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## **Scalable production and purification of Adeno-associated viral vectors (AAV)**

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### **Abstract**

Here we describe methods for the production of adeno-associated viral (AAV) vectors by transient transfection of HEK293 cells grown in serum-free medium in orbital shaken bioreactors and the subsequent purification of vector particles. The protocol for expression of AAV components is based on polyethyleneimine (PEI) mediated transfection of a 2-plasmid system and is specified for production in milliliter- to liter-scales. After PEI and plasmid DNA (pDNA) complex formation the diluted cell culture is transfected without a prior concentration step or medium exchange. Following a three-day batch process, cell cultures are further processed using different methods for lysis and recovery. Methods for the purification of viral particles are described, including iodixanol gradient purification, immunoaffinity chromatography and ultrafiltration, as well as quantitative PCR to quantify vector titer.

Keywords: Transient transfection, HEK293, adeno-associated viral vector, orbital shaken bioreactors, suspension cell culture, affinity chromatography purification

Running title: AAV vector production with HEK293 suspension cells

## 1. Introduction

Adeno-associated viral (AAV) vectors are amongst the most popular viral vectors for gene therapy applications in pre-clinical and clinical research (1). Discovered in 1965, AAV are non-pathogenic members of the *Dependovirus* genus of the parvoviruses and are endemic in humans (2, 3). Besides the numerous clinical trials that have been conducted to date, the recent approval by the FDA of Luxturna™, an AAV-based gene therapy to treat an inherited condition leading to blindness (Spark Therapeutics), demonstrates the popularity and feasibility of AAV-based gene therapy approaches. For many years, optimizing transgene delivery and expression, and the cell or tissue specificity of AAV variants, has been the focus of translational research. It is now that this technology reaches the market when needs and challenges concerning manufacturing and quality become most apparent. As a consequence, the focus of research and development is shifting into process development.

Today, one commonly used AAV production platform is based on plasmid transfection of adherent HEK293 cells grown in serum-containing media. This production method is difficult to scale-up, labor intensive and disadvantageous for manufacturing. Therefore cost-effective and scalable protocols are needed for large-scale AAV vector production. Recent research has validated the use of orbital shaking technology (OSR) for the cultivation of HEK293 cells in suspension, using disposable vessels up to a 1,000-Liter scale (4). Key features of the OSR technology are high gas transfer rates, low sheer stress for cells, low mixing times and low specific power consumption (5-7). These characteristics make the OSR technology one of the most promising disposable bioreactor platforms for animal cell culture for small- and large-scale operations. In parallel, different groups have demonstrated the technical feasibility to scale-up AAV production by transfecting suspension adapted HEK293 cells without prior concentration of cells or exchange of medium (8, 9). Here, we describe the transient transfection of suspension-adapted HEK293 cells in orbital shaken bioreactors for the production of AAV vectors.

Next to the upstream processing for vector manufacturing, scalable methods are also required for the downstream processing in order to generate AAV vectors that satisfy the requirements for clinical application. In many labs, the purification via gradient centrifugation is commonly used for lab-scale isolation of AAV vectors harvested from cell pellets. This density-based method has the advantage that it allows the removal of empty AAV particles. Nevertheless, as this operation is very laborious to process larger liquid volumes, it would not be suitable at a scale relevant for studies in large animal models or for clinical trials. As an alternative to centrifugation, chromatographic purification procedures can be applied. Various methods have been described for AAV purification in the past including ion exchange chromatography, hydrophobic interaction chromatography, and size exclusion chromatography (10-13). These have been applied successfully to achieve high purity, but often suffer from low recovery rates. The recent development of affinity ligands and resins offers a solution to this issue. Thanks to their high selectivity, affinity ligands allow the efficient removal of impurities while maximizing vector recovery (14, 15). The possibility to scale-up and to process large liquid volumes makes affinity chromatography an ideal option for downstream processing of AAV vectors at all scales.

