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Supplemental information

Development of a scalable and robust AEX method for enriched rAAV preparations in genome-containing VCs of serotypes 5, 6, 8, and 9

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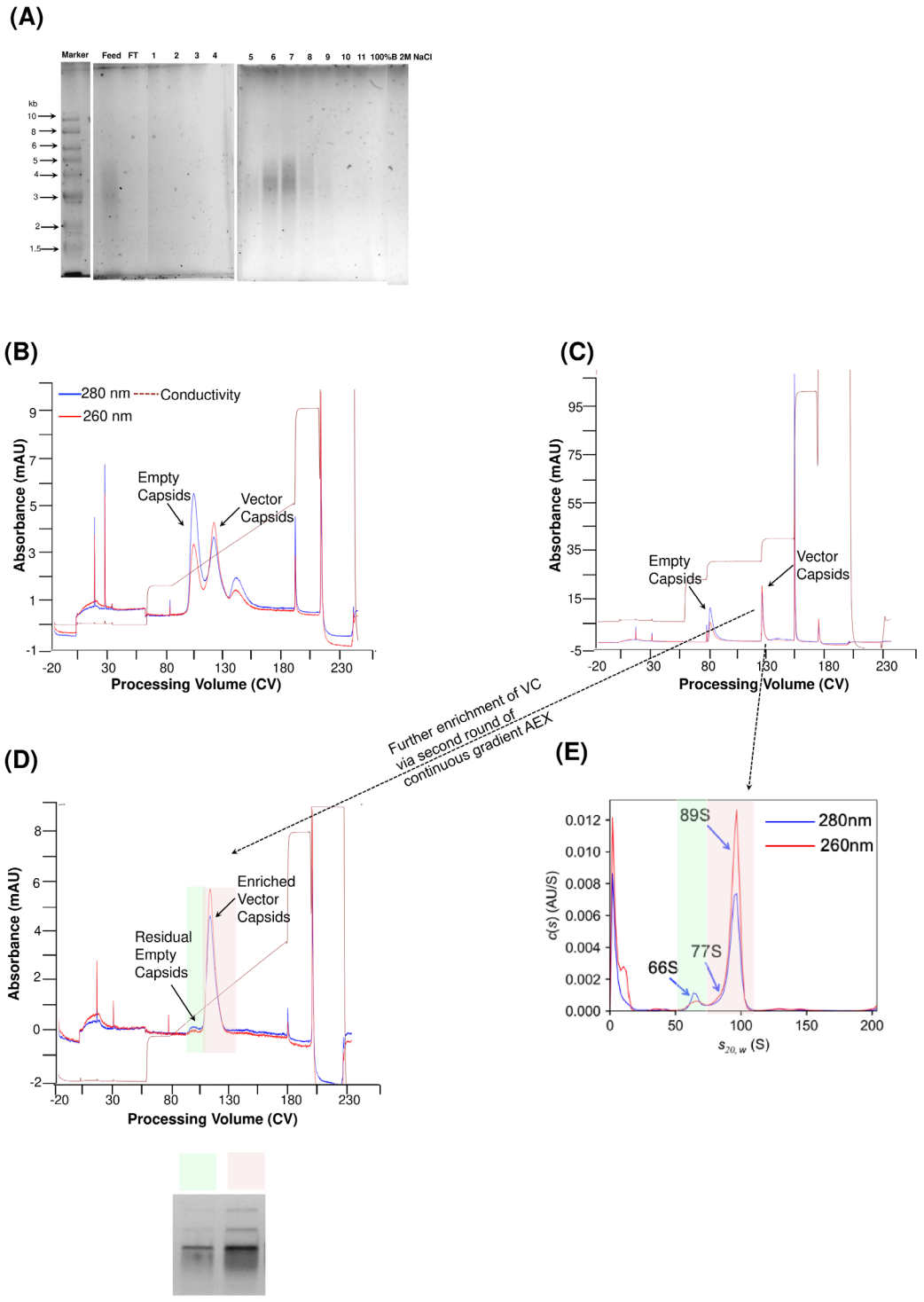
Supplemental Data

