

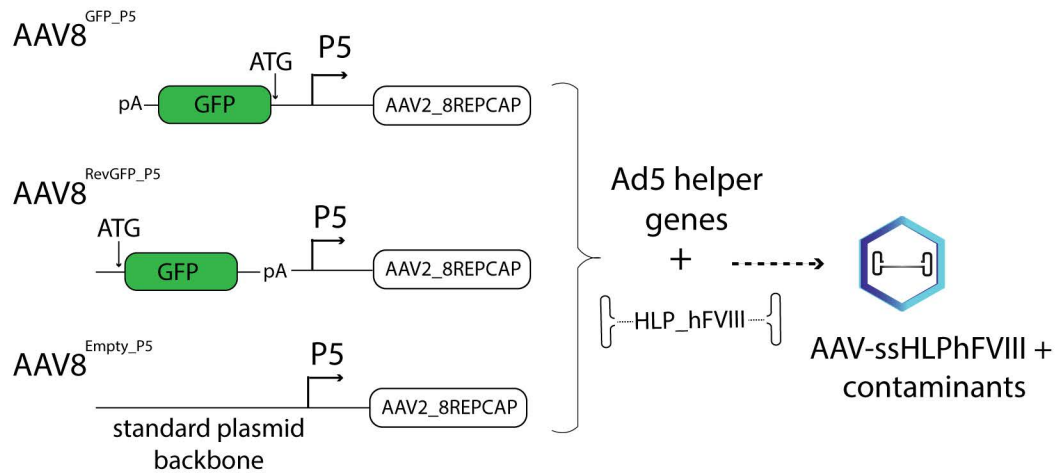
Supplemental information

Preventing packaging of translatable

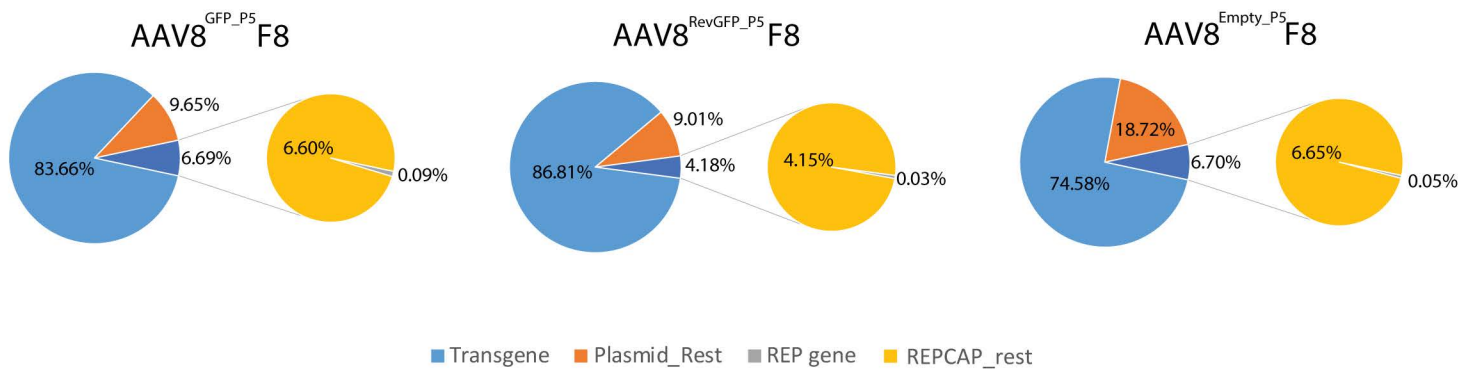
P5-associated DNA contaminants

in recombinant AAV vector preps

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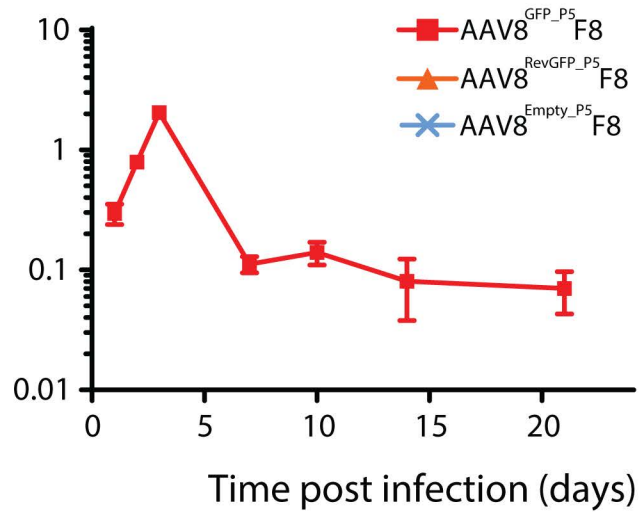