## **2. Materials**

### **2.1 Cell culture**

1. HEK293 cells (ExcellGene SA, Monthey, Switzerland) adapted to serum-free medium and cultivated in orbital shaken bioreactors.
2. TubeSpin 50 bioreactor and TubeSpin 600 bioreactor tubes (TPP, Trasadingen, Switzerland) (see Note 1)
3. Serum-free Freestyle F17 Expression medium (Thermo Fisher Scientific AG, Reinach, Switzerland) (F17) is supplemented with L-alanyl-L-glutamine (Gibco Glutamax, Thermo Fisher Scientific) by adding 20 mL of a 200 mM stock solution to 1 L of F17 medium. Additionally Pluronic F-68 (Thermo Fisher Scientific) was added to a final concentration of 0.1 % (10 mL of a 10% stock solution in 1 L F17 medium).

4. 0.4 % Trypan Blue solution
5. Phosphate-buffered saline (PBS).
6. ISF1-X or ISF4-X incubator shaker (Adolf Kühner AG, Birsfelden, Switzerland)
7. Phase contrast microscope

## **2.2 Transfection**

1. Linear 25 kDa polyethyleneimine (PEI) (Polysciences, Eppenheim, Germany): to prepare the 1 mg/mL PEI stock solution, 1 g of PEI is weighed and transferred in 800 mL water. To dissolve the PEI, the pH is lowered to about 3 by adding 1 N HCl. Once PEI is dissolved, the pH is increased to 7.0 with 1 N NaOH and the volume adjusted to 1 L with water. Next, the solution is sterile-filtered using a bottle-top filter (0.2  $\mu$ m) and aliquoted into sterile 50-mL tubes for storage at  $-20^{\circ}\text{C}$ .
2. Shuttle plasmid containing the sequence encoding the gene of interest flanked by two ITRs, typically derived from AAV serotype 2 (see Note 2).
3. pDP-helper plasmid encoding the rep (AAV2) and cap genes (serotype dependent), and adenoviral helper genes (VA, E2A, E3, and E4) (see Note 3).
4. Plasmid DNA (pDNA) obtained from contract manufacturer. Adjusted to concentrations of 1 mg/mL with distilled water for long-term storage ( $-80^{\circ}\text{C}$ ).

## **2.3 Harvest and lysis**

1. Triton X-100 (Merck, Zug, Switzerland)
2. 10x Lysis buffer: 1% Triton X-100, 20 mM  $\text{MgCl}_2$  in 500 mM Tris buffer pH 7.4
3. Dry ice
4. 70% ethanol
5. 5 M NaCl, sterile filtered
6. Benzonase 25 kU/mL (Merck)

7. 10% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (Axon Lab AG, Le Mont-sur-Lausanne, Switzerland)
8. Supracap™ depth filter, 0.4 - 1.0 µm, Seitz BIO 20 media (Pall, Basel, Switzerland)
9. AcroPak™ 0.8/0.45 µm membrane filter (Pall)
10. Hermle Z 513 K centrifuge and Hermle 220.70 rotor with tube holder for TubeSpin 50 and Tubespin 600 (Hermle Labortechnik GmbH, Wehingen, Germany) (for cell harvest at 1,000xg)
11. Sorvall Evolution RC centrifuge and Sorvall SLA-600TC Super-Lite rotor (50 mL centrifugal tubes) (Thermo Fisher Scientific) (for removal of cell debris at 10,000xg)

## **2.4 Iodixanol gradient centrifugation**

1. Optiprep (Axon Lab AG, Baden-Dattwil, Switzerland), sterile and endotoxin tested multi-purpose medium based on 60% iodixanol in water.
2. 0.5 % Phenol red solution
3. PBS-MK buffer: DPBS (500 mL) supplemented with 50 mg MgCl<sub>2</sub> × 6H<sub>2</sub>O.
4. PBS-MK 1M NaCl: obtained by addition of 36 g NaCl to 1 L PBS-MK
5. Preparation of 4 gradient solutions, 50 ml final volume:
  - 15%: 12.5 ml Optiprep, 37.5 ml PBS-MK 1 M NaCl
  - 25%: 20.8 ml Optiprep, 29.2 ml PBS-MK and 125 µL Phenol red
  - 40%: 33 ml Optiprep and 17 ml PBS-MK
  - 60%: 50 ml Optiprep and 125 µL Phenol red
6. Beckman Coulter ultracentrifuge Optima 90-K (Beckman Coulter, Nyon, Switzerland)
7. Rotor 70Ti (Beckman Coulter)
8. OptiSeal polypropylene tubes (32.4 mL) (Beckman Coulter)
9. Spacer (Adapter for tubes in Rotor 70Ti) (Beckman Coulter)

