in which the *Rep* and *Cap* genes and Ad helper genes (E1A, E1B55K, E2A, E4orf6, and VA RNA) are contained within one plasmid (pHelper), has some practical advantages over the common three-plasmid (triple) transfection method, including reduced cost for plasmid production[23–24]. The AAV genome plasmid containing the transgene expression cassette (pTransgene), must be flanked by ITRs, and must not exceed ~4.7 kb in length. Vector titer and purity can be affected by the transgene due to potential cytotoxic effects during transfection. Assessment of vector purity is described herein. Vectors produced using this method, which yield a 1 x 10^{13} vg/mL for each, were evaluated in mice, hamsters, and ovine animal models.

[Insert **Table 1** here]

PROTOCOL:

1. Double plasmid transfection of HEK293 cells in cell stacks

1.1 Thaw a cryo-vial of HEK293 cells in a bead bath set at 37 °C.

NOTE: Pre-warm complete DMEM to 37 °C while cells are thawing to ensure the cold temperature does not shock cells when plating. Ensure the cells have a low passage number, ideally less than 20, to ensure optimal growth and transfection efficiency. Ensure that the cells are certified to be mycoplasma-free.

1.2 Transfer the contents of the cryo-vial dropwise into a 15 mL conical tube containing 10 mL of pre-warmed complete DMEM and centrifuge the cells at 500 x *g* for 5 min.

1.3 Aspirate the media, and then resuspend the HEK293 cells in 20 mL of pre-warmed complete DMEM. Seed the cells in a 15 cm plate and incubate at 37 °C, with 5% CO_2.

1.4 Split the cells from one 15 cm plate into three for seeding in the cell culture chamber.

1.4.1 Once cells are 80% confluent, aspirate the media and gently wash the plate with 3 mL of PBS to not disrupt the monolayer. Then, aspirate PBS and add 3 mL of trypsin.

1.4.2 Incubate for 2 min at 37 °C until the cells lift from the plate, and then neutralize trypsin by adding 7 mL of complete DMEM to the plate.

1.4.3 Collect all the media and cells into a 15 mL tube and pellet the cells by centrifuging at 500 x *g* for 5 min.

1.4.4 Aspirate the supernatant from the 15 mL tube and resuspend the cell pellet in 3 mL of complete DMEM. Add 1 mL to each 15 cm plate containing 20 mL of complete DMEM; gently rock the plates to distribute the cells evenly, and incubate at 37 °C, with 5% CO_2.

1.5 Once the cells are 80% confluent, repeat steps 1.4.1 and 1.4.2. Collect the supernatant in 50 mL conical tubes, and gently invert the tube to ensure the cells are homogeneous.

1.6 Determine the cell density by mixing 10 µL of the samples of cells with 10 µL of trypan blue and adding the mixture to a cell counting slide for analysis in the cell counter.

1.7 Mix 1 L of pre-warmed complete DMEM with the needed cell suspension to seed the cell culture chamber (surface area of 6360 cm^2) with 1 x 10^4 cells/cm^2. Pour the cell mixture into the cell culture chamber and gently rotate to evenly distribute the cells across each monolayer (**Figure 1**) and incubate at 37 °C, with 5% CO_2.

1.8 In addition to the cell culture chamber, plate a 15 cm plate with 1 x 10^4 cells/cm^2 as a reference for confluency.

1.9 Following ~65-h incubation, check the reference plate for confluency—ideally ~80%–90% confluent.

NOTE: Pre-warm complete DMEM for adding to the cell culture chamber at 37 °C.

[Insert **Figure 1** here]

1.10 Prepare polyethyleneimine (PEI)/DNA mixture at a concentration ratio of 3:1 (w/w).

1.10.1 Prepare the DNA mixture in a 50 mL conical tube by adding 475 µg of pTrangene and 1425 µg of pHelper to 40 mL of reduced-serum medium to create a 3:1 ratio of pHelper:pTrangene.

NOTE: The PEI/DNA mixture calculator can be found using **Table 2**.

1.10.2 Add 5.7 mL of PEI (1 g/L) to the reduced-serum medium and the DNA mixture dropwise. Then, vortex briefly and incubate for 10 min at room temperature.

NOTE: As PEI/DNA incubates at room temperature, it will become slightly cloudy.

1.11 After 8 min of PEI/DNA incubation, remove the media from the cell culture chamber.

NOTE: Ensure to loosen both orange caps to maintain a smooth flow of media to avoid dislodging of cells.

1.12 Add PEI/DNA to 1 L of pre-warmed complete DMEM, and slowly pour the mixture into the cell culture chamber port. Distribute the liquid evenly to all the rows (**Figure 1**) and incubate for 72 h at 37 °C, with 5% CO_2.

2 Harvesting AAV and chemical lysis of the transfected HEK293 cells

2.1 Shake the cell culture chamber vigorously to dislodge the cells until the media appears cloudy from dislodged cells and pour into four 500 mL centrifuge tubes.

2.2 Centrifuge the tubes at 18,000 x *g* for 30 min at 4 °C to pellet the cells. Pour the clarified supernatant into 1 L polyethylene terephthalate copolyester (PETG) bottle.

NOTE: If one does not have access to a high-speed centrifuge, centrifuge at 12,000 x *g* for 40 min. The pelleted cells may not be solid at this speed and will slide as pouring out supernatant.

2.3 Resuspend the cell pellets in 500 mL centrifuge tubes with 50 mL of lysis buffer and incubate for 60 min at 37 °C.

2.4 Centrifuge the tubes at 18,000 x *g* for 30 min, and then transfer the supernatant into the same 1 L PETG bottle. Discard the pelleted cell debris.

NOTE: Purify the clarified supernatant immediately and store at 4 °C for up to 72 h, or at -80 °C for long-term storage. Do not store at -20 °C.

3 AAV Vector purification using heparin affinity chromatography

3.1 Remove the crude lysate from -80 °C and leave at 4 °C overnight to thaw. Once thawed, use a 0.22 µM filter to filter the crude lysate.

3.2 To passivate the centrifugal concentrator, add 4 mL of filter pre-treatment buffer to a centrifugal concentrator for each heparin sepharose column being used. Passivate the centrifugal concentrator at room temperature for 2–8 h. Set up passivation immediately before the purification steps.

3.3 Set up the tubing and the pump (**Figure 2**).

3.3.1 Place the tubing in a peristaltic pump and run 20 mL of 1 M NaOH. Next, run 50 mL of molecular grade water, and then run 50 mL of basal DMEM.

3.3.2 Attach 5 mL heparin sepharose column to tubing and run 25 mL of basal DMEM to remove the preservative.

3.4 Run 0.2 µM of the filtered crude lysate through the column at a flow rate of 1–2 drops/s.

[Insert **Figure 2** here]

NOTE: Ensure not to introduce bubbles or allow the column to run dry, as this will compromise the column and prevent the elution of AAV. Discard the column if it runs dry and use a new column for the remainder of crude lysate.

3.5 Load all of the crude lysate onto the heparin column and use the following solutions to wash the column.

3.5.1 Wash using 50 mL of 1x Hank's Balanced Salt Solutions (HBSS) without Mg^{2+} and Ca^{2+}.

3.5.2 Wash using 15 mL of 0.5 % N-Lauroylsarcosine in HBSS without Mg^{2+} and Ca^{2+}.

3.5.3 Wash using 50 mL of HBSS without Mg^{2+} and Ca^{2+}.

3.5.4 Wash using 50 mL of HBSS with Mg^{2+} and Ca^{2+}

3.5.5 Wash using 50 mL of 200 mM NaCl/HBSS with Mg^{2+} and Ca^{2+}.

3.5.6 Elute 5 x 5 mL (25 mL total) with 300 mM of NaCl/HBSS with Mg^{2+} and Ca^{2+} and label the elutions as E1–E5 (each elution is of 5 mL).

3.6 Concentrating the virus using a centrifugal concentrator

3.6.1 Spin the centrifugal concentrator containing the pre-treatment buffer at 900 x *g* for 2 min. Discard the flow-through.

3.6.2 Wash the centrifugal concentrator filter with 4 mL of HBSS with Mg^{2+} and Ca^{2+} and centrifuge at 1000 x *g* for 2 min; discard the flow-through.

3.6.3 Add the elution E2 to the centrifugal concentrator. Spin at 1000 x *g* for 5 min and discard the flow-through.

3.6.4 Finish adding E2 and then add E3 to the centrifugal concentrator and spin at 1000 x *g* for 5 min until the concentrated virus is approximately 1 mL.

NOTE: Avoid centrifuging the vector such that the volume is below the level of the filter. Do not concentrate E1, E4, or E5 in the centrifugal concentrator, as they contain very little to no vector and contain contaminants.

3.6.5 Remove the concentrated virus from the centrifugal concentrator using a p200 filtered tip and place it into a sterile 1.5 mL centrifuge tube.

3.6.6 Rinse the centrifugal concentrator with 200 µL of HBSS with Mg^{2+} and Ca^{2+} to dislodge any remaining AAV from the filter. Pipette up and down vigorously multiple times (for ~30 s) to dislodge any virus adhered to the membrane and place in the 1.5 mL centrifuge tube with the remainder of the virus. Mix the tube well.

3.6.7 Aliquot 5 µL for DNA extractions and store the purified vector at -80 °C.

3.7 Wash the column using 25 mL of 2 M NaCl. Further, use 25 mL of 0.1% Triton X-100, pre-heated to 37 °C to wash the column. Next, wash the column using 50 mL of sterile dH_2O, and then wash using 25 mL of 20% ethanol.

3.8 Ensure the column membrane is fully saturated in 20% ethanol, as this is the storage solution. Seal the column with plugs provided and store at 4 °C.

3.9 Store the tubing in 1 M NaOH.

NOTE: If cleaned properly, heparin sepharose columns can be re-used up to five times.

4 AAV genomic DNA extraction

4.1 Prepare the reaction mix mentioned in **Table 3** in a PCR tube for DNase treatment.

[Insert **Table 3** here]

4.2 Vortex the PCR tube to mix and pulse the PCR tube to spin down the contents.

4.3 Using a thermocycler, incubate at 37 °C for 20 min followed by 75 °C for 15 min to heat inactivate the DNase.

4.4 Add 5 µL of Proteinase K.

4.5 Using a thermocycler, incubate at 50 °C for 60 min and then at 95 °C for 30 min to heat inactivate Proteinase K.

4.6 Use a DNA clean-up kit to remove potential contaminants.

NOTE: This step was performed using a commercially available blood and tissue clean up kit (**Table of Materials**).