Supplementary Figure 1. Contaminant reporter cassettes in AAV REPCAP plasmids. Schematic of designed P5-associated contaminant AAV REPCAP constructs used for production of AAV8ssHLP_hFVIII. Forward facing arrows denote transcriptional start site. Downward arrows denote location of start codon

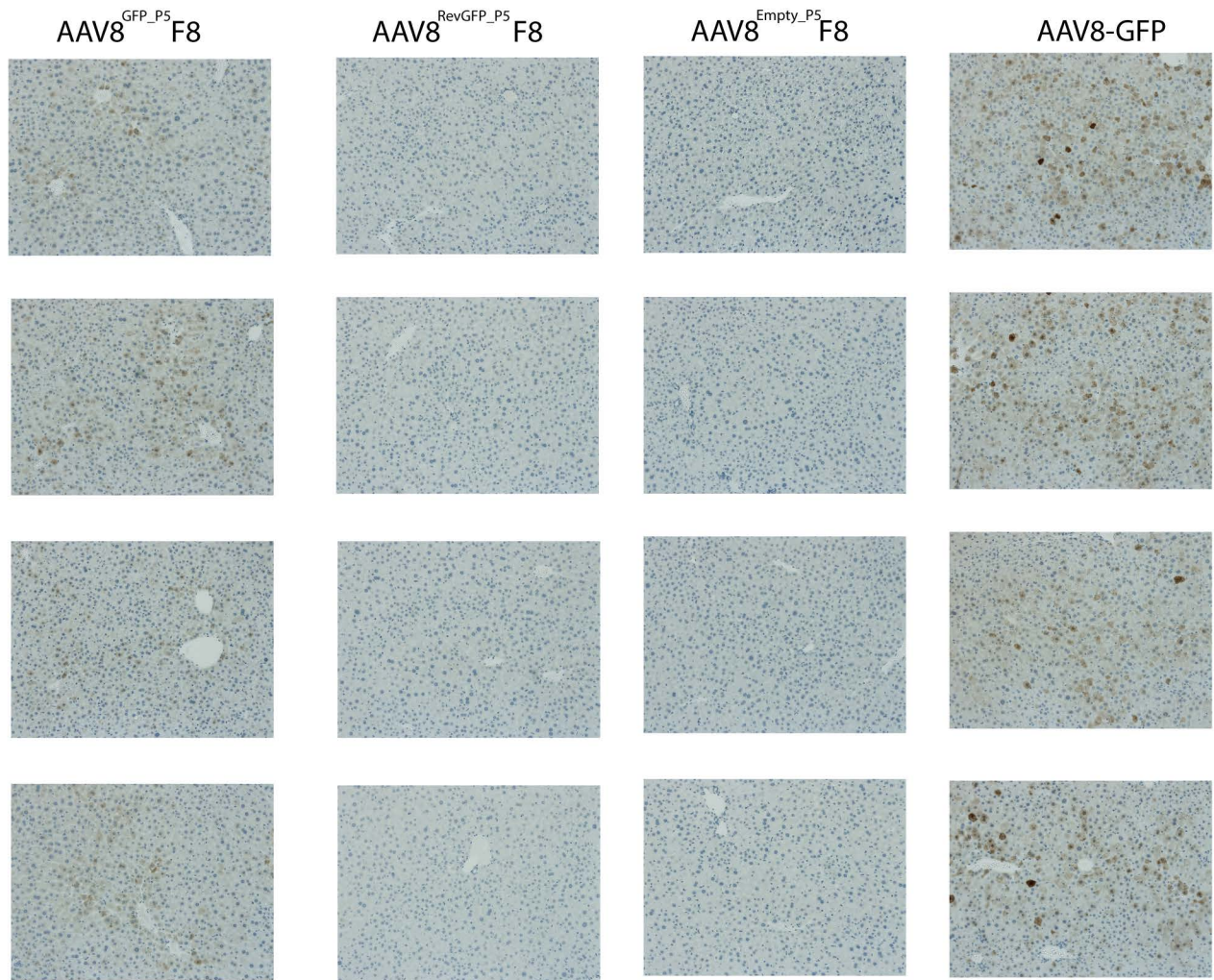


Supplementary Figure 2. Incorporation of designed P5-associated contaminants in rAAV.