10. Syringes 10 ml (B.Braun, Sempach, Switzerland)
11. Needle 18 Gauge (Thermo Fisher Scientific)
12. Syringe equipped with spinal needle (1.27 x 89 mm) (Becton Dickinson, Allschwil, Switzerland)

## **2.5 Immunoaffinity chromatography**

1. POROS™ CaptureSelect™ AAV9, AAV8 and AAVX resin (Thermo Fisher Scientific) (see Note 4)
2. Omnifit® column (Diba Industries Inc, Danbury, CT, USA) with movable end piece.
3. Equilibration buffer: PBS, pH 7.4
4. 0.1 M citric acid monohydrate solution
5. 0.1 M trisodium citrate dehydrate solution
6. 50 mM citrate elution buffer: citric acid buffer pH 3 is prepared by mixing 82 mL of the 0.1 M citric acid monohydrate solution and 18 mL of the 0.1 M trisodium citrate dihydrate solution. The resulting solution is diluted two fold to obtain 50 mM citrate elution buffer (see Note 5)
7. Regeneration buffer: 5 M guanidine hydrochloride
8. Cleaning buffer: 0.1 M phosphoric acid
9. ÄKTA Pure chromatography controller (GE Healthcare, Glattbrugg, Switzerland)
10. Fraction collector F9-R (GE Healthcare)
11. UNICORN 7 control software for ÄKTA purification systems (GE Healthcare)

## **2.6 Ultrafiltration**

1. DPBS (without calcium and magnesium) (Thermo Fisher Scientific)
2. DPBS (Thermo Fisher Scientific)

3. Amicon<sup>®</sup> Ultra-15 centrifugal filter units, molecular weight cutoff (MWC) 100 kDa (Merck)

4. Amicon<sup>®</sup> Ultra-4 centrifugal filter units, MWC 100 kDa (Merck)

## **2.7 Quantitative Polymerase chain reaction (qPCR)**

1. Rotor gene probe qPCR kit (Qiagen AG, Hombrechtikon, Switzerland)

2. Forward primer (4  $\mu$ M) (see Note 6)

3. Reverse primer (4  $\mu$ M) (see Note 6)

4. qPCR probe (2  $\mu$ M) (see Note 6)

5. QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific)

## **Methods**

### **3.1 Cell culture**

1. Frozen HEK293 cells are thawed at 37°C and immediately transferred into a centrifuge tube containing 10 mL pre-warmed F17 medium.

2. After quick shaking by hand the tube is centrifuged at 500xg for 3 min.

3. The supernatant is discarded and cells are resuspended with pre-warmed 10 mL F17 medium and transferred into a TubeSpin 50 bioreactor tube. Henceforth, the cultures are maintained at 37°C, 80% humidity, 5% CO<sub>2</sub> and constant shaking at 180 rpm (shaking diameter 5 cm). (see Note 7)

4. After 3-4 days cells are counted using the Trypan blue staining, a Neubauer hemocytometer and an inverted phase contrast microscope. (see Note 8)

5. Based on the viable cell density, the cell suspension is diluted to a final concentration of  $1 \times 10^6$  cells/mL by transferring the according cell culture volume into fresh and pre-warmed F17 medium. (see Note 8)