4.6.1 Add 200 µL of AL buffer (Blood and tissue clean up kit, **Table of Materials**) to the PCR tube containing the DNase/Proteinase K treated vector.

4.6.2 Vortex the PCR tube and incubate at 56 °C for 10 min in a thermocycler.

4.6.3 Pipette the liquid from the PCR tube into a sterile spin column sitting in a collection tube.

4.6.4 Add 200 µL of 100% ethanol to the column and mix thoroughly by vortexing.

4.6.5 Centrifuge at 6,000 x *g* for 1 min and discard the flow through.

4.6.6 Add 500 µL of buffer AW1 (Blood and tissue clean up kit, **Table of Materials**) to the spin column.

4.6.7 Centrifuge at 6,000 x *g* for 1 min and discard the flow through.

4.6.8 Add 500 µL of buffer AW2 (Blood and tissue clean up kit, **Table of Materials**) to the spin column.

4.6.9 Centrifuge at 15,000 x *g* for 3 min and discard the flow through.

4.6.10 Place the spin column into a sterile 1.5 mL centrifuge tube and add 200 µL of buffer AE (Blood and tissue clean up kit, **Table of Materials**) directly to the spin column membrane.

4.6.11 Incubate at room temperature for 1 min.

4.6.12 Centrifuge at 6,000 x *g* for 1 min to elute the DNA.

4.6.13 Store the DNA at -20 °C.

5 Titration of AAV vector genomes using quantitative polymerase chain reaction and a Simian Virus 40 (SV40) probe

NOTE: Perform all qPCR work in a PCR hood using filtered pipette tips to avoid external DNA contamination. If the AAV genome does not encode an SV40 polyA sequence, use a probe against the ITR described elsewhere[25]. Ensure the plasmid DNA selected as standard contains SV40 polyA sequence.

5.1 Stock standard preparation

5.1.1 Dilute the stock plasmid DNA standard (pTransgene plasmid containing SV40 polyA sequence) to a final concentration of 10 µg/µL and store at -20 °C in 6 µL aliquots.

5.1.2 Determine the copy number present in plasmid DNA standard using the following online calculator[26].

NOTE: Use a plasmid DNA used for the standard produced by a commercial vendor to ensure quality and correct concentration. Prepare a large quantity of standard (e.g., 10 mL) to conduct bridging studies when transitioning to a newly prepared standard.

5.2 Prepare the following reagent mix mentioned in **Table 4** for both the samples and the standard in a 1.5 mL centrifuge tube.

NOTE: Prepare sufficient overage of master mix. See **Table 5** for primer/probe sequences.

[Insert **Table 4** here]

[Insert **Table 5** here]

5.3 Pipette the master mix up and down to mix.

5.4 Set up the dilution plate.

5.4.1 Use a clear 96-well plate to prepare standard and sample dilutions, add 45 µL of molecular grade water to each well in every other column starting with column 1 (columns 1, 3, 5, 7, 9, and 11).

5.4.2 Add 5 µL of the standard to well A1 and pipette to mix.

5.4.3 Use a new filtered pipette tip to create a 1/10 dilution from well A1 to B1.

5.4.4 Continue a series of 10-fold dilutions down the column until reaching G1.

5.4.5 Do not add to H1, as this will act as a negative control.

5.4.6 Apply the first sample (S1) by adding it to well A3, forming a 1/10 dilution. Pipette this mixture and transfer 5 µL to well B3. Discard the pipette tip after this transfer.

5.4.7 With a new pipette tip, mix the solution in well B3 and form a 1/100 dilution. Transfer 5 µL of this mixture to well C3 and discard the tip after the transfer.

5.4.8 With a new pipette tip, pipette up and down the solution in well C3 to make a 1/1000 dilution. Discard the tip.

5.4.9 Continue diluting samples without adding any samples to columns 2, 4, 6, 8, 10, or 12.

5.4.10 Once all samples are diluted, mix the contents in wells of column 1, and then transfer 20 µL to column 2.

5.4.11 Repeat this for columns 3, 5, 7, 9, and 11 to create replicates of each standard and sample dilution. Refer to **Figure 3** for plate layout.

NOTE: When following plate set-up in **Figure 3**, samples diluted in rows G and H will only have 1/10 and 1/100 dilutions.

[Insert **Figure 3** here]

5.5 Titration by SV40 polyA detection-based qPCR

5.5.1 Add 15 µL of qPCR master mix to each well of a white-semi skirted 96-well qPCR plate.

5.5.2 Transfer 5 µL of each sample from the clear 96-well plate to the white-semi skirted 96 well qPCR plate.

5.5.3 Use a multichannel pipette to ensure adequate mixing of the qPCR master mix and sample.

5.5.4 Seal the plate with a sealing film and centrifuge the qPCR plate at 1500 x *g* for 30 s.

5.5.5 Run the qPCR reaction on plate-based real-time PCR amplification and detection instrument, using the suggested conditions in **Table 6**.

[Insert **Table 6** here]

NOTE: For qPCR AAV titration worksheet see **Table 7**.

5.6 Data analysis to determine AAV genome copy numbers.

5.6.1 Fill in the spreadsheet data cells (**Table 7A**) with the concentration values obtained from the qPCR run for both standard and sample dilutions.

5.6.2 Use concentration values from **Table 7A** to produce a standard curve (**Table 7B**).

NOTE: The standard curve will be shown as a natural logarithm ($y = a \ln(x) + b$) along with R^2 efficiency. A standard curve must have an efficiency close to 100 % and R^2 close to 1.0 (≥0.99).

5.6.3 Fill in the slope efficiency by filling in this online calculator[27].

NOTE: An efficiency between 90%–110% is acceptable. If the efficiency of qPCR is outside of this range, re-run the qPCR.

5.6.4 Use concentration values from **Table 7A** to average the dilutions of each sample and determine the standard deviation of each sample (**Table 7C**).

NOTE: Exclude dilutions from samples that are more than one standard deviation away from the average of the sample dilutions.

5.6.5 Using the mean concentration of each dilution, multiply by the dilution factor, and then divide by five to get the vector genomes (vg)/µL of each sample (**Table 7C**).

5.6.6 Calculate the vg/mL of each sample by multiplying the mean of each sample's concentrations by 80,000 (**Table 7C**).

5.6.7 Average the vg/mL of each dilution to produce the final vg/mL of each sample (**Table 7C**).

NOTE: The user must divide the mean concentration of each dilution by a factor five to account for the 5 µL loaded into each well for the qPCR run to produce the concentration in vg/µL. The factor of 80,000 accounts for the transition from each samples' mean concentration value to vg/mL. First, the mean of each sample's concentration value must be multiplied by 2 to account for the single-stranded genomes, as the primer-probe set only quantifies positive-sense, single-stranded DNA (ssDNA), and the AAV genome exists in an approximate 1:1 ratio between positive and negative-sense ssDNA[25,28]. The mean of each sample's concentration value must be multiplied x40 to account for the sample dilution from 5 µL of purified vector (section 4.1) to 200 µL of extracted DNA (section 4.6.12). Lastly, the mean of each sample's concentration value must be multiplied x1000 to convert from vg/µL to vg/mL.

6 Assessment of vector quality and purity

6.1 Quality Control – Western Blot

6.1.1 Prepare a 12% SDS PAGE gel.

6.1.2 Perform polyacrylamide gel electrophoresis.

NOTE: Load 6 x 10^{10} vg of samples per well.

6.1.3 Transfer the proteins to polyvinylidene difluoride (PVDF) membrane.

6.1.4 Blocking PVDF membrane

6.1.4.1 Remove the membrane from the transfer apparatus and rinse in 0.1% PBST to remove loose acrylamide.

6.1.4.2 Place the membrane in blocking solution for at least 1 h at room temperature or overnight at 4 °C.

NOTE: Blocking buffer can be further supplemented with 2% goat serum.

6.1.5 Incubation with primary antibody

6.1.5.1 Decant the blocking buffer and add the primary antibody, an anti-AAV mouse monoclonal antibody at a 1:200 dilution.

6.1.5.2 Incubate overnight at 4 °C.

6.1.5.3 Decant the primary antibody and wash five times with 0.1% PBST for 5 min at room temperature with agitation.

6.1.6 Incubation with secondary antibody

6.1.6.1 Decant the wash solution and add HRP conjugated secondary antibody, diluted at a 1:7500 in blocking buffer, and incubate for 1 h at room temperature with agitation.

6.1.6.2 Decant the secondary antibody and wash five times with 0.1% PBST for 5 min at room temperature with agitation.

6.1.6.3 Perform a final wash with PBS at room temperature with agitation.

6.1.7 Detect the proteins using enhanced chemiluminescent (ECL) substrate.

6.1.8 Image the gel to visualize the viral proteins (VP1, VP2, and VP3 subunits) (**Figure 4**).

[Insert **Figure 4** here]

6.2 Purity Control – SDS PAGE and Coomassie Stain

6.2.1 Prepare SDS-PAGE gel and samples as described from step 6.1.1 and 6.1.2.

6.2.2 Fix the gel in fixing solution for 1 h or overnight with gentle agitation. Change the fixing solution once during the first hour.

6.2.3 Stain the gel in staining solution for 2–4 h with gentle agitation.

6.2.4 Destain the gel with a destaining solution. Replenish the destaining solution several times until the background of the gel is fully destained (4–24 h).

6.2.5 Store the destained gel in a storage solution.

6.2.6 Image the gel to visualize all proteins stained by Coomassie staining solution.

[Insert **Figure 5** here]

6.3 Alternative purity control assay – HEK293 host cell protein detection ELISA

6.3.1 Perform the HEK293 host cell protein detection via ELISA as per the manufacturer's instructions.

NOTE: Use dilutions of 5 x 10^{-2} and 1 x 10^{-3} for purified rAAV samples. Once TMB is added to the well, incubate away from light. Linear regression cannot be used to analyze the results.

6.3.2 Perform a point-to-point analysis, cubic spline, or four-parameter logistic fit method to interpolate concentrations of unknowns and multiply by the dilution factor to determine original sample concentration.