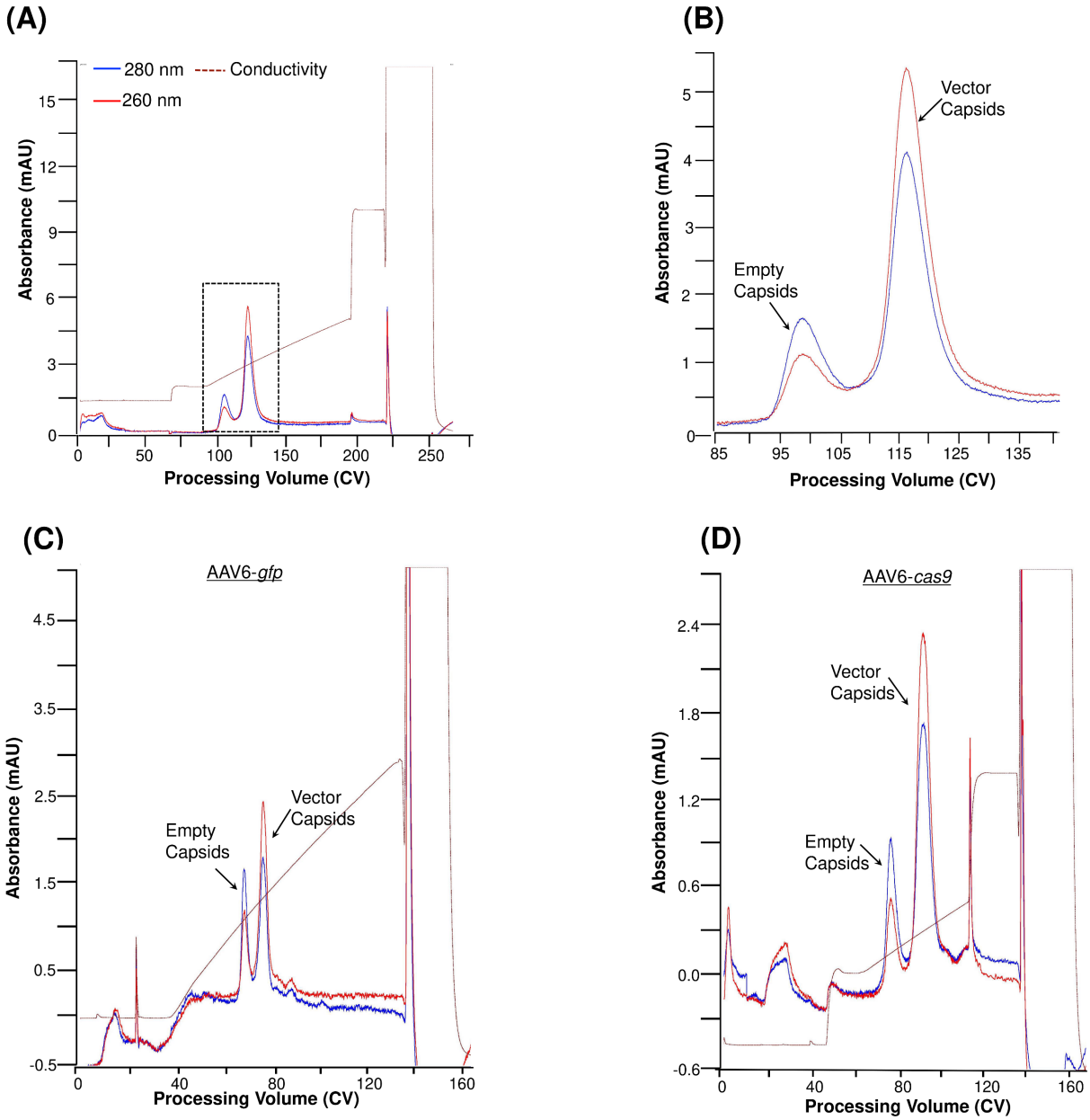
Figure S1



AAV5 AEX process characteristics

(A) Alkaline agarose gel profile of fractions collected from continuous gradient elution run of AAV5. **(B)** Continuous gradient elution profile and **(C)** optimized step-gradient elution profile of AAV5 AEX run. **(D)** Second round of AEX for near-complete removal of residual empty capsids and further enrichment of vector capsids in the VC fraction collected from step-gradient elution run **(C)**. Residual empty capsids separated in the second round of AEX and enriched vector capsids are shown in different color shades. The SDS-PAGE/silver staining profile of AAV5 empty capsids and vector capsids fractions are shown. **(E)** Representative sv-AUC profile of VC fraction of step-gradient elution run **(C)**. AEX, anion-exchange chromatography; CV, column volume; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; sv-AUC, sedimentation velocity analytical ultracentrifuge; VC, vector capsids.