6. To maintain the cell culture at high viability, the cells are passaged twice per week.



### 3.2 Transient transfection

1. Three days before transfection, cells are seeded at  $1 \times 10^6$  cells/mL in a volume corresponding to the desired scale of vector production (TubeSpin 50 or TubeSpin 600 bioreactor tube). (see Note 9)
2. On the day of transfection, cells are counted as previously described (see 3.1) and diluted to  $1 \times 10^6$  cells/mL in fresh F17 medium (TubeSpin 50 or TubeSpin 600 bioreactor tube).
3. The helper and shuttle plasmids are pre-mixed at equimolar ratios, with the total amount of DNA and volume depending on the scale of transfection and the total cell number (Table 1).
4. A volume of F17 medium (room temperature) corresponding to 5% of the cell culture is added to a sterile polystyrene tube (depending on scale of transfection use 5 mL or 19 mL tube).
5. A total of 1.5  $\mu$ g of pDNA per million cells is added to the F17 medium. The tube is then shaken briefly. (see Note 10)
6. 3  $\mu$ g of PEI per million cells is added to the tube (DNA:PEI = 1:2 [w/w]). (see Note 10)
7. At this point the tube is gently vortexed.
8. The tube is then incubated for 10 min at room temperature for pre-complex formation. (see Note 11)
9. The whole content of the tube is then transferred to the cell suspension for transfection.
10. The cell culture is subsequently incubated at standard conditions described above (3.1, step 3.) for 48-72 h before vector harvest (Figure 1). (see Note 12)

Table 1: Parameters for transfections at different scales using TubeSpin bioreactors.

Culture vessel	Culture Volume [mL]	Cell density Transfection	Cells total	Pre-mix volume [mL]	DNA [ $\mu$ g]	PEI [ $\mu$ g]
TubeSpin 50	10	$1 \times 10^6$	$10 \times 10^6$	0.5	15	30
TubeSpin 600	100	$1 \times 10^6$	$100 \times 10^6$	5	150	300
TubeSpin 600	250	$1 \times 10^6$	$250 \times 10^6$	12.5	375	750

### 3.3 Harvest

If purification via gradient centrifugation is intended as a one-step purification, only the cells are processed to isolate intracellular AAV particles. For immunoaffinity chromatography, it is optional to collect cells and/or culture medium. As it is possible to process larger liquid volumes with this method, cells can also be lysed in the cell culture directly. (see below 3.4.2)

1. The cells are harvested by centrifuging the TubeSpin 50 bioreactors at 1,000xg for 5 min or TubeSpin 600 bioreactor tubes at 1,000xg for 10 min. It is recommended to collect and store at  $-20^{\circ}\text{C}$  a sample of the supernatant at this point to determine the amount of particles released by the packaging cells (e.g. by quantifying the amount of genome-containing particles via qPCR).
2. Discard the supernatant and gently resuspend the pellet in 10 mL PBS.
3. Transfer in a 50 mL polypropylene centrifuge tube and centrifuge at 1,000xg for 7 min.
4. After discarding the remaining supernatant, the cell pellet is further processed, continuing with the steps described in 3.4.1 or 3.4.2. The cell pellet can be stored at  $-20^{\circ}\text{C}$  before further processing.

### 3.4 Lysis

#### 3.4.1 Freeze-thaw lysis (see Note 13)

1. Mix dry ice with 70% ethanol.
2. If required, thaw the pellet initially in a water bath at  $37^{\circ}\text{C}$ .

3. Resuspend the pellet in PBS with a volume corresponding to 2% of the initial cell culture volume.
4. Then freeze (10 min) in dry ice-ethanol mix and thaw (10 min) in 37°C water bath. After thawing, the suspension is vortexed vigorously for 30 sec. This procedure is repeated three times (Figure 1).
5. Add Benzonase at 50 U/mL and incubate 30 min at 37°C under agitation (100 rpm).
6. Add 0.5% CHAPS, incubate 30 min at 37°C and 100 rpm.
7. Add 5 M sodium chloride to a final concentration of 0.1 M and vortex briefly. The volume can be estimated based on the graduation displayed on the 50 mL polypropylene centrifuge tube.
8. Centrifuge for 10 min at 10,000xg, 4°C.
9. Pass the supernatant through a 0.45 µm filter (see Note 14). The lysate is ready for iodixanol gradient centrifugation or immunoaffinity chromatography. (see Note 15)