Pie charts of NGS confirmation of designed P5-associated contaminants within AAV. For AAV8^{GFP_P5}F8 (left), AAV8^{RevGFP_P5}F8 (middle), and AAV8^{Empty_P5}F8 (right). Reads mapped as in Fig. 1d.

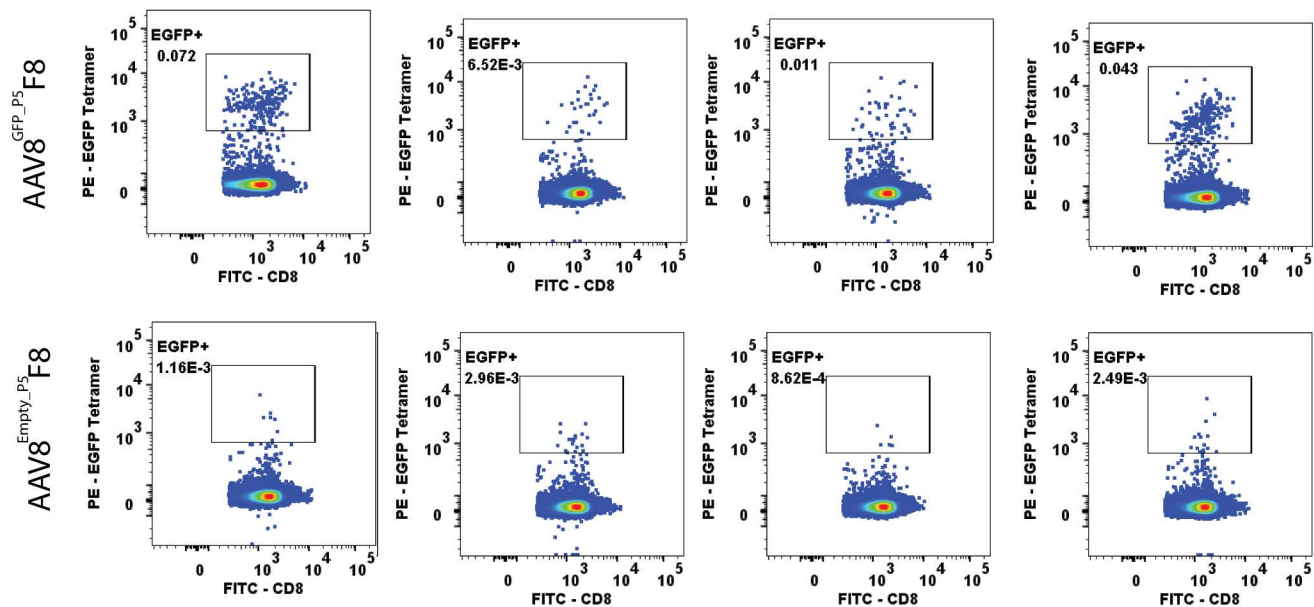


Supplementary Figure 3. Contaminant translation in continually passaged 293T cells. Logarithmic representation of data depicted in figure 2d: Time course graph depicting detectable GFP positive cells in AAV8ssHLPPhFVIII post infected (5e6 MOI) with AAV8^{GFP_P5}F8 (red), AAV8^{REV GFP_P5}F8 (Orange) or AAV^{Empty_P5}F8 (Blue). (Values for AAV8^{REV GFP_P5}F8 and AAV^{Empty_P5}F8 all below y-axis limit).