REPRESENTATIVE RESULTS:

Translation from small rodent models to larger animal models and eventual clinical application presents a significant challenge due to the large amount of AAV required to transduce larger animals and achieve therapeutic effects. To compare transduction efficiency of the rationally designed AAV6.2FF capsid, previously demonstrated a 101-fold increase in transduction efficiency in murine muscle cells compared to AAV6[3], mice, hamsters, and lambs were all administered AAV6.2FF expressing a human monoclonal antibody (hIgG). The AAV6.2FF-hIgG-expressing vector contained a CASI promoter[4], a Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and a SV40 polyA sequence. Six-week-old female BALB/c (n = 4) mice were intramuscularly (IM) administered 1 x 10^{11} vg of AAV6.2FF-hIgG in 40 µL, and blood was collected on days 0, 7, 14, 21, and 28 post AAV administration for hIgG monitoring. Four-week-old Syrian hamsters (two females and two males) were IM administered 1 x 10^{12} vg of AAV6.2FF-hIgG in 40 µL, and blood was collected weekly to monitor for hIgG expression. Lastly, 10-day-old male Dorset lambs (n = 3) were IM administered 1 x1 0^{13} vg /kg of AAV6.2FF-hIgG in two to three 1 mL injections in the rump. Weekly blood collection was completed by jugular bleeds and hIgG was monitored weekly for 28 days. All animal experiments were approved by the Institutional Animal Care Committee of the University of Guelph.

Mice IM administered 5 x 10^{12} vg/kg of AAV6.2FF-hIgG expressed between 171–237 µg/mL of hIgG in the serum by day 28 post-administration (**Figure 6**). Hamsters IM administered 2 x 10^{13} vg/kg of AAV6.2FF-hIgG expressed much higher levels than the mice, with serum hIgG levels of 495–650 µg/mL by day 28 post-administration. Lastly, sheep IM administered 1 x 10^{13} vg/kg expressed serum hIgG levels of 21–46 µg/mL by day 28 post-administration. Across all animal

models, the vector did not appear to impede any health indices, such as weight, proving the safety and quality of the produced vectors. It is well known that AAV-mediated monoclonal antibody (mAb) expression varies considerably depending on the species and the mAb. To the best of the authors' knowledge, there are no reports of AAV-mAb expression in ovine species thus, there is no benchmark for expected expression levels. It is noteworthy that the sheep in this study doubled in weight over the 28-day post-injection period and that the animals in **Figure 6** were all transduced with different AAV-mAbs and thus cannot be compared.

[Insert Figure 6]

FIGURE AND TABLE LEGENDS:

Figure 1: Maneuvering of cell stack for cell seeding and transfection. For seeding cell stack, start by removing one of the vent caps and pouring in 1 L of pre-warmed complete DMEM with needed quantity of HEK293 cells (**A**). Evenly distribute cells and media by tightening both vent caps and bring all media to the corner of the cell stack with one of the vent caps and place it in that corner (**B**), place the cell stack on its side (**C**), and then turn the cell stack 90° (**D**) so that the vent ports are up (**E**). Gently lower the cell stack to its normal horizontal position and ensure all the chambers of the cell stack are completely covered in media (**F**). When transfecting, unscrew both vent caps and slowly pour out old media into a waste sterile waste container for even flow not to disturb the monolayer of the cells (**G**).

Figure 2: Set up for peristaltic pump for AAV purification. Run the tubing from the crude lysate, through the peristaltic pump, and into the heparin matrix column.

Figure 3: Plate layout for qPCR AAV titration. Blue indicates the placement of the serial dilution of the standard; green indicates the placement of the negative control; purple indicates the placement of the dilution of the samples. Each standard, negative, or sample is added in replicate. An example for the concentration of the standard has been added to show the dilution series of the standard, and placement of sample dilutions have been added to their respective wells.

Figure 4: Western blot showing AAV capsid proteins. Lane A; MW ladder, Lane B; AAV6.2FF-hIgG01, Lane C; AAV6.2FF-hIgG02, Lane D; AAV6.2FF-hIgG03, and Lane E; AAV6.2FF-hIgG04. 6 x 10^{10} vg of each AAV6.2FF-hIgG was loaded into their respective lanes.

Figure 5: Coomassie-stained gel. Lane A; MW ladder, Lane B; AAV6.2FF-hIgG01, Lane C; AAV6.2FF-hIgG02, Lane D; AAV6.2FF-hIgG03, Lane E; AAV6.2FF-hIgG04, Lane F; AAV6.2FF-hIgG05, and Lane G; AAV6.2FF-hIgG06. 6 x 10^{10} vg of each AAV6.2FF-hIgG was loaded into their respective lanes.

Figure 6: Intramuscular delivery of AAV6.2FF-hIgG leads to sustained serum hIgG expression in mice, hamsters, and sheep. Female BALB/c mice (n = 4) were IM administered 5 x 10^{12} vg/kg of AAV6.2FF-hIgG, Syrian hamsters (2 male, 2 female), were IM administered 1 x 10^{13} vg/kg of AAV6.2FF-hIgG, and 10-day-old male Dorset lambs (n = 3) were IM administered 1 x 10^{13} vg/kg.

Serum was monitored for hIgG expression over a period of 28 days. Data are represented as the mean ± standard deviation.

Table 1: Composition of required solutions. Necessary information, including percentages and volumes, of components needed for various solutions throughout the protocol.

Table 2: Transfection calculator for cell culture chamber. Interactive worksheet to determine correct concentration of pTransgene, pHelper, and PEI for transfection of cell culture chamber.

Table 3: DNase treatment master mix formula. Recommended components and volumes required for DNase treatment of AAV viral vectors during DNA extraction.

Table 4: qPCR master mix for AAV titration. Recommended components and volumes required for qPCR of DNA extracted from AAV viral vectors.

Table 5: Primer sequences against the SV40 polyA DNA sequence. Sequences of the primers and probe used for qPCR titration, which bind to specific areas of AAV viral vectors that contain the SV40 polyA sequence.

Table 6: Thermocycler protocol for hydrolysis probe-based qPCR titration. Recommended thermocycler protocol for use of probe-based qPCR titration of DNA extracted purified AAV vectors.

Table 7: AAV Titration Calculator. Interactive worksheet to determine final concentration of AAV samples from qPCR, expressed as vg/mL.

DISCUSSION:

The production of recombinant AAV (rAAV) vectors described in this paper uses common materials, reagents, and equipment found in majority of the molecular biology research labs and facilities. This paper allows for high-quality *in vitro* and *in vivo* grade rAAV to be produced by the reader. Above all, this protocol for rAAV production, compared to more tedious protocols involving cesium chloride purification, is efficient and avoids the use of ultracentrifugation. Once HEK293 cells have been transfected, purified AAV is ready to use within 5 working days.

During the rAAV production and purification process, many steps can influence the purity and final titer of the vector. The first and most critical step is the health of the HEK293 cells, which has a direct effect on the vector titer. The use of HEK293 cells as opposed to other cell types or systems, such as HEK293T cells, is advantageous in that HEK293 cells have a greater ability to adhere to the plastic coating of plates and cells creating robust cell networks for continual cell growth. It is recommended to monitor cells for mycoplasma contamination and to avoid passaging HEK293 cells past ~40 passages or once their doubling time appears to be slowing down as this will lead to lower AAV yields. Furthermore, confirming cells are approximately 80% confluent prior to transfection ensures that cells are not too sparse or overgrown. With regard to the transfection components, the use of high-quality plasmid DNA (e.g., commercially

prepared plasmid DNA) is highly recommended to ensure DNA remains in solution and interacts properly with chemical transfection delivery components, such as PEI. Lastly, the transfection reagent has a significant impact on rAAV yields. Here, PEI is used as the transfection agent. PEI is a stable cationic polymer that delivers exogenous DNA to the nucleus of cells through production of plasmid-polymer complexes, which are then taken up by cells and trafficked to the nucleus through host-cell processes[29]. PEI-based transfections are quick and easy in comparison to other methods of DNA delivery, including calcium phosphate or lipofectamine. However, the ratio of PEI:DNA must be optimized.

The use of cell stacks opposed to traditional adherent plates provides a more convenient and consistent method for the production of rAAV. Cell stacks involve less technical manipulation during transfection, mitigating the possible dislodgement of cells from the adherent monolayer, allowing for stronger cell networks, and better production of rAAV. Post transfection, the collection of supernatant and lysis of cells is efficient and consistent. To harvest rAAV from cells, physical cell lysis methods such as freeze-thawing are inefficient and inconsistent as there is no way to ensure that all the cells are lysed. Here, a chemical lysis procedure is described. The use of chemical lysis buffer ensures that all the cells are exposed to the lysis agent at a specific concentration, as well as additives such as protease inhibitor to prevent capsid degradation. This method more consistently lyses cells allowing for a more efficient harvest of rAAV. Furthermore, the pelleting and removal of cellular debris eliminates possible contaminants from the crude lysate, which can increase the time and cost of filtering lysate prior to purification.

Though rAAV might be present in high amounts in the crude lysate, the act of capturing and cleaning the rAAV can differ among various purification methods, such as cesium chloride gradients. Here, the use of heparin sepharose affinity chromatography purification provides a rapid and easy method for vector purification that results in ultrapure virus and does not require gradient ultracentrifugation. However, not all AAV serotypes contain heparin-binding domains, so for those serotypes this purification method would not be appropriate. For example, while AAV2 and AAV6 bind heparin sulfate, AAV4 and AAV5 do not[30]. Though this method is not universal, it is efficient and easy for those capsids that do bind heparin sulfate. Unlike ultra-centrifugation gradients such as iodixanol, all rAAV particles are bound to the membrane of the column until they are eluted using high salt concentration washes, avoiding issues such as mixing of gradients and skill needed to recover fractions from the gradient. Elution through high salt concentration washes allows for controlled and precise elution of the virus into fractions that can be further concentrated. Only concentrating certain fractions of the eluted virus further removes potential contaminants while recovering >97% of the eluted virus. One limitation of heparin sepharose affinity chromatography purification is that it does not distinguish between empty and full particles. Additional analytical ultracentrifugation steps would need to be incorporated in order to remove empty capsids[31].

qPCR is a cost-effective and rapid method for determining the number of vector genomes in an AAV vector prep. Though qPCR is a sensitive quantification method, it does not provide any information about the number of infectious particles in the prep. Moreover, the sensitivity of this assay can result in variability as technical errors during pipetting can lead to inter-assay

variability. Thus, for any experiment that involves comparing different AAV vectors, it is critical that the vectors be titered on the same qPCR plate. Despite these limitations, qPCR is the most accurate method developed for titering AAV and is currently the most widely used and accepted method for quantification of AAV vectors[32]. The use of a primer/probe that binds to the SV40 polyA of a rAAV genome is based on the fact that this sequence is conserved across many AAV genome plasmids engineered in the laboratory. Beyond choosing a conserved sequence among the reader's rAAV genome plasmids, qPCR probes can be designed for all parts of the rAAV genome, including, but not limited to, promoters, transgenes, or post-transcriptional factors.