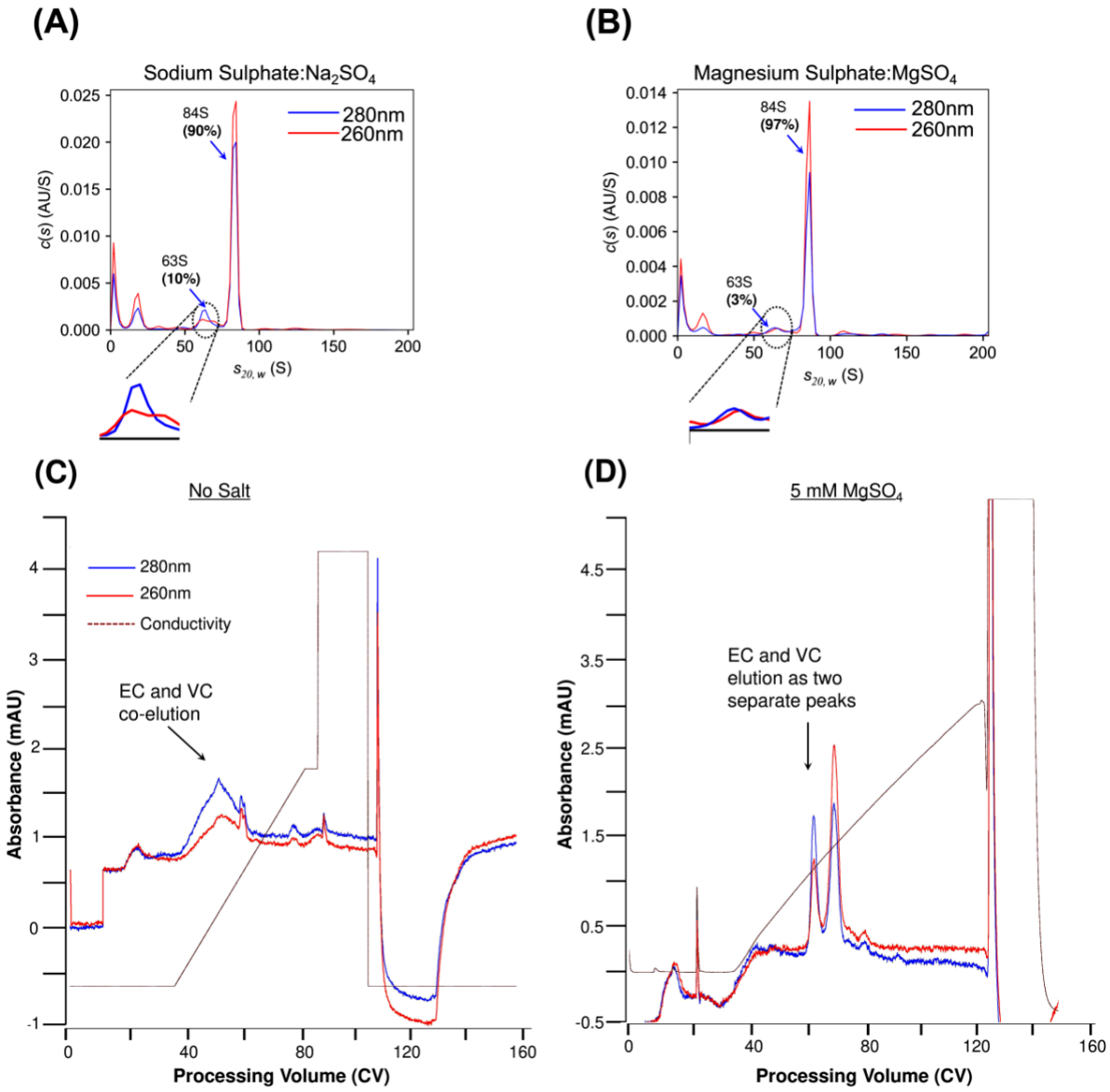
Figure S2



AAV6 and AAV8 Serotypes elution profiles under optimal conditions of continuous gradient AEX process

