# STAR Protocols

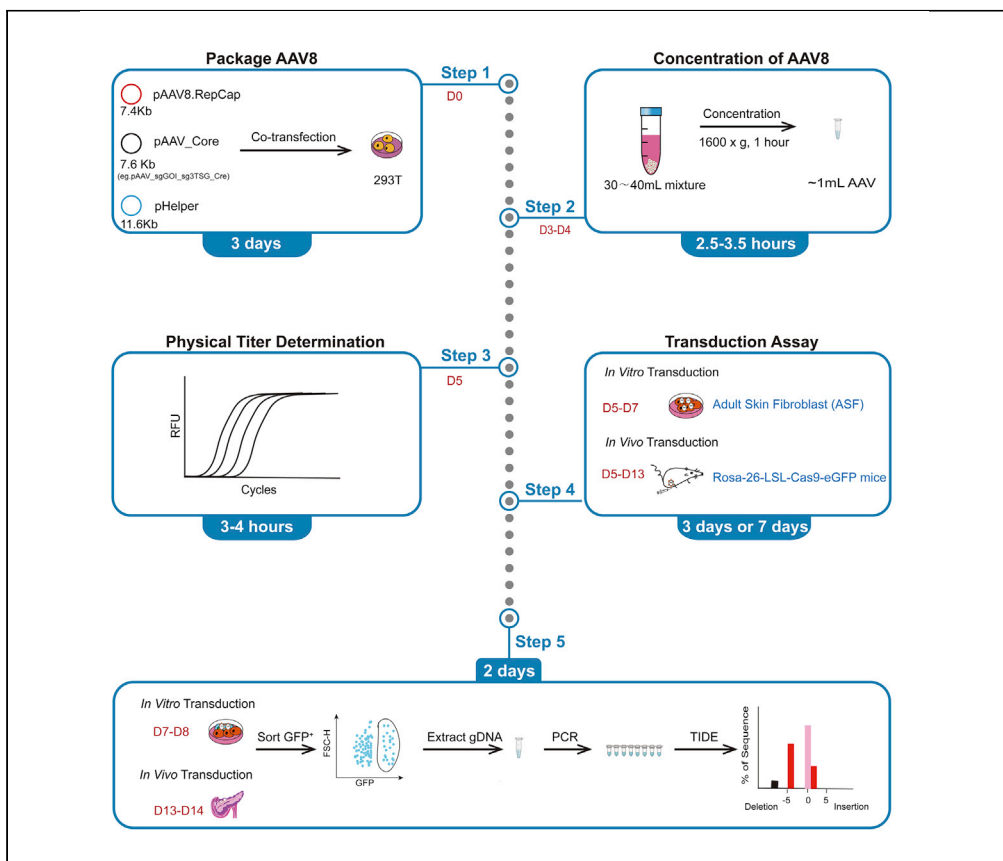**Protocol**

# Protocol to package and concentrate adeno-associated virus serotype 8 for use in autochthonous mouse models of pancreatic cancer



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### Highlights

A straightforward method for assessing gene function in the pancreas

Manipulation of AAV genomes for gene overexpression and CRISPR-Cas9 knockout

Detailed steps for packaging and concentrating AAV8 for use in mice

Method to determine AAV8 physical titer and functional titer

We have developed an economical and rapid protocol to package and concentrate adeno-associated virus serotype 8, allowing production of high-titer virus for use *in vivo* within 1 week. When combined with the CRISPR-Cas9 system, this provides a straightforward method for knockout of genes of interest in the pancreas. The method can also be used to express cDNAs in the pancreas. This method shows great potential to accelerate pancreatic cancer research in autochthonous models.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

**Protocol**

# Protocol to package and concentrate adeno-associated virus serotype 8 for use in autochthonous mouse models of pancreatic cancer

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## SUMMARY

**We have developed an economical and rapid protocol to package and concentrate adeno-associated virus serotype 8, allowing production of high-titer virus for use *in vivo* within 1 week. When combined with the CRISPR-Cas9 system, this provides a straightforward method for knockout of genes of interest in the pancreas. The method can also be used to express cDNAs in the pancreas. This method shows great potential to accelerate pancreatic cancer research in autochthonous models.**
**For complete details on the use and execution of this protocol, please refer to Li et al. (2021).[1]**