Assessing the purity and quality of the AAV product is an important step in the production process. Western blotting can be used to detect the VP1, VP2, and VP3 structural proteins that make up the capsid of AAV, as well as any altered forms of VP. VP1, VP2, and VP3 are typically present at a ratio of 1:1:10, but this can vary from 1:1:5 to 1:1:20 depending on the serotype. Therefore, it is critical to determine the ratio for each system empirically, particularly because the N-terminal region of VP1 has been shown to be important for infectivity and transduction[33]. SDS-PAGE coupled with Coomassie staining is a rather straightforward method for detecting VP1, VP2, and VP3, as well as host cell protein contaminants. The use of fluorescent protein stains such as SYPRO Ruby offer higher sensitivity detection of host cell protein contaminants than Coomassie; however, not all laboratories have access to the imaging equipment required to visualize the gel. Finally, commercial ELISAs can be used to quantify host cell protein contaminants in AAV vectors produced in HEK293 cells.

This protocol provides an in-depth overview of producing high titer, high purity rAAV for serotypes that bind to heparin. In the representative results section, the use of novel AAV6.2FF expressing hIgG in a variety of animal models shows the safety and efficiency of this rAAV *in vivo*. Though the AAV6.2FF vector was administered IM, these high-quality, high purity viruses can be administered via a variety of different routes *in vivo*, as possible inflammatory contaminants, such as host-cell protein, have been eliminated through our purification processes.

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DISCLOSURES:

Sarah K. Wootton is an inventor on a US patent US10806802B2 for the AAV6.2FF capsid.

REFERENCES:

1. Hastie, E., Samulski, R. J. Adeno-associated virus at 50: A golden anniversary of discovery, research, and gene therapy success—a personal perspective. *Human Gene Therapy*. **26** (5), 257–265 (2015).

2. Wang, D., Tai, P. W. L., Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nature Reviews Drug Discovery*. **18** (5), 358–378 (2019).
3. Nathwani, A. C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *The New England Journal of Medicine*. **371** (21), 1994–2004 (2014).
4. Kuzmin, D. A. et al. The clinical landscape for AAV gene therapies. *Nature Reviews Drug Discovery*. **20** (3), 173–174 (2021).
5. Ylä-Herttuala, S. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Molecular Therapy: The Journal of the American Society of Gene Therapy*. **20** (10), 1831–1832 (2012).
6. FDA approves novel gene therapy to treat patients with a rare form of inherited vision loss. FDA News Release, *FDA*. at https://www.fda.gov/news-events/press-announcements/fda-approves-novel-gene-therapy-treat-patients-rare-form-inherited-vision-loss (2020).
7. FDA approves innovative gene therapy to treat pediatric patients with spinal muscular atrophy, a rare disease and leading genetic cause of infant mortality. FDA News Release, *FDA*. at https://www.fda.gov/news-events/press-announcements/fda-approves-innovative-gene-therapy-treat-pediatric-patients-spinal-muscular-atrophy-rare-disease (2020).
8. Statement from FDA Commissioner Scott Gottlieb, MD and Peter Marks, MD, Ph.D., Director of the Center for Biologics Evaluation and Research on new policies to advance development of safe and effective cell and gene therapies. FDA Statement, *FDA*. at https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics (2020).
9. Asokan, A., Schaffer, D. V., Samulski, R. J. The AAV vector toolkit: poised at the clinical crossroads. *Molecular Therapy: The Journal of the American Society of Gene Therapy*. **20** (4), 699–708 (2012).
10. Rose, J. A., Hoggan, M. D., Shatkin, A. J. Nucleic acid from an adeno-associated virus: chemical and physical studies. *Proceedings of the National Academy of Sciences of the United States of America*. **56** (1), 86–92 (1966).
11. Lusby, E., Fife, K. H., Berns, K. I. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *Journal of Virology*. **34** (2), 402–409 (1980).
12. Masat, E., Pavani, G., Mingozzi, F. Humoral immunity to AAV vectors in gene therapy: challenges and potential solutions. *Discovery Medicine*. **15** (85), 379–389 (2013).
13. Ling, C. et al. Enhanced Transgene Expression from Recombinant Single-Stranded D-Sequence-Substituted Adeno-Associated Virus Vectors in Human Cell Lines In Vitro and in Murine Hepatocytes In Vivo. *Journal of Virology*. **89** (2), 952–961 (2014).
14. Cathomen, T., Stracker, T. H., Gilbert, L. B., Weitzman, M. D. A genetic screen identifies a cellular regulator of adeno-associated virus. *Proceedings of the National Academy of Sciences of the United States of America*. **98** (26), 14991–14996 (2001).
15. McCarty, D.M. Self-complementary AAV vectors; advances and applications. *Molecular Therapy*. **16** (10), 1648–1656 (2008).
16. Aschauer, D. F., Kreuz, S., Rumpel, S. Analysis of transduction efficiency, tropism and axonal transport of aav serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PLOS One*. **8** (9), e76310 (2013).
17. Zincarelli, C., Soltys, S., Rengo, G., Rabinowitz, J. E. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Molecular Therapy*. **16** (6), 1073–1080 (2008).

18. Pillay, S. et al. Adeno-associated virus (AAV) serotypes have distinctive interactions with domains of the cellular AAV receptor. *Journal of Virology*. **91** (18) (2017).
19. Merkel, S. F. et al. Trafficking of adeno-associated virus vectors across a model of the blood-brain barrier; a comparative study of transcytosis and transduction using primary human brain endothelial cells. *Journal of Neurochemistry*. **140** (2), 216–230 (2017).
20. van Lieshout, L. P. et al. A novel triple-mutant AAV6 capsid induces rapid and potent transgene expression in the muscle and respiratory tract of mice. *Molecular Therapy. Methods & Clinical Development*. **9**, 323–329 (2018).
21. Wu, Z., Asokan, A., Grieger, J. C., Govindasamy, L., Agbandje-McKenna, M., Samulski, R. J. single amino acid changes can influence titer, heparin binding, and tissue tropism in different adeno-associated virus serotypes. *Journal of Virology*. **80** (22), 11393–11397 (2006).
22. Liu, J., Moon, Y.-A. Simple purification of adeno-associated virus-DJ for liver-specific gene expression. *Yonsei Medical Journal*. **57** (3), 790–794 (2016).
23. Grimm, D., Kern, A., Rittner, K., Kleinschmidt, J. A. Novel tools for production and purification of recombinant adeno-associated virus vectors. *Human Gene Therapy*. **9** (18), 2745–2760 (1998).
24. Kimura, T. et al. Production of adeno-associated virus vectors for in vitro and in vivo applications. *Scientific Reports*. **9** (1), 13601 (2019).
25. Aurnhammer, C. et al. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. *Human Gene Therapy Methods*. **23** (1), 18–28 (2012).
26. dsDNA copy number calculator. at <http://cels.uri.edu/gsc/cndna.html>.
27. qPCR Efficiency Calculator - CA. at <//www.thermofisher.com/ca/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html>.
28. Lamb, R. A., Kolakofsky, D. Paramyxoviridae: The viruses and their replication. *Fields Virology*. at https://www.scholars.northwestern.edu/en/publications/paramyxoviridae-the-viruses-and-their-replication (1996).
29. Boussif, O. et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America*. **92** (16), 7297–7301 (1995).
30. Kaludov, N., Brown, K. E., Walters, R. W., Zabner, J., Chiorini, J. A. Adeno-associated virus serotype 4 (AAV4) and AAV5 Both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *Journal of Virology*. **75** (15), 6884–6893 (2001).
31. Burnham, B. et al. Analytical ultracentrifugation as an approach to characterize recombinant adeno-associated viral vectors. *Human Gene Therapy Methods*. **26** (6), 228–242 (2015).
32. Dobnik, D. et al. Accurate quantification and characterization of adeno-associated viral vectors. *Frontiers in Microbiology*. **10**, 1570 (2019).
33. Backovic, A. et al. Capsid protein expression and adeno-associated virus like particles assembly in Saccharomyces cerevisiae. *Microbial Cell Factories*. **11**, 124 (2012).