#### **3.4.2 Triton X-100 lysis**

1. Add 10% (v/v) lysis buffer to the cell culture (Figure 1). If only the cell pellet is processed, resuspend the pellet in PBS with a volume corresponding to 2% of the initial cell culture volume. (see Note 16)
2. Add Benzonase at 50 U/mL.
3. Incubate at 37°C, 100 rpm shaking for 30 min.
4. Add 5 M sodium chloride to a final concentration of 0.1 M and briefly vortex. The volume can be estimated based on the graduation displayed on the 50 mL centrifuge tube.
5. If only the cell pellet is processed and the volume does not exceed 15 mL, centrifuge for 10 min at 10,000xg, 4°C (Figure 1). For larger volumes, proceed with step 6.
6. Pass the lysate through a depth filter for clarification and removal of debris.

7. Pass the supernatant through a 0.8  $\mu\text{m}$  pre-filter and then through a 0.45  $\mu\text{m}$  filter. It is also possible to use filter capsules with a built-in pre-filter. (see Note 14)

8. The lysate is ready for iodixanol gradient centrifugation or immunoaffinity chromatography. (see Note 15)

### **3.5 Iodixanol gradient centrifugation**

1. Rinse ultracentrifuge tube with sterile PBS to remove electrostatic charge that may cause air bubbles otherwise.

2. Place sterile OptiSeal tube, washed in PBS, in the assembly rack.

3. Pipette the lysate (6 mL maximal volume) at the bottom of the 30 ml ultracentrifuge tube.

4. Using a needle connected to a syringe, underlay the lysate with 15% (7 mL), 25% (5 mL), 40% (5 mL) and 60% (8 mL) iodixanol solutions (Optiprep). This can be achieved by carefully placing the tip of the needle at the very bottom of the tube for each of the different solutions sequentially added according to increasing iodixanol concentrations.

5. The tube is then sealed before centrifugation.

6. Centrifuge in 70Ti rotor at 350,000 $\times$ g for 1 h at 4°C.

7. Fix the tube on holder, remove the rubber lid from top and insert syringe with 18G needle at the border between the 60% and the 40% iodixanol fractions, with the needle facing upwards to the lower portion of the 40% iodixanol fraction where the viral particles accumulate.

8. Carefully remove the entire 40% iodixanol layer (transparent), without disturbing the layer of protein contaminants that accumulate at the interphase between the 40% and 25% iodixanol layers. It is typically possible to collect 4-5 mL of suspension.

9. Dilute 1:1 in PBS pH 7.4.

10. Continue with the ultrafiltration described in 3.7.

### 3.6 Immunoaffinity chromatography

This method has been implemented using the Poros™ CaptureSelect™ AAV9 and AAV8 resins. The AAVX resin has recently been introduced. Together, these three commercially available resins allow the purification of capsids of the AAV1 through AAV9 serotypes, and possibly a variety of modified and chimeric serotypes. (see Note 17)

1. After gently mixing the bottle containing the resin (resins are supplied as approximately 56% slurry in 18% ethanol), the desired amount of slurry is transferred to an Omnifit® column with a serological pipette.
2. After transferring the slurry, the resin is allowed to settle by gravity. Do not let the resin dry out.
3. Once the resin is not compacting any further, the column is filled with distilled water. A convex meniscus should form at the very top of the column.
4. The removable column end piece is screwed back on the column without introducing any air bubble into the column. The part with the 25 µm PTFE frit is moved on top of the resin leaving 1-2 mm space between resin and frit (watch out for liquid that may spill out of the column!).
5. Once the end piece is in place, the column is connected to the chromatography controller.
6. A flow rate of 10 mL/min (linear velocity  $\approx$  763 cm/h) is applied to pack the column. Water is used for this step. Using 10 mL of slurry, the volume of the packed column should be approximately 6 mL under these conditions.
7. The column is either equilibrated with 10 column volumes (CVs) at a flow rate of 2 mL/min (linear velocity  $\approx$  152 cm/h) with equilibration buffer for immediate use, or a 20% ethanol solution for storage at 4-8°C.
8. The clarified lysate (see 3.4) is loaded with a flow rate of 1 ml/min (linear velocity  $\approx$  76 cm/h).