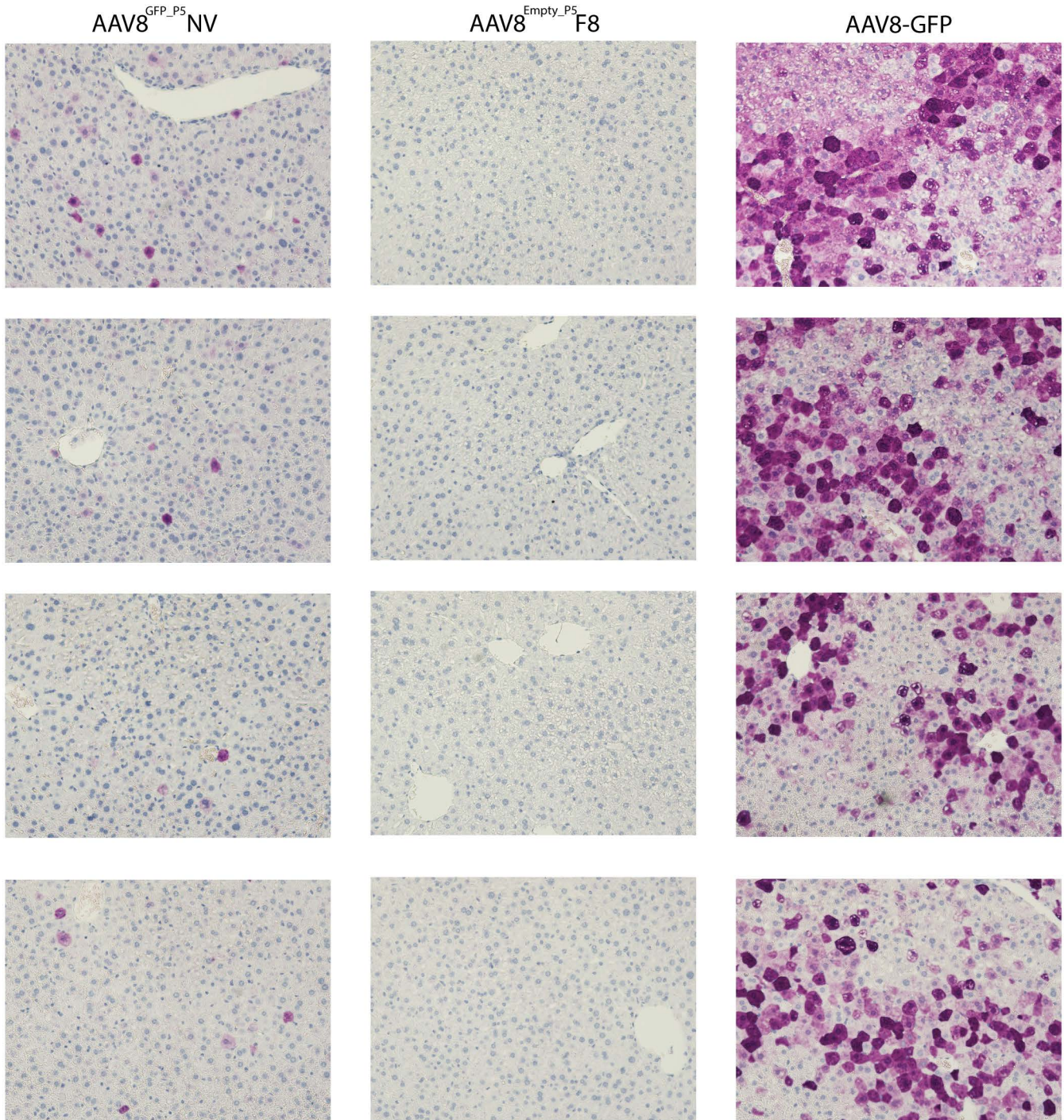
Supplementary Figure 4. Translation of P5-associated rAAV contaminants.

Representative IHC images of C57BL/6J mouse liver sections stained for GFP protein 1-week post 9.4×10^{11} vg AAV8ssHLPHFVIII infection from each additional mouse in treatment groups from experiment shown in Fig. 1h.



Supplementary Figure 5. Immunogenicity of P5-associated rAAV contaminants.

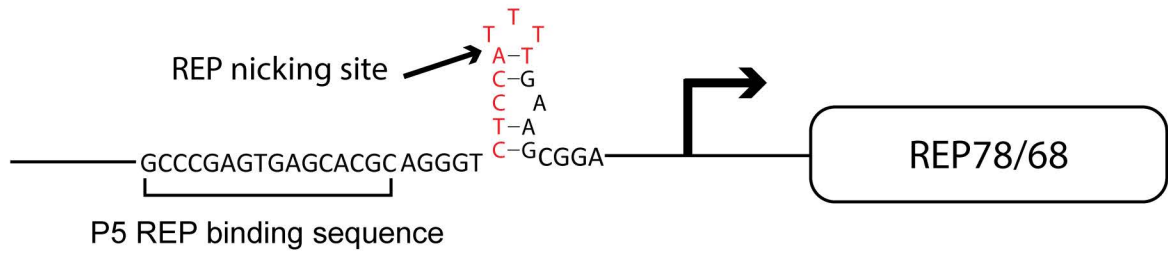
FACS plots for BALB/cJ GFP reactive splenic CD8⁺T cells 9 days post infection from each additional mouse in experiment shown in Fig. 2f. Top row: AAV8^{GFP_P5}F8; bottom row: AAV8^{Empty_P5}F8.