Figure 1

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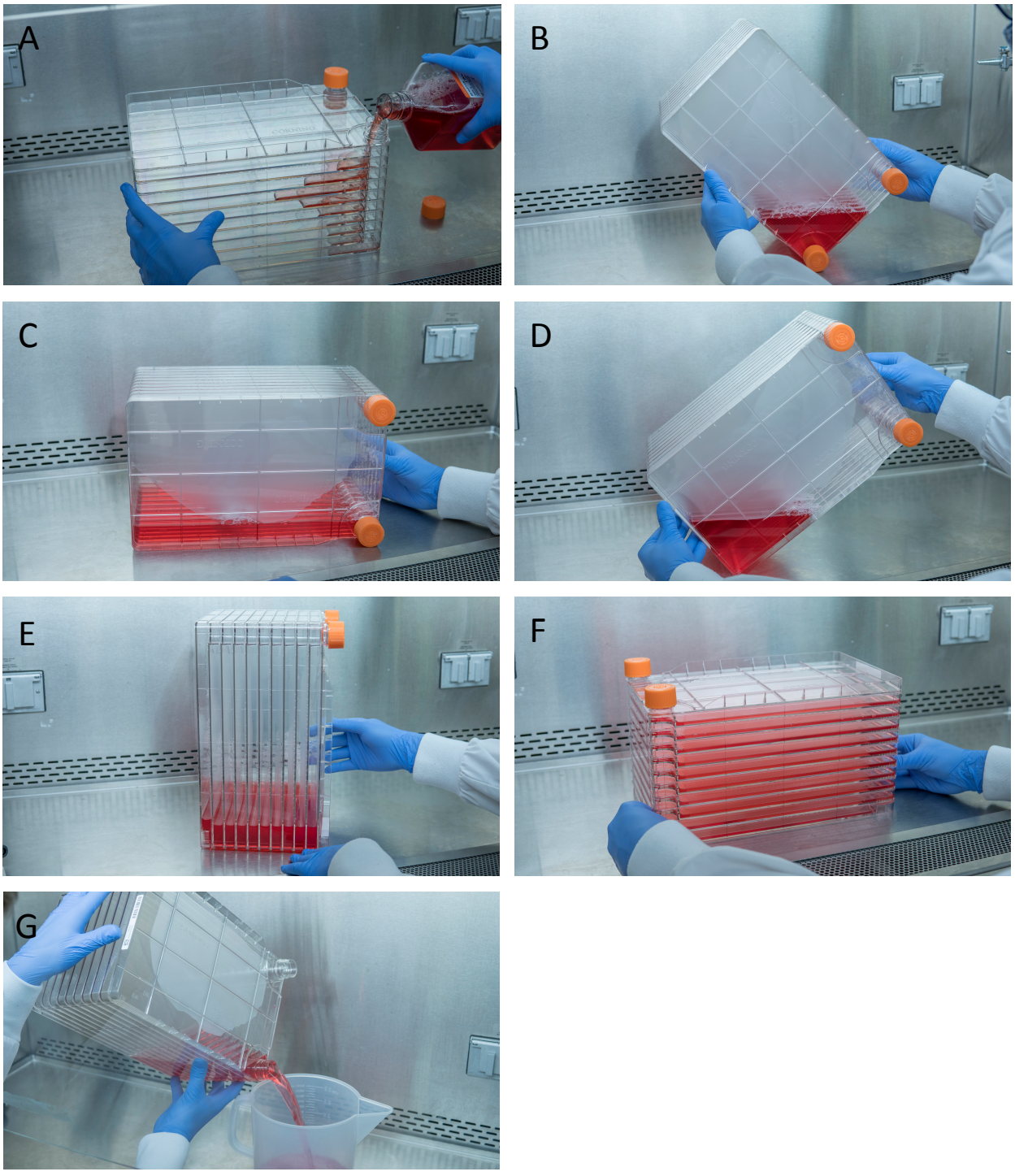

Figure 2

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Figure 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	$5x10^{8}$	$5x10^{8}$	$1x10^{-1}$	$1x10^{-1}$	$1x10^{-1}$	$1x10^{-1}$						
B	$5x10^{7}$	$5x10^{7}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$						
C	$5x10^{6}$	$5x10^{6}$	$1x10^{-3}$	$1x10^{-3}$	$1x10^{-3}$	$1x10^{-3}$						
D	$5x10^{5}$	$5x10^{5}$	$1x10^{-1}$	$1x10^{-1}$	$1x10^{-1}$	$1x10^{-1}$						
E	$5x10^{4}$	$5x10^{4}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$						
F	$5x10^{3}$	$5x10^{3}$	$1x10^{-3}$	$1x10^{-3}$	$1x10^{-3}$	$1x10^{-3}$						
G	$5x10^{2}$	$5x10^{2}$										
H	NC	NC										

Standard Curve
Negative Control
Sample 1
Sample 2
Sample 3
Sample 4

Figure 4

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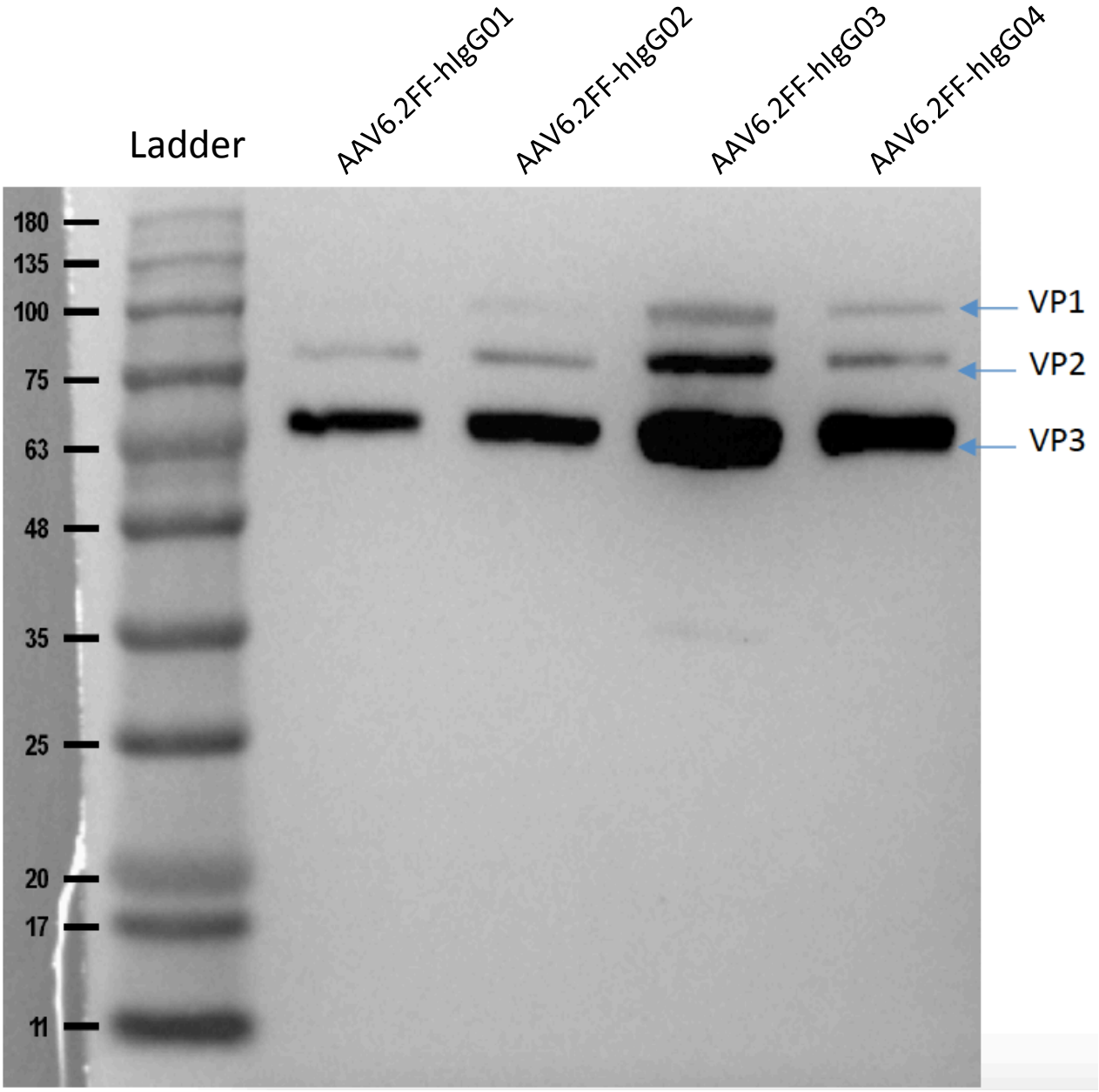


Figure 5

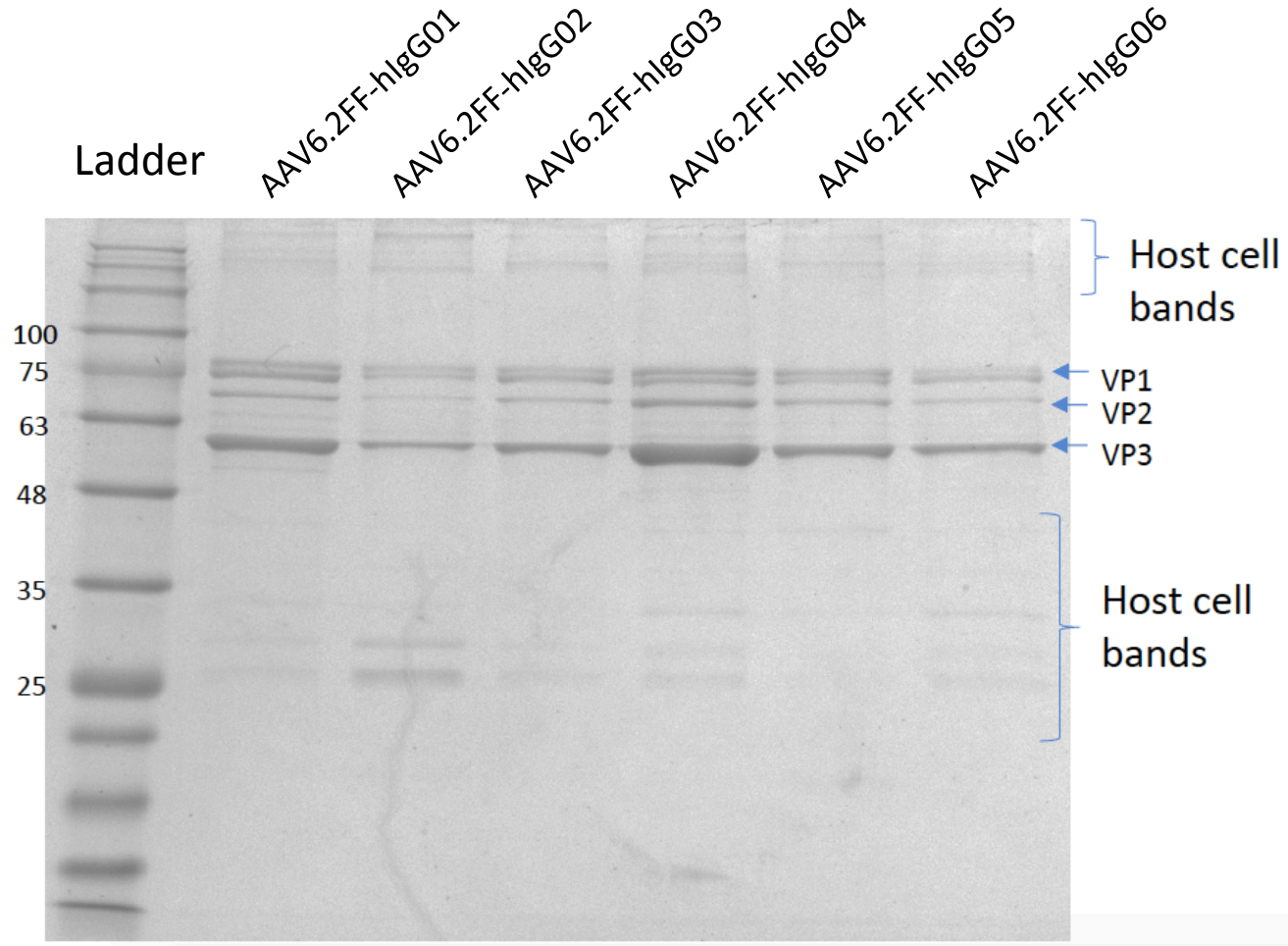
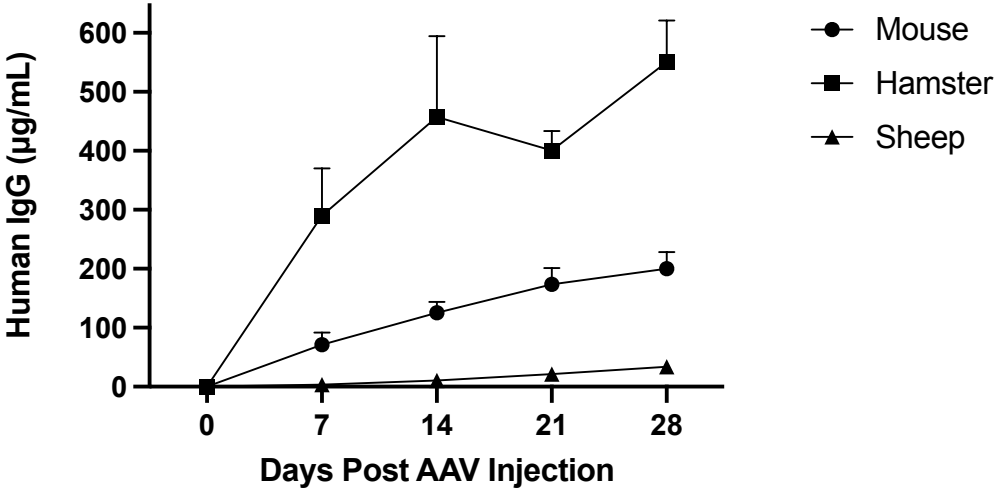