(A) AAV8-*gfp* elution profile under optimal conditions of AEX processing (10 mM BTP, pH 9.0, and $MgSO_4$) operated under a shallow continuous gradient of salt (0.4 mM salt/CV). **(B)** Enlarged image of two distinctly separate peaks corresponding to AAV8 EC and VC. **(C)** AAV6-*gfp* and **(D)** AAV6-*cas9* elution profiles under optimal conditions of the AEX process (10 mM BTP, pH 9.0, and $MgSO_4$) operated under a shallow gradient of the salt (0.4 mM salt/CV).

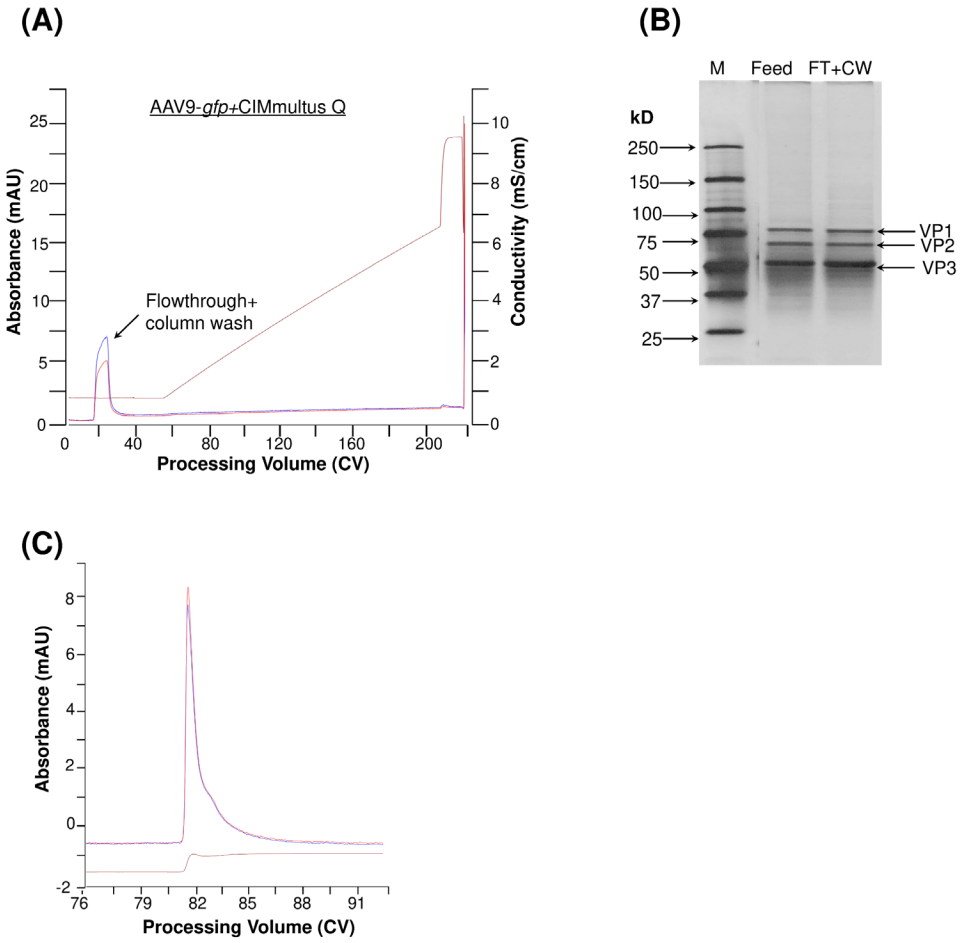
Figure S3



Effect of buffer composition on AAV VC enrichment and separation

sv-AUC profile of AAV8-*gfp* VC peak fraction collected under Na_2SO_4 **(A)** and $MgSO_4$ **(B)** salt gradient. In Na_2SO_4 elution fraction, the relative proportion of EC (63S) and VC (84S) are 10% and 90%, respectively. The peak at 63S (enlarged) also shows a higher 280 nm signal profile and $A_{260/280}$ =0.69. In the $MgSO_4$ elution fraction, the peak at 63S, though present, does not represent the $A_{260/280}$ profile representative of EC, indicating a possibility of UV signal noise in *sv*-AUC. Nevertheless, upon calculation, the relative proportions of 63S and 84S peaks were 3% and 97%, respectively. AAV6-*gfp* vector elution profile without **(C)** and with **(D)** additional salt in the exchange buffer and column equilibration buffer. $A_{260/280}$, the ratio of peak area measured at 260 nm and 280 nm absorbance: *sv*-AUC, sedimentation velocity analytical ultracentrifugation.

Figure S4



AAV9-gfp AEX characteristics

(A) AAV9-gfp AEX continuous gradient elution run profile with CIMmultus Q-1mL column. **(B)** SDS-PAGE/silver staining profiles of feed and FT+CW fraction collected from AAV9- *gfp*/CIMmultus Q run (A). **(C)** Enlarged image of AAV9-gfp vector capsid fraction collected from AAV9-gfp/POROS HQ run (Figure 5E). CW, column wash; FT, flowthrough; M, protein molecular weight marker.