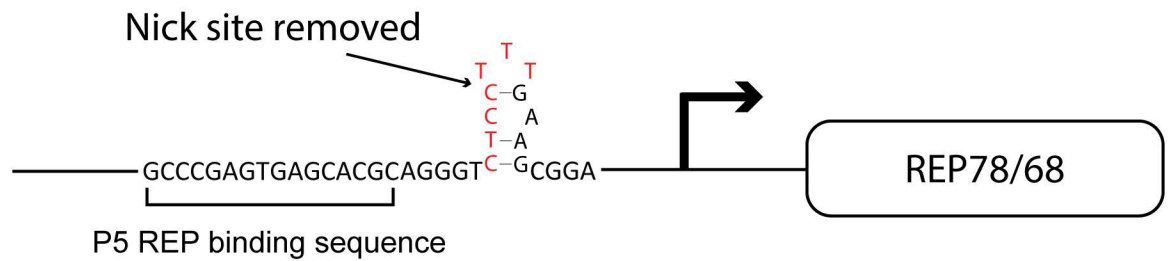
Supplementary Figure 6. Translation of P5-associated rAAV contaminants in absence of expression cassette.

Representative IHC images of C57BL/6J mouse liver sections stained for GFP protein 1-week post 2.7×10^{11} vg GFP copy infection from each additional mouse in treatment groups from experiment shown in Fig. 4d.

a P5 promoter



b P5 Δ nick



c P5-HS5 promoter sequence

TAGAGGTCCTGTATTAGAGGTCACGTGAGTGTTTTGCGACATTTTGCACACCATGTGG
 TCACGCTGGGTATTTAAGCCCGAGTGAGCACGCAGGGT**GCAACCTCCATTT**TGAAGCG
 GGAGGTTTGAACGCGCAGCCACC