Figure 6



Solution	Composition	Comments
Cell Culture and Transfection		
Complete DMEM	DMEM 1x	
	7% FBS	
	1% L-glutamine	
	1% Penicillin-Streptomycin	
PEI (1 mg/mL)	900 mL of molecular grade water	0.22 µm filter. CAUTION.
	1 g of PEI powder	
	pH to 7.0 using HCl	
	Up to 1 L of molecular grade water	
	Store at 4 °C for up to 6 months.	
Chemical Lysis of HEK293 Cells		
1M Tris Base	121.14 g of Tris base	
	Up to 1 L of molecular grade water	
1 M NaCl	58.44 g of NaCl	
	Up to 1 L of molecular grade water	
100 mM $MgCl_2$	9.52 g of $MgCl_2$	
	Up to 1 L of molecular grade water	
10 % Triton X-100	10 mL of Triton X-100	
	Up to 100 mL of molecular grade water	
Lysis buffer	500 mL of molecular grade water	0.22 µm filter
	30 mL of 1 M Tris base	
	100 mL of 1 M NaCl	
	20 mL of 100 mM $MgCl_2$	
	5 mL of 10% Triton X-100	
	Up to 1 L of molecular grade water	

	Adjust pH to 8.0. Store at 4 °C for up to 1 month. Immediately prior to use aliquot the required volume of lysis buffer and add 10 U/mL of nuclease (i.e., to 100mL of lysis buffer, add 1,000 units of nuclease).	
AAV Purification		
1 M NaOH	40 g of NaOH	
	Up to 1 L of molecular grade water	
0.5 % N-Lauroylsarcosine HBSS without Mg^{2+} and Ca^{2+}	2.5 g of N-Lauroylsarcosine sodium salt	0.22 µm filter
	Up to 500 mL of HBSS without Mg^{2+} and Ca^{2+}	
200 mM NaCl HBSS with Mg^{2+} and Ca^{2+}	1.7 g of NaCl	0.22 µm filter
	Up to 500 mLof HBSS with Mg^{2+} and Ca^{2+}	
300 mM NaCl HBSS with Mg^{2+} and Ca^{2+}	4.6 g of NaCl	0.22 µm filter
	Up to 500 mL of HBSS with Mg^{2+} and Ca^{2+}	
Filter pre-treatment	5 mL of Polysorbate 20	CAUTION, 0.22 µm filter
	Up to 100 mL of HBSS Mg^{2+} and Ca^{2+}	
2 M NaCl	58.44 g of NaCl	0.22 µm filter
	Up to 500 mL of molecular grade water	
0.1% Triton X-100	1 mL of Triton X-100	CAUTION, 0.22 µm filter
	Up to 100 mL of molecular grade water	
20% Ethanol	100 mL of 95 % ethanol	0.22 µm filter
	Up to 500 mL of molecular grade water	
AAV Purity Control		
Stacking Gel	1.98 mL of 30% Acrylamide/Bis	CAUTION
	3.78 mL of 0.5 M Tris-HCl pH 6.8	CAUTION

	150 µL of 10% SDS	CAUTION
	9 mL of molecular grade water	
	15 µL of TEMED	CAUTION
	75 µL of 10% APS	CAUTION
	Add TEMED and 10% APS immediately prior to pouring gel into casting tray.	
Resolving Gel	6 mL of 30% Acrylamide/Bis	CAUTION
	3.75 mL of 1.5 M Tris-HCl pH 8.8	CAUTION
	150 µL of 10% SDS	CAUTION
	5.03 mL of molecular grade water	
	7.5 µL of TEMED	CAUTION
	75 µL of 10% APS	CAUTION
	Add TEMED and 10 % APS immediately prior to pouring gel into casting tray.	
1x running buffer	3 g of 25 mM Tris base	CAUTION
	14.4 g of glycine	
	1 g of 0.1% SDS	CAUTION
10% APS	1 g of APS	
	Up to 10 mL of molecular grade water	
Western blot sample buffer	1 mL of 0.5 M Tris-HCl pH 6.8	
	2 mL of 25% glycerol	
	80 µL of 1% bromophenol blue	
	1.6 mL of 10% SDS	
	2.92 mL of molecular grade water	
	of β-mercaptoethanol prior to using aliquot.	CAUTION
Transfer buffer	2.9 g of Tris base	CAUTION
	14.5 g of glycine	
	200 mL of methanol	CAUTION

Blocking solution	25 g of skim milk powder	
	500 µL of Polysorbate 20	CAUTION
	Up to 500 mL of 1x PBS	
Fixing solution	50 mL of methanol	CAUTION
	10 mL of glacial acetic acid	CAUTION
	Up to 100 mL of molecular grade water	
Staining solution	100 µL of Coomassie Brilliant Blue R-250	
	50 mL of methanol	CAUTION
	10 mL of glacial acetic acid	CAUTION
	Up to 100 mL of molecular grade water	
Destaining solution	40 mL of methanol	CAUTION
	10 mL of glacial acetic acid	CAUTION
	Up to 100 mL of molecular grade water	
Sorage solution	5 mL of glacial acetic acid	CAUTION
	Up to 100 mL of molecular grade water	
CAUTION: Refer to the Materials Table for guidelines on the use of dangerous chemicals.		

Transfection of Cell Culture Chamber

Protocol

1. Allow DNA, OptiMEM and PEI to warm to room temperature prior to transfection
2. Input the number of cell stacks to be transfected (no overage required)
3. Ensure the DNA concentrations are correct as this will change the volumes required

Number of Cell Culture Chambers	1

Concentration of transgene plasmid	1 mg/mL
Concentration of pDGM6.2FF plasmid	1 mg/mL

		Amount Per cell culture chambers	**Transfection Mastermix**
1:3 ratio pTransgene:pHelper	**OptiMEM**	48.1 mL	48.1 mL
	pTransgene	475 µg	475 µL
	pHelper	1425 µg	1425 µL

4. Add required volumes of OptiMEM and plasmid DNA to a 50 mL conical tube
5. Invert 10 times to mix

3:1 PEI:DNA ratio	**PEI Max**	5.7 mL	5.7 mL

6. Add the required amount of PEI to the 50 mL tube containing the OptiMEM and plasmid DNA
7. Immeditely close the 50 mL tube and vortex and invert 3–5 times to mix.
8. Set a timer for 10 min for the PEI complexes to incubate
9. Move cell stacks to be transfected into the BSC
10. After 10 min, pour the trasnfection mix into one orange port.
11. Gently mix liquid throughout the cell stack
12. Return the transfected cells stack to the incubator ensuring equal volume on each layer
13. Harvest cell culture chamber 72 h later

Component	**Volume**
Purified AAV vector	5 µL
10x DNase Buffer	2 µL
DNase	1 µL
ddH_2O	12 µL
Final Volume	**20 µL**

Component	**Volume**
Universal qPCR master mix (2X)	10 µL
Molecular grade water	4.5 µL
40x SV40 polyA primer/probe	0.5 µL
Final Volume	**15 µL**

Table 5 Click here to access/download;Table;Table 5.xlsx

Component	Sequence
Forward primer	5’-AGCAATAGCATCACAAATTTCACAA-3’
Reverse primer	5’-CCAGACATGATAAGATACATTGATGAGTT-3’
Probe	/56-FAM/AGCATTTTT/Zen/TTCACTGCATTCTAGTTGTGGTTTGTC/3IABkFQ

Table 6 Click here to access/download;Table;Table 6.xlsx

Section	Cycles	Time	Temperature	Description
Pre-incubation	1x	5 min	95 °C	DNA denaturation.
Amplification	38x	15 s	95 °C	Amplification of DNA. Settings can be modified if using alternative primers with different annealing temperatures.
		60 s	60 °C	
Cooling	1x	60 s	40 °C	Plate Cooling. End of run.

Table 7 Click here to access/download;Table;Table 7.xlsx

Section A. Input Raw Data

Replace the current example data with the user's concentration value in the highlighted cells below.