## BEFORE YOU BEGIN

In addition to its great potential in gene therapy, AAV has become an important tool in basic research.[2–4] However, the complex purification procedure to obtain virus impedes accessibility for many researchers.[5] Here, we developed a convenient method to acquire high quality AAV8 particles for *in vivo* study. Our method involves packaging of an easily modified AAV backbone in 293T cells, followed by precipitation-based concentration to prepare high-titer AAV8 particles for infection of the pancreas via intraperitoneal injection. The procedure requires less than one week to obtain AAV8 suitable for animal studies. Unlike most existing methods, this protocol does not employ ultracentrifugation or the use of toxic reagents.[6–8] Our method has great potential to accelerate research using autochthonous models of pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDA) is a lethal disease with few existing therapies.[9] Most human PDA tumors are initiated by oncogenic *KRAS* mutations while alterations in tumor suppressor genes such as *CDKN2A* and *TP53* promote tumor progression.[10–12]

To overcome this, we have harnessed AAV to provide an alternative for gene knockout and overexpression in the pancreas. AAV8 efficiently infects both acinar and endocrine cells in the pancreas,[13–15] and we have harnessed this to develop AAV8-driven mouse models of pancreatic cancer driven by KRAS[G12D]. To achieve this, we have engineered AAV genomes to express up to four sgRNAs, including sgRNAs targeting the key tumor suppressors (TSGs) *Cdkn2a*, *Trp53*, and *Smad4*.[16] These AAV particles are then used to infect mice harboring LSL-Kras[G12D]; LSL-Cas9-eGFP alleles. To activate mutant KRAS and Cas9/eGFP, Cre-expressing AAV can be used, or mice bearing *Pdx1-Cre* or *Ptf1a-Cre*[ERT2] alleles can be infected to achieve pancreas- or acinar-specific knockout respectively. In the case of infection of Cre-bearing mice, the AAV is engineered to express
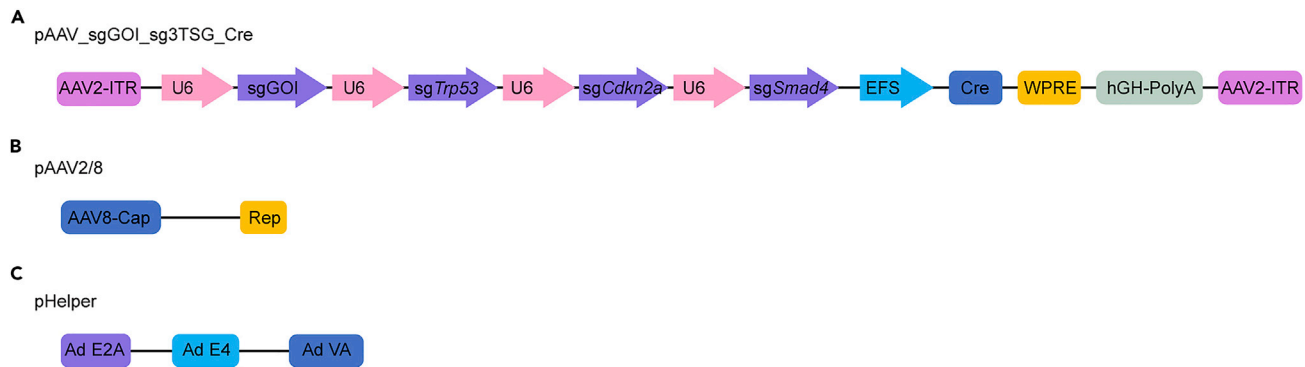
**Figure 1. Plasmids used in AAV preparation**
(A) Schematic of pAAV_core plasmid used for AAV packaging. Here, a Cre- and 3TSG-expressing plasmid is shown. See key resources table for cloning details.
(B) Schematic of pAAV2/8 plasmid used for AAV packaging.
(C) Schematic of pHelper plasmid used for AAV packaging.

a compatible fluorescent protein (for example, tBFP) instead of Cre to track infected cells. These TSG sgRNAs can be combined with control sgRNAs or sgRNAs targeting a gene of interest (GOI) to rapidly assess the role of that factor in PDA tumorigenesis. This system can also be used to express transgenes of interest in the pancreas.

The protocol below describes the specific steps for using three plasmids to prepare AAV8: one containing the Ad E2A, E4, and VA RNA helper genes (pHelper), another expressing the AAV8 rep/cap genes (pAAV8.RepCap), and the transgene construct flanked by AAV inverted terminal repeats (ITRs) (pAAV_Core; Figure 1). Here, we will take AAV_sgGOI_sg3TSG_Cre, a Cre-expressing AAV8 containing an sgRNA targeting a gene of interest (in this case, a control sgRNA targeting the *Rosa26* locus) and TSG sgRNAs to initiate PDA, as an example to illustrate how to package AAV8 based