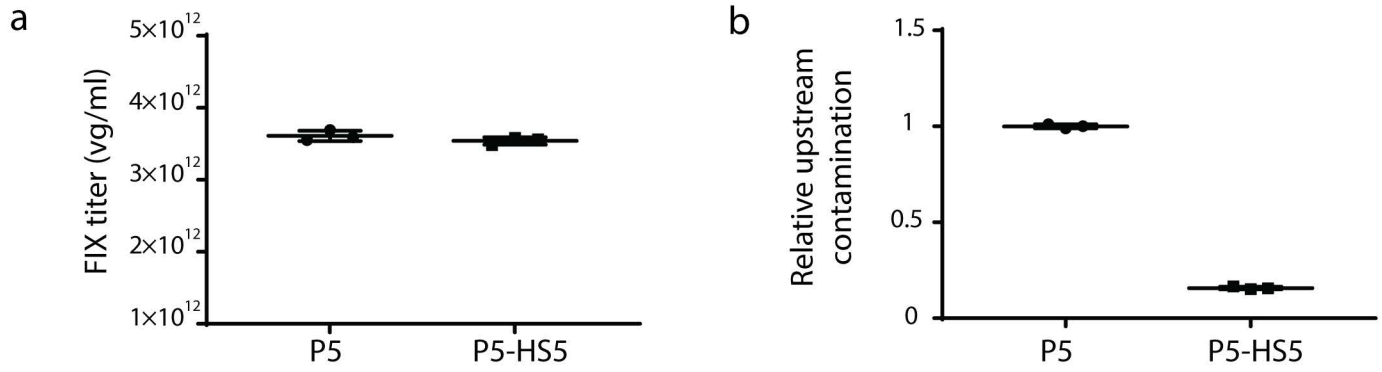
d P5-HS100 promoter sequence

TAGAGGTCCTGTATTAGAGGTCACGTGAGTGTTTTGCGACATTTTGCACACCATGT
 GGTCACGCTGGGTATTTAAGCCCGAGTGAGCACGCAGGGT**AGGTAGCAACTATCAC**
TCAAGAAGCAGACACAGTAAGACACGGTCTAGCTGACTGTCTATCGGCTAG-
GTCAA ATAGAGAGCTTTGATATCTGCATGTCTACCTCCATTTTGAAGCGGGAG-
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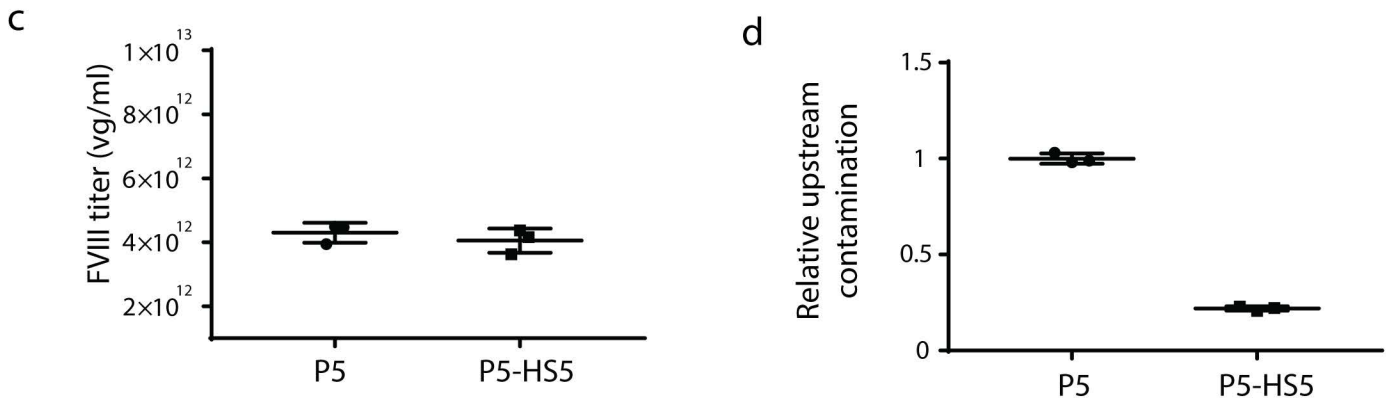
Supplementary Figure 7. Alternative P5 promoter designs.

(a-b) Schematics of AAV production REP78/68 promoters (a) P5 promoter detailing REP binding site in relative positioning to YY1+1 binding site (red), (b) P5 Δ nick promoter in which the AT dinucleotide within the YY1+1 binding site (red) has been deleted. Forward facing arrows denote transcriptional start site. **(c-d)** P5-HS promoter designs: **(c)** Sequence of P5-HS5 promoter with spacer sequence (blue) and YY1+1 binding site (red) highlighted. **(d)** Sequence of P5-HS100 promoter with spacer sequence (blue) and YY1+1 binding site (red) highlighted.

AAV8scLP1hFIXco



AAV8ssHLPhFVIII



Supplementary Figure 8. Cell factory scale P5-HS production.

(a+b) AAV8scLP1hFIXco and (c+d) AAV8ssHLPhFVIII analyzed by qPCR for titer (a+c) and relative contamination upstream of the REP78/68 promoter (b+d).

Contaminant FISH probe sequence:

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CTACAGGGCGCGTC

Supplementary Figure 9. Contaminant FISH probe sequence used in Fig. 2a.

Supplementary Table 1. Addgene AAV plasmids with P5 promoter downstream of CAP gene.

AAV serotype REPCAP plasmids available from plasmid repository addgene containing the P5 promoter downstream of the capsid gene. (Analysed for P5 presence in snap-gene).

Plasmid	Serotype	Addgene ID	Notes
pAAV2_1	AAV1	112862	
pAAV2_2	AAV2	104963	2 copies of P5
pAAV2_5	AAV5	104964	2 copies of P5
pAAV2_7	AAV7	112863	
pAAV2_8	AAV8	112864	
pAAV2_9n	AAV9	112865	
pAAV2_rh10	rh10	112866	
rAAV2-retro helper	AAV2-retro	81070	2 copies of P5
7M8	7M8	64839	2 copies of P5
shh10	shh10	64867	2 copies of P5
pAnc80L65	Anc80L65	68837	2 copies of P5