## **Expanded View Figures**



Figure EV1. Application of AAVPure<sup>Mfg</sup> 1.0 in different AAV serotypes.

(A) Schematics of plasmid components in triple transfection (left panel) and AAVPure<sup>Mig</sup> 1.0 (right panel) used to produce AAV9.EGFP and AAV8.EGFP vectors. (B) Comparison of AAV9.EGFP produced by either triple transfection or AAVPure<sup>Mig</sup> 1.0. (C) Comparison of AAV8.EGFP produced by either triple transfection or AAVPure<sup>Mig</sup> 1.0. The procedure of rAAV production and vector characterization in (B, C) were the same as shown in Fig. 2. In (B, C), data are mean ± s.d. of biological replicates, n = 4 for each group. Statistical analysis was performed using unpaired *t* test. Exact *P* value was indicated in the figure.



Figure EV2. Lower VP expression increased the full capsid ratio with a marked decrease in vector genome titer.

(A) Schematic showing the experimental procedure. (B) Construct illustrations of the plasmids used in quadruple transfection to produce AAV9.EGFP. (C) Representative western blotting images (left) and the quantification (right) of viral proteins (VP1, 2, 3) at indicated cumate concentrations. (D-F) Comparison of rAAV genome titer, capsid titer, and full capsid ratio in cleared lysates with different cumate concentrations. In (C-F), data are mean  $\pm$  s.d. of biological replicates, n = 3 for each group. Statistical analysis was performed using unpaired *t* test. Exact *P* value was indicated in the figure.



## Figure EV3. Application of AAVPure<sup>Mfg</sup> 2.0 in producing AAV vectors with different serotypes, transgenes, and ITR configurations.

(A, C, E) Schematics of plasmid components in AAVPure<sup>Mfg</sup> 2.0 in producing ssAAV2.EGFP, ssAAV9.N.Cas9, and scAAV9.EGFP. In (E), the gray bar indicates mutant ITR that results in self-complementary (sc) vector genome. (B, D, F) Comparison of rAAV genome titer, capsid titer, full capsid ratio, and plasmid backbone DNA levels in cleared lysates between triple transfection and AAVPure<sup>Mfg</sup> 2.0. In (B, D, F), data are mean ± s.d. of biological replicates, n = 4 for each group. Statistical analysis was performed using unpaired *t* test. Exact *P* value was indicated in the figure.



## Figure EV4. Developing AAVPure<sup>Mfg</sup> 2.1 with pCAG-Bxb1 spike-in.

(A) Schematics of plasmid components in AAVPure<sup>Mg</sup> 2.1. (B) AAV9.EGFP genome titer produced by triple transfection or AAVPure<sup>Mg</sup> 2.1 with different spike-in amount of pCAG-Bxb1. The rAAV production and titering procedures were the same as Fig. 2B. (C-E) Comparison of AAV9 capsid titer (C), full capsid ratio (D), and plasmid backbone DNA levels (E) in cleared lysates between triple transfection and AAVPure<sup>Mg</sup> 2.1 with 1% pCAG-Bxb1 spike-in. The detailed plasmid usage is described in Table EV1. In (B-E), data are mean ± s.d. of biological replicates, n = 4 for each group. Statistical analysis was performed using unpaired t test (C-E) or one-way ANOVA followed by Dunnett's multiple comparisons test against the triple transfection group (B). Exact *P* value was indicated in the figure.



## Figure EV5. Generating a monoclonal HEK293-Bxb1 cell line.

(A) Schematic diagram illustrating *Bxb1* knock-in into the *AAVS1* site in HEK293 cells. (B) Schematic diagram showing the workflow to engineer and select HEK293-Bxb1 cells. (C) Procedure of HEK293-Bxb1 monoclonal cell line generation and screening. (D) Schematics of a reporter assay to determine Bxb1 recombination activity.
(E) Representative fluorescence images showing naive HEK293 cells or HEK293-Bxb1 monoclonal cell lines that were transfected with the reporter plasmids, pCMV-attP and pattB-EGFP. Images were taken 1 day post transfection. Scale bar, 100 µm.