9. The resin is then washed with equilibration buffer at a flow rate of 2 mL/min (linear velocity  $\approx$  152 cm/h). Washing is made with at least 5 CVs or until the 280 nm absorbance reaches baseline value or remains stable.
10. Bound AAV9 particles are recovered by a one-step elution using 50 mM citrate buffer (pH 3) with a flow rate of 2 mL/min. (see Note 18)
11. Peak fractions are collected with a fraction collector and immediately after collection adjusted to a neutral pH by adding 50  $\mu$ L neutralization buffer per mL elute (1M Tris, pH 9). (see Note 19)
12. The peak fractions are pooled based on the recorded 280 nm absorbance. (see Note 20)
13. At this point continue with Section 3.7 for further sample processing. The steps below describe the procedure for column regeneration and storage.
14. After the elution step, the column is regenerated with 5 M guanidine hydrochloride buffer.
15. The chromatography system and resin can be cleaned using 0.1 M phosphoric acid. (see Note 21)
16. The column is re-equilibrated with equilibration buffer (5 CVs)
17. For storage at 4-8°C repeat step 7 using 20% Ethanol.

### **3.7. Ultrafiltration**

With this final step the preparations deriving from the gradient centrifugation (3.5) or the immunoaffinity chromatography (see 3.6) can be subjected to buffer exchange and concentrated (Figure 1).

1. Add 12 mL sterile water to the Amicon centrifugal filter unit and centrifuge at 3,000xg.
2. Discard the flow-through.
3. Add 12 mL sterile DPBS (without calcium and magnesium) to the Amicon centrifugal filter units and centrifuge at 3,000xg. (see Note 22)
4. Discard the flow-through.

5. Add gradient fraction or the pooled elution fractions to the Amicon Ultra-15 (a maximum of 12 mL)
6. Centrifuge at 3,000xg until volume is reduced to 1 mL (the volume depends on the desired AAV particle concentration).
7. Discard the flow-through.
8. Add DPBS (without calcium and magnesium) to the concentrated particles to a final volume of 15 mL.
9. Repeat steps 6-8 up to 3 times.
10. Add DPBS to the concentrated particles to a final volume of 15 mL.
11. Centrifuge at 3,000xg until volume is narrowed down to 500  $\mu$ L.
12. The AAV particle suspension can at this point be aliquoted in low retention tubes for storage at  $-80^{\circ}\text{C}$ . (see Note 23)
12. For further concentration, transfer the vector suspension to the Amicon Ultra-4 and add DPBS to a final volume of 4 mL.
12. Centrifuge at 3,000xg until the desired volume is reached (the volume depends on the desired AAV particle concentration) and discard the flow-through.
13. Aliquot the concentrated AAV particle suspension in low retention tubes for storage at  $-80^{\circ}\text{C}$  (Figure 1).

### **3.8 Determination of the amount of genome-containing AAV particles**

There is a variety of methods that are frequently used to quantify AAV vectors. The most commonly used method is based on qPCR and determines the number of vector particles that contain a genome (Figure 1). The advantage of this method is that it is fast and inexpensive and it can be applied independently of the vector serotype. However, this method does not provide any measurement for the infectivity of vectors or the amount of empty particles in a preparation.