Well		Concentration
1	A1	530000000
2	A2	615000000
3	A3	68000000
4	A4	67700000
5	A5	
6	A6	
7	A7	
8	A8	
9	A9	
10	A10	
11	A11	
12	A12	
13	B1	55800000
14	B2	58400000
15	B3	6470000
16	B4	6230000
17	B5	
18	B6	
19	B7	
20	B8	
21	B9	
22	B10	
23	B11	
24	B12	
25	C1	7330000
26	C2	7390000
27	C3	667000
28	C4	676000
29	C5	
30	C6	
31	C7	
32	C8	

Well		Concentration
33	C9	
34	C10	
35	C11	
36	C12	
37	D1	294000
38	D2	388000
39	D3	
40	D4	
41	D5	
42	D6	
43	D7	
44	D8	
45	D9	
46	D10	
47	D11	
48	D12	
49	E1	71900
50	E2	73500
51	E3	
52	E4	
53	E5	
54	E6	
55	E7	
56	E8	
57	E9	
58	E10	
59	E11	
60	E12	
61	F1	4960
62	F2	6880
63	F3	
64	F4	

Standard concentrations

Negative control concentratio

Section B. Standard curve concentrations and calculation

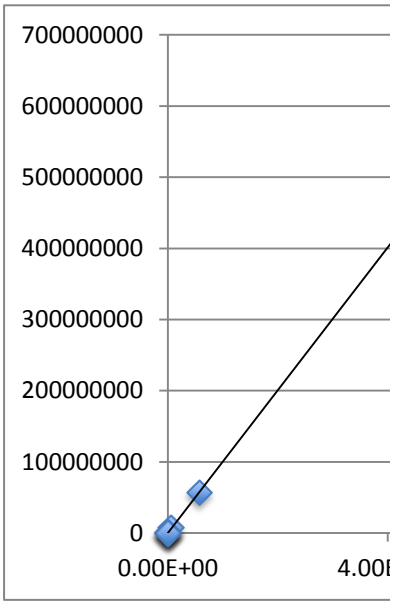
qPCR plate Set-up including concentrations

	1	2	3	4	5	6	7	8
A	530000000	6.15E+08	###	67700000	0	0	0	0
B	55800000	58400000	###	6230000	0	0	0	0
C	7330000	7390000	###	676000	0	0	0	0
D	294000	388000	0	0	0	0	0	0
E	71900	73500	0	0	0	0	0	0
F	4960	6880	0	0	0	0	0	0
G	573	573	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0

Create Standard Curve

NOTE: The Theorectical copy number for the standard will change based on the standard created by the user.

Theoretical Copy Number	Standard Curve Concentrations		Concentration Mean
	1	2	
5.71E+08	530000000	6.15E+08	572500000
5.71E+07	55800000	58400000	57100000
5.71E+06	7330000	7390000	7360000
5.71E+05	294000	388000	341000
5.71E+04	71900	73500	72700
5.71E+03	4960	6880	5920
5.71E+02	573	573	573
0	0	0	0



Efficiency of qPCR run: 96%

NOTE: This link in the protocol to produce this number

NOTE: The qPCR plate should be re-done should the efficiency of the plate fall outside of the 90%-1

Section C. AAV Titration Results

Sample	Dilution Factor	Concentration 1	Concetration 2	Mean	Dilution Correction	vg/µL
1	1.00E+01	6.80E+07	6.77E+07	6.79E+07	######	#####
	1.00E+02	6.47E+06	6.23E+06	6.35E+06	######	#####
	1.00E+03	6.67E+05	6.76E+05	6.72E+05	######	#####

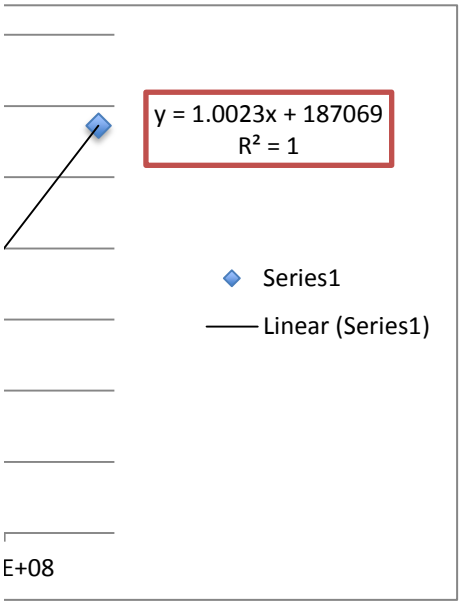
				STDEV:	3.73E+07		Averag

Sample	Dilution Factor	Concentration 1	Concetration 2	Mean	Dilution Correction	vg/µL
1	1.00E+01	0.00E+00	0.00E+00	0.00E+00	######	#####
	1.00E+02	0.00E+00	0.00E+00	0.00E+00	######	#####
	1.00E+03	0.00E+00	0.00E+00	0.00E+00	######	#####
			STDEV:	0.00E+00		Averag

Well		Concentration
65	F5	
66	F6	
67	F7	
68	F8	
69	F9	
70	F10	
71	F11	
72	F12	
73	G1	573
74	G2	573
75	G3	
76	G4	
77	G5	
78	G6	
79	G7	
80	G8	
81	G9	
82	G10	
83	G11	
84	G12	
85	H1	
86	H2	
87	H3	
88	H4	
89	H5	
90	H6	
91	H7	
92	H8	
93	H9	
94	H10	
95	H11	
96	H12	

ns Sample concentrations

9	10	11	12
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0



10% range.

vg/mL
1.09E+13
1.02E+13
1.07E+13

1.06E+13

vg/mL
0.00E+00
0.00E+00
0.00E+00
0.00E+00

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   The manuscript has been thoroughly proofread.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
   Numbering of protocol has been formatted to reflect instructions for authors.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: OptiMEM, PEI MAX, Qiagen, Taqman, Kimwipe, Whatman, etc.
   All commercial language has been removed from the manuscript.

4. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
   Various steps in the protocol have been combined to fit the suggested formatting.

5. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
   Adequate spacing has been added and 3 pages of protocol for filming have been highlighted.

6. Please include a title and a description of each figure and/or table in the Figure legends section. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
   All figure legends have been added to the section after the Representative Results.

7. Figure 2: Please remove the commercial terms from the figure (e.g., Masterflex)
   Figure 2 image has been altered to remove commercial terms.

8. Figure 3: Please specify what the numbers in the colored boxes indicate in the figure legends.
   Figure legend has been updated to indicate what colouring in Figure 3 indicates.

9. Table 2: Please replace the commercial terms with generic terms in the table (e.g., OptiMEM). Use standard abbreviations for the units (replace “ug” and “ul” with “µg” and “µL”, respectively).
   Commercial terms have been used and appropriate units have been added.

10. Please sort the Table of Materials in alphabetical order.
    Table of materials has been sorted by alphabetical order.

Reviewers' comments:

Reviewer #1:

1. It is not clear only cell lysate was used to purify the viruses or culture media was used as well, although it is indicated "supernatant can be immediately purified or stored..." A large part of AAVs are released in the culture media (e.g. ref 29) and using the culture media will increase the yield.
   In this protocol AAV is purified from both the cell lysate and supernatant. The cell lysate is clarified and combined with the supernatant before purification.

2. The source of HEK293 cells should be described. Is it HEK293T or HEK293? HEK293T should be used for helper-free system.
   The manuscript mentions use of HEK293 cells in the introduction, only HEK293 cells are referenced throughout protocol.

3. Table of Materials contains unnecessary detail information. The important source (e.g. anti-AAV antibody, HEK) should be written in the text. Figure 4 (how to transfer blot) is not necessary for this paper.
   The protocol for western blot has been simplified, and as a result, the original Figure 4 has been removed.

4. Western blot will detect VP1-3, but not contamination of other proteins. What is the ELISA to detect cell protein? The protein gel followed by staining can detect purity. It is not clear how they purify AAVs to apply in vivo.
   The Western blot can be used to demonstrate that the VP proteins are intact (i.e. no evidence of degraded VPs) and that the ratio of VP1:VP2:VP3 is correct. The HCP ELISA can be used to *quantify* the amount of HEK293 host-cell protein contamination, which is known to increase the immunogenicity of AAV vectors in vivo.

5. Why intramuscular injection of AAV-hIgG shows much lower plasma human IgG levels in sheep compared to mouse or hamster? Large animals need higher dose, or im does not go to liver efficiently, or sheep does not have much receptor to AAV - anything known?

AAV-mediated mAb expression varies considerably in different species. As far as we know, there are no reports of AAV-mAb expression in ovine species thus there is no "benchmark" for expression. It is noteworthy that sheep almost double in weight over the 28-day post-injection period.

6. Minor Concerns: Ln 334-335; Figure 4 does not make sense. Figure 3 does not match. No legends for Table 2,6,7.
   We have removed Figure 4. Figure 3 has been revised and a more explanatory figure legend included.

Reviewer #2:

7. The protocol describes AAV production for use in large animals, yet the data in figure 6 suggest that the vector product does not transduce large animals well. Because these experiments did not include vectors purified by other methods (or other controls or serotypes), one cannot conclude whether the low transduction is what should be expected for this capsid/transgene/animal combination or whether the method presented is inefficient. While this reviewer can appreciate that it is unrealistic to request additional large animal experiments for a methods paper, at minimum an explanation of these seemingly poor results is required. For example, the authors could state that this level of expression for a secreted product, such as factor iX, could be therapeutically beneficial for a hemophilia patient.
   As mentioned above, AAV-mediated mAb expression varies considerably depending on the species and the mAb. As far as we know, there are no reports of AAV-mAb expression in ovine species thus there is no "benchmark" for expression. It is noteworthy that sheep almost double in weight over the 28-day post-injection period. It is important to note that the animals in Figure 6 were all transduced with different AAV-mAbs and that thus cannot be compared. If the reviewer's would prefer that this figure be removed or only the ovine expression levels be shown we can certainly do that.

8. Sufficient information on how to obtain the plasmids pTransgene and pHelper is lacking.
   These plasmids can be obtained from Addgene (https://www.addgene.org/search/all/?q=AAV).

9. Affinity purification is convenient and accessible, but it does not enrich for full capsids like density gradient ultracentrifugation. Titration by qPCR will only quantify encapsidated genomes, and if the vector prep has a large proportion of empty capsids, delivery of the desired dose will introduce a large amount of immunogenic material that is not capable of gene transfer. This can lead to stronger than expected immune responses and have a significant effect on experimental outcomes. These issues should be covered in the discussion.
   These are great issues that definitely should be brought up in the discussion and have now been added. Please see the discussion for this added insight.