Table S1. Chromatographic parameters of AAV5 elution under different salts

Condition	Peak resolution (R_s)	$A_{260/280}$		% Peak area[d]			
		Empty Capsid	Vector Capsid	Empty Capsid (280 nm)	Vector Capsid (280 nm)	Empty Capsid (260 nm)	Vector Capsid (260 nm)
AAV5/*sv*-AUC	NA	0.53	0.97[c]	61[e]	39[e]	48[e]	52[e]
AAV5/NaCl	0.31[a]/0.5[b]	0.63	0.89	72	28	24	76
AAV5/Na_2HPO_4	0.55 [a]/1.06[b]	0.71	1.21	38	62	28	72
AAV5/Na_2SO_4	0.49 [a]/0.90 [b]	0.62	1.19	53	47	35	65

NA, not applicable; $A_{260/280}$, the ratio of peak area measured at 260 and 280 nm absorbance

[a] Calculation of resolution of based on the equation for peak width and peak distance: [$R_s=2(V_2- V_1)/(W_1+W_2)$]. V_1 and V_2 are the elution points of peak 1 and 2, respectively. W_1 and W_2 are peak width of peak 1(empty capsid) and 2 (vector capsid), respectively; see figure below. The retention time was converted to retention volume by multiplying with the flow rate: 0.5 mL/min.

[b] Calculation of resolution based on an alternative equation.
Rs = $(\sqrt{N}/4)*((\alpha-1)/\alpha)*(k'/(k'+1))$
Where α = selectivity factor, k' = retention factor of peak 2, N = theoretical plates.
$N= 16\ (tr/tw)^2$, where tr is retention time (or volume) and tw is peak width (in time or volume).
The retention time was converted to retention volume by multiplying with the flow rate: 0.5 mL/min.

[c] calculation based on the combined peak area of 79S and 95S peaks.

[d] calculation based on peak area from valley-to-valley integration of the peaks shown in the HPLC chromate gram (see figure below).

[e] calculation based on peak area determined using Gussi software applying baseline peak integration of peaks shown in AUC histogram.

(To Fig. 1)