1. Thaw an aliquot of purified vector at room temperature and pipette twice 2  $\mu\text{L}$  of the purified vector to low retention reaction tubes.
2. Dilute 1:10<sup>4</sup> and 1:10<sup>5</sup> with PBS using serial dilution steps.
3. Prepare pDNA standards containing the same template DNA used to amplify and quantify the vector genomes. Establish a standard curve based on a known number of pDNA copies, ranging from 10<sup>3</sup> to 10<sup>7</sup> copies. Note that each plasmid copy is equivalent to two single stranded AAV vector genomes.
4. Prepare master mix for PCR by combining 5  $\mu\text{L}$  of 2x Rotor-Gene probe PCR master mix, 1  $\mu\text{L}$  forward primer (4  $\mu\text{M}$ ), 1  $\mu\text{L}$  reverse primer (4  $\mu\text{M}$ ), 1  $\mu\text{L}$  probe (2  $\mu\text{M}$ ). Vortex the master mix before further use. (see Note 25)
5. Distribute reaction master mix to reaction tubes (8  $\mu\text{L}$ /tube).
6. Prepare triplicates for each sample. 2  $\mu\text{L}$  of the diluted samples or standards are transferred to each of the three tubes containing the reaction master mix (10  $\mu\text{L}$  total reaction volume). The tubes are then quickly centrifuged for 5-10 sec.
7. To lyse the AAV capsid and release the vector genome (PCR template) the reaction tubes are heated to 95°C for 20 min in the PCR cycler before initiating the PCR cycling (40 cycles of 3 sec at 95°C followed by 10 sec at 60°C).
8. Determine the number of genome-containing AAV particles, taking into account the quantification according to the standard curve, sample dilution, and the volume of vector suspension added to the PCR. Of note, a factor 2 correction has to be applied when the single-stranded AAV genome is compared to double-stranded plasmid DNA.

## Notes

1. OSRs are available as single-use bioreactor systems up to a scale of 1,000 L (4). The mixing principle of these reactors is based on



horizontal movement and shaking of the entire reactor in contrast to the mixing of stirred tank bioreactors with an impeller. Key features of the OSR technology are high gas transfer rates, low mixing times and low specific power consumption (5-7). These characteristics make the OSR technology one of the most promising disposable bioreactor platforms for animal cell culture for small- and large-scale operations.

2. The backbone of the shuttle plasmid can be a standard vector containing a bacterial origin of replication and a bacterial selection gene. The sequence flanked by the ITRs will be incorporated into the AAV capsid as the recombinant genome and has to be designed to encode the protein or the small RNA of interest.
3. The pDP helper plasmid is a part of a two-plasmid system typically used to produce rAAV vectors. As an alternative, a three-plasmid system is commercially available. This system encodes the AAV essential genes (rep and cap) and the adenoviral helper genes (VA, E2A, E3, and E4) on two separate plasmids. We have compared both systems and did not observe a significant impact on the overall vector yield between them.
4. The nature of the Poros™ CaptureSelect™ AAV resins and their mechanical resistance to pressures up to 10 MPa allows the application of high linear velocities. Hence, these resins are ideally suited to process large liquid volumes in a manufacturing scale.
5. The buffer pH should be around 3 but should be checked using a pH probe and be adjusted, if necessary.
6. The primers are designed according to the recombinant AAV genome (pPCR template).
7. Using the TubeSpin 50 bioreactor a working volume of 20 mL should not be exceeded. The ideal working volume is 10-15 mL. Above this, the formation of cell clusters can be observed with some cell lines.
8. When HEK293 cells are seeded in F17 medium at  $1 \times 10^6$  cells/mL, cells have to be passaged after a maximum of 5 days. Seeding at a lower

cell density ( $0.3-0.5 \times 10^6$  cells/mL) allows prolonging the cultivation time to 6-7 days.

9. Based on the growth characteristics of the HEK293 cells the dilution factor on the day of transfection is approximately 5 - 6 fold. For a 1-liter transfection, a minimum of 200 mL of cell suspension at  $1 \times 10^6$  cells/mL will need to be seeded three days before the transfection. As the cells are typically maintained in 10 mL cultures, it might be necessary, depending on the scale of production, to expand the cell culture volume already one week (2 passages) before the planned transfection.
10. The parameters for transient transfection of HEK293 cell suspensions are based on protocols optimized in our lab, which are used to define total pDNA amount per cell and the w/w ratio of pDNA:PEI (16, 17). In combination with the findings from other groups we adapted protocols towards the transfection of HEK293 cells in conditioned Freestyle-F17 medium (8, 9). Depending on several parameters including the cell line, the plasmids used for vector production, and the type of AAV, the amounts of pDNA and PEI have to be optimized on a case-by-case basis.
11. After a short incubation time (30 sec), an increased turbidity can be observed. This is an indicator of complex formation. The tube with medium containing pDNA and PEI appears “milky” compared to a tube with medium only.
12. The incubation time depends on the cell line, the medium and the process strategy (e.g. batch, fed-batch or perfusion). Under standard batch conditions we have observed a maximum accumulation of AAV vectors (intracellular) between 48 h and 72 h post-transfection. Furthermore, we did not observe any significant increase of the overall vector yield for longer process times. After 72 h, the number of particles in the culture medium may increase due to a loss of cell viability.
13. Freeze-thaw lysis is mostly used when lysates are directly purified via gradient centrifugation. We have observed that detergents in the

lysate, in particular sodium deoxycholate, can interfere with gradients and separation.