10. There are several major concerns with section 6:
    a) The description of how to pour a gel and perform a western blot takes up over two pages of the protocol. This is a standard technique that feels out of place at this level of detail in a vector production and purification protocol written for molecular biology laboratories (as mentioned in the abstract), particularly when considering that a western blot absolutely does not assess purity of the vector. This space would be better used demonstrating what western blots of different serotypes compatible with the purification method should look like (see related point (c) below). Line 481 states "also check for possible protein contamination" which is impossible when the only bands the user will see are VP proteins (the ~30 kDa band is a product of VP proteolysis that occurs during any purification protocol, this is not a contaminant). A better method for these purposes would be standard SDS PAGE and staining with a sensitive dye such as SYPRO Ruby. This is much more straightforward, and will show contaminating proteins as well as the VP1, VP2, and VP3 bands and allow for quality assessment based on their ratios.
    We have greatly simplified this section.
    Although the AAV VP western blot will not detect host cell protein contamination, it is important to ensure the three structural proteins critical for AAV encapsidation are present in the correct ratio, and that there is little to no VP protein degradation. To evaluate host cell protein contamination, SDS PAGE followed by Coomassie or SYPRO Ruby staining (if the appropriate imaging equipment is available) is an easy method for evaluating the purity of your AAV preparation. This information has been added.

    b) Use of the Host-Cell Protein detection ELISA kit, which costs almost $800, seems ridiculous after pouring your own gel, which is time consuming, toxic, and inconsistent yet only costs $100 for ten pre-cast gels. The authors need to make a strong case for why this kit is suggested over standard SDS-PAGE, which would yield the same results with less cost and hands-on time. Moreover, there are no data demonstrating purity of the vector product by this method; as mentioned previously, a western blot does not demonstrate this, and representative results from the HCP ELISA are not included. The simplest solution would be to show stained SDS PAGE gels of the vector product, and it would greatly strengthen the article to include other serotypes that the authors have purified by this method.
    The host cell protein ELISA is a highly sensitive kit that allows the researcher to not only detect contaminants, but to quantify HEK293 host cell protein contaminants. We are not proposing that this ELSIA be used as a routine quality control assay. If one wants to more carefully scrutinize their purification process, especially if one is considering clinical translation of their AAV vector and/or process, this assay may be more appropriate. We can remove any reference to the kit if the reviewer would prefer that.

    c) The example prep in figure 5 has a very low level of VP1 protein, yet the authors specifically mention a 1:1:10 ratio should be seen (Line 480). This disparity should be resolved by providing a better example western blot (high MW bands transfer less efficiently, perhaps this is the problem?) or provide a discussion why the VP1 protein is almost nonexistent here. Is this typical for the AAV6.2FF variant?

A new image of a western blot has been added, which shows the VP1:VP2:VP3 proteins with VP3 at the highest protein density. Though the packaging of the AAV virion is typically a 1:1:10 ratio, different AAV serotypes may have different VP ratios ranging from 1:1:5 all the way to 1:1:20. Furthermore, crude extracts can yield as low as a 1:1:5 ratio (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3539887/#B21). The fact that there is variability in the ratio of VP1:VP2:VP3 has been added to the discussion.

11. The introduction discusses tropisms and kinetics of many AAVs, but several of the serotypes discussed cannot be purified by heparin column. The authors should explicitly state which of the listed serotypes cannot be purified by this method, not only those that can. Although AAV4 and 5 are mentioned in the discussion, it would strengthen the article to include an even more exhaustive list or table of serotypes compatible and incompatible with the protocol presented, in one cohesive location rather than several.
    The introduction has been modified to focus more on the AAV serotypes that would be suitable for the heparin sulfate. The serotypes that can be purified use heparin sulfate chromatography are mentioned in the introduction as a convenient way to introduce the methods and to ensure the reader is aware of the serotypes appropriate for this method prior to reading the protocol. Furthermore, the reintroduction of the appropriate/inappropriate serotypes is mentioned in the discussion when describing the strengths, weaknesses and limitations of the protocol.

12. Line 118: 20 mL of media will not fit into a 15 mL conical.
    Protocol has been corrected to read 10 mL of media.

13. Line 148: The cell suspension should first be diluted into the 1L of warm media, then added to the flask, but the wording here sounds like the media and cells are added separately. This is important for even cell seeding; consider revising.
    This is a good point and has been reflected in step 1.7.

14. A 3:1 PEI/DNA ratio is quite high. Considering lab to lab differences in the optimal ratio, perhaps it's worth mentioning that the PEI:DNA ratio can be adjusted as a way to troubleshoot low transfection efficiency.
    Experiments have been performed in regard to optimal PEI:DNA ratio for HEK293 transfections with pHelper and pTransgene. Though this ratio has worked for us, it has been added to the discussion that this ratio should be optimized for each plasmid set constructed.

15. Line 229: elution's should not have an apostrophe.
    This has been corrected.

16. The excel sheet (Table 7) is very convenient! (no concerns with it, just wanted to compliment it).
    Thank you very much!

17. There is a complete lack of troubleshooting advice. While not required, it would be more useful to the reader and a more appropriate use of space to offer suggestions on how to address low yield, high levels of impurities, etc. than how to cast a gel.
A small section has been added to the discussion to mention various methods of assessing the purity, including western blot, SDS-PAGE with Coomassie staining and SYPRO Ruby, and host cell protein ELISA. A few manuscripts have been references in the discussion to assist researchers with where to go to revise the protocols for reducing contamination. We are limited for room in the discussion, which has a 6 paragraphs limit.

Reviewer #3:

18. Please check the line spaces in table1. Some words are hided in reviewer's PDF version.
Table 1 has been updated to fit nicely into the reviewer's PDF.

19. Re-format Table 2 to make it concise and straightforward.
Simple instructions have been added to Table 2 for optimal use and simplicity.

20. Parts of Table7 are not visible in reviewer's PDF version, please check and re-organize the tables.
Table has been revised to fit properly.
21. Please double check whether 1 plate of 15cm at 80% confluence could provide enough cells for seeding a CCS10 cell stack at the 1E4 cell/cm^2.
The initial 15 cm plate is split into three 15 cm plates, and once at least 80% confluent, these cells are collected and together yield more than enough for seeding the cell stack at $1x10^4$ cells/cm^2.

22. Line188: keep the number format and the aberration consistent.
The note made on line 188 has been updated.

23. Line 193 & 173: check grammar.
Grammar on line 173 and 193 have been corrected.

24. Line 258: please specify the DNase classification, manufacture information.
The DNase brand and catalog number are in the materials/reagent list, which can be searched for all specifications in regard to the product. This is an RQ1 RNA-free DNase.

25. Line 258: what's the volume of sample for AAV titer? How to define the dilution fold "x40" (no potential loss of AAV 's vg?) to calculate the AAV titer if you choose to use the step4.6's clean-up procedure? Please clarify the if step 4.6 is alternative.
A note describing the breakdown of AAV titration calculations is made after step 5.6.7 in the "Data analysis to determine AAV genome copy numbers." We assume no loss of DNA in the DNA clean up step of 4.6. To ensure that no background binding in the AAV titration occurs, contaminants in the DNA extraction protocol are removed using a DNA cleanup kit. This is a

highly reputable kit (refer to materials/reagents table for exact kit), but this step is non-avoidable to ensure limiting of contaminants in qPCR.

26. For step6. Please describe the sample preparation, including the loading volume of AAV vectors.
    Sample buffer, which is described in Table 1, is made up in a 4x concentration, and is added to sample to create a 1x concentration. We have indicated how many vector genomes (VG) should be loaded to ensure good visualization by Western blot.

27. Step 6.1.8: How to identify the possible protein contamination (if any) without a standard as shown in Fig.5?
    A new figure has been added, along with a Coomassie stain to show difference between the VP1/VP2/VP3 AAV structural proteins, and additional proteins, which would be considered contaminants. Similar comments have been posed and addressed above.

28. How many independent experiments were fulfilled to show the "consistently yields >1E13 vector genomes/mL"? What's the total vg from each CCS10?
    This protocol has built on numerous years of AAV6.2FF vector production, with no changes to the protocol since June 2020. Since then, approximately 20 different batches of AAV6.2FF have been made, which yielded greater than $1x10^{13}$ vg/mL (ranging from from $1x10^{13}$ vg to $4x10^{13}$ total vg). Since we typically end up with a final volume of 1mL, we report that our protocol yields $>1x10^{13}$ vg/mL.
29. Line205: interpret "RT".
    This has been corrected to room temperature.

30. Please double check line 305-310, move them to Table 4.
    Lines 305-310 that refer to the preparation of the mastermix for AAV titration have been moved into the protocol, instead of between Table 4 and Table 5 for flow of protocol.

31. It is interesting to see similar dose applied in different animals lead to different transduction efficacy. Could author discuss this data?
    Similar comments were posed by Review 1 and 2 and have been addressed above. As well, points of discussion have been added to both the representative data section and discussion.

Editorial Comments:

1. The protocol step numbers have been formatted. Please check and ensure they are correct.
    Numbering of protocol has been formatted to reflect instructions for authors and is correct.
2. Lines 402-407: Please ensure that the Table and the Figure numbers referenced in the text are correct (e.g., The text “Figure 4” was changed to “Figure 3”)

The text now refers to the correct figures for each section of the protocol.

3. Section 4.6: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm) (lines 321, 326, 331, 338, etc.)

Centrifuge speeds have now been updated to x g for Section 4.6.

4. Line 356/438/: Please remove the link from the manuscript text and include it as a reference.

Hyperlinks have been removed and links have been added as references.

5. Line 518: Please check and correct the step numbers mentioned in the line.

This has been correctly to include the correct steps, 6.1.1 and 6.1.2.

6. Figure 4/5: Please insert the labels of the lanes in the figures to make them more informative.

Labels for samples in each lane have been updated in both the figure and the legend.

7. Please add a brief description for the Tables in the Figure legends section.

Brief descriptions have been added to each Table and Figure legend.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

3 pages of protocol have been highlighted for video.

UNIVERSITY of GUELPH

ONTARIO VETERINARY COLLEGE

DEPARTMENT OF PATHOBIOLOGY

May 19, 2021

RE: Manuscript ID JoVE62727

Dear Editor,

Thank you for considering our revised manuscript entitled "Production of Adeno-Associated Virus Vectors in Cell Stacks for Preclinical Studies in Large Animal Models" for publication in JoVE. We have carefully addressed all of the reviewer's comments and these corrections can be identified by the highlighted text within the body of the manuscript as well as in our detailed response to reviewers attached.

Thank you for considering our manuscript for publication. I look forward to your final decision in due course.

Sincerely,

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