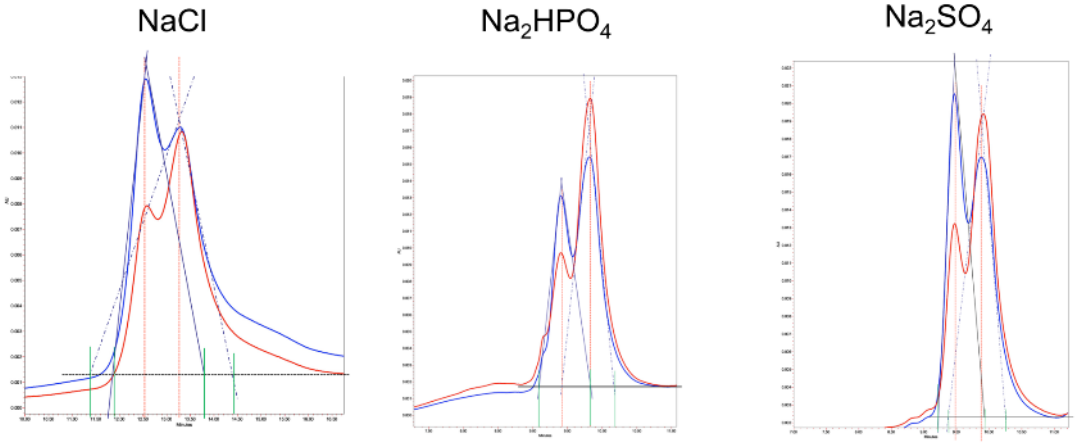


Table S2. AAV5 vector capsid recovery under sodium sulfate continuous gradient elution

Fraction	Fraction number	% VG eluted	% VG Recovery
Empty capsid fractions	1	0.87	4.79%
	2	1.3	
	3	1.11	
	4	1.51	
Overlapped fractions	5	8.11	19.51%
	6	11.4	
Vector capsid fraction	7	22.8	57.09%
	8	18.05	
	9	10.2	
	10	6.04	

(To Fig 1)

Table S3 Summary of AEX step gradient process characteristics

Sample	VG/mL	Volume (mL)	Total VGs	% VG Recovery[a]
AAV5-*gfp*[b]				
Feed	8.50×10^{11}	10	8.50×10^{12}	-
Flowthrough and column wash	1.98×10^{09}	30	5.94×10^{10}	0.7
EC peak fraction	2.42×10^{10}	45	1.09×10^{12}	12.8
VC peak fraction	4.12×10^{11}	15	6.18×10^{12}	72.7
High salt washes:100%B and 2 M NaCl[c]	1.58×10^{10}	30	4.74×10^{11}	5.6
AAV8-*gfp*[b]				
Feed	2.0×10^{11}	10	2.0×10^{12}	-
Flowthrough and column wash	2.0×10^{08}	30	6.0×10^{09}	0.3
EC peak fraction	1.50×10^{09}	50	7.5×10^{10}	3.7
VC peak fraction	1.09×10^{11}	15	1.6×10^{12}	81.7
High salt washes:100%B and 2 M NaCl[c]	2.13×10^{09}	30	6.4×10^{10}	3.2
AAV6-*gfp*				
Feed	1.5×10^{11}	10	1.5×10^{12}	-
Flowthrough and column wash	2.5×10^{08}	30	7.5×10^{09}	0.5
EC peak fraction	1.6×10^{09}	25	4.0×10^{10}	2.7
VC peak fraction	8.0×10^{10}	15	1.2×10^{12}	79.9
High salt washes:100%B and 2 M NaCl[c]	9.0×10^{08}	30	2.7×10^{10}	1.8
AAV6-*cas9*				
Feed	3.0×10^{11}	10	3.0×10^{12}	-
Flowthrough and column wash	7.0×10^{08}	30	2.1×10^{10}	0.7
EC peak fraction	1.97×10^{09}	35	6.9×10^{10}	2.3
VC peak fraction	1.66×10^{11}	15	2.5×10^{12}	83.0
High salt washes:100%B and 2 M NaCl[c]	1.7×10^{09}	30	5.1×10^{10}	1.7
AAV9-*gfp*[d]				
Feed	5.5×10^{10}	10	5.5×10^{11}	-
Flowthrough and column wash	6.0×10^{7}	30	1.8×10^{9}	0.33
EC peak fraction	1.2×10^{9}	35	4.4×10^{10}	7.9
VC peak fraction	3.1×10^{10}	15	4.7×10^{11}	84.7
High salt washes:50%B+100%B and 2 M NaCl[c]	1×10^{8}	20	2×10^{9}	0.3

EC, empty capsids; VC, vector capsids; VG, vector genome copies

[a] VGs recovery reported with respect to the total VGs loading in feed being 100%. The difference in the overall VG mass balance may represent the error associated with ddPCR analyses.