14. The choice of filter depends on the production scale and the particle burden of the lysate. At a liter-scale of production, syringe filters are not suitable anymore. At this scale, membrane filter capsules connected to a peristaltic pump are best suited. With an increasing scale of production the usage of membrane filters becomes too expensive and inefficient. A solution at this scale is the application of depth filtration to remove most of the insoluble particles followed by a membrane filtration (0.45  $\mu\text{m}$ -0.8  $\mu\text{m}$ )
15. The vectors are usually not purified when transfections are carried out at a small scale (10-15 mL in TubeSpin 50 bioreactors) for screening purposes in order to optimize the transfection efficiency, AAV yields or other process parameters. Indeed, depending on the number of transfections, this would be very laborious. Also, the quantities produced at this scale could not be efficiently recovered. To quantify the vector yield, cells are collected and lysed in 1.5 mL tube following the same protocol as described in 3.4.1 or 3.4.2. A sample of the supernatant (1 mL) is taken as well. The amount of intracellular vector and the amount of vector in the cell culture supernatant can be quantified using qPCR as described in 3.8.
16. Triton X-100 addition as compared to the freeze-thaw method is very simple to perform and less time consuming. The method is applied when lysates are directly purified using immunoaffinity chromatography.
17. The purification of AAV vectors via gradient centrifugation (iodixanol or CsCl) has long been used to produce research grade vector preparations for pre-clinical studies (18). The combination of gradient centrifugation and chromatographic procedures results in a higher purity and can help to decrease possible adverse effects of protein impurities during *in vivo* studies.

18. The elution conditions have been optimized for the step gradient elution of AAV9. We found that elution was most efficient at a pH of 3. Instead of citrate buffer other acidic buffers can be used for elution.
19. The AAV capsids are in general relatively heat and pH stable. Nevertheless, extended storage under extreme conditions (pH or temperature) will result in the denaturation of AAV capsids and a decreased vector amount. To avoid this, the pH is immediately adjusted to 6.5-7.5 after fraction collection.
20. The peak is not defined very stringently. One fraction before the A280 nm signal starts increasing and one fraction after the signal reaches baseline are included in the pooled fractions, with the aim to increase vector recovery.
21. It is important to clean the system and resin sufficiently in order to avoid residual contaminations.
22. Above vector particle concentrations of  $10^{13}$  vectors/mL, aggregation of AAV particles can occur. Previous studies have demonstrated that by increasing ionic strength of the suspension buffer, it is possible to limit AAV aggregation (19).
23. The aliquot volume is chosen based on the application and the vector amount needed for an experiment. Re-freezing a vial should be avoided, as repeated freeze-thaw cycles might cause vector degradation.

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**Figure 1: Process-scheme for the transient production of AAV vectors with HEK293 cells in suspension.** (1.) PEI-mediated transfection of HEK293 cells in suspension using a 2-plasmid system for AAV expression. (2.a) Collection of cells via centrifugation and subsequent cell lysis via repeated freeze-thaw cycles. (2.b) Cell lysis by direct addition of Triton X-100 to the cell suspension. (3.a) Removal of cell debris (clarification) via centrifugation (supernatant contains AAV vectors). (3.b) Clarification by filtration (depending on production scale, use membrane and/or depth filtration) (filtrate contains AAV vectors). (4.a) Immunoaffinity chromatography purification. (4.b) Purification via density gradient centrifugation. (5.) Buffer exchange and concentration using ultrafiltration (retentate contains AAV particles). (6.) Retentate is aliquoted for storage. The number of genome-containing particles in the purified AAV preparation is quantified via qPCR.