[b] Values reported as an average of triplicate runs

[c] VGs reported combinedly for both wash fractions

[d] AEX run performed using pre-packed POROS-HQ 1mL column

Table S4. Chromatographic characteristics of AAV capsids elution under continuous salt gradient elution

rAAV Vector	Peak Resolution[a] (R_S)
AAV5-*gfp*	0.78
AAV8-*gfp*	1.27
AAV6-*gfp*	1.16
AAV6-*cas9*	1.10
AAV9-*gfp*/POROS™ HQ	0.93
AAV8-*gfp,* Monolith[b] Vs. Packed-bed[b]	
AAV8-*gfp*/ POROS™ HQ	0.91
AAV8-*gfp*/ CIMmultus™ QA	1.59

[a, b] calculations based on data from continuous gradient run with a gradient slope value of 0.16 mM salt/CV and 0.25 mM salt/CV, respectively. CV, column volume:1 mL.

[a] Calculation of resolution of based on the equation for peak width and peak distance: $[R_s=2(V_2-V_1)/(W_1+W_2)]$. V_1 and V_2 are the elution points of peak 1 (empty capsid) and 2 (vector capsid), respectively. W_1 and W_2 are peak width of peak 1and 2, respectively.

Table S5 CIMmultus QA monolith column characteristics at different scales

Parameters	Comparison of monolith columns								
Column volume (mL)	1	4	8	40	80	400	800	4000	8000
Channel size (nm)	950-1150								
Ligand density (mmol/mL of wet support)	0.45-0.65								
Dynamic binding capacity (BSA)	≥20 mg/mL under standard testing conditions								

The values presented above are extracted from the product literature provided by the supplier of the CIMmultus monolith column used in this study.

Supplemental calculations

1. Parameters for *sv*-AUC-based calculation

Parameters for capsids containing packaged DNA				
Capsid population	Size of the packaged DNA (kb)	Molecular weight	Molar absorption coefficient at 260nm: ε_{260}	Molar absorption coefficient at 280nm: ε_{280}
79S	1.770	5.37×10^5	1.63×10^7	8.87×10^6
84S	2.400	7.28×10^5	2.21×10^7	1.20×10^7
95S	3.790	1.15×10^6	3.49×10^7	1.90×10^7
Parameters for empty capsids				
65S	NA	3.74×10^6	3.72×10^6	6.31×10^6

2. Sample calculation for affinity-purified AAV5 material based on *sv*-AUC data

Capsid population	Integrated peak area from 260nm *sv*-AUC profile	Integrated peak area from 280nm *sv*-AUC profile	$A_{260/280}$	% relative proportion (based on 260nm data)	% relative proportion (based on 280nm data)
65S	0.162	0.28	0.58	91.84%	88.48%
79S	0.022	0.029	0.76	2.32%	3.81%
95S	0.107	0.098	1.09	5.84%	7.72%

3. Sample calculation for optical-density based method (Sommer et al., 2003)

Parameters	Affinity-purified AAV5 (mixture of EC and VC)	Empty capsid reference standard	Vector capsid reference standard
A_{260}	0.64	0.143	0.228
A_{280}	0.80	0.177	0.166
$A_{260/280}$	0.80	0.80	1.37
VG/mL	$6.72x10^{12}$	$1.49x10^{12}$	$5.04x10^{12}$
Cp/VG	9.27	9.33	1.12
% Filled capsids	10.78	10.71	89.42
% Empty capsids	89.22	89.28	10.58

Calculation method

3.1 Calculation of the titer

$$(\mathrm{VG/mL}) = \frac{4.47 \times 10^{19}(\mathrm{A_{260}} - 0.59\mathrm{A_{280}})}{\mathrm{MW_{DNA}}}$$

Size of the expression cassette (ITR-to-ITR): 3.79 kb

Molecular weight of the expression cassette (MW_{DNA})= $1.15x10^6$

3.2 Calculation of the total capsids (Cp) to viral genome (VG) ratio

$$\text{Capsid ratio (Cp/VG)} = \mathrm{MW_{DNA}} \times \frac{1.76 \times 10^{-6}(1.80 - \mathrm{A_{260}/A_{280}})}{\mathrm{A_{260}/A_{280}} - 0.59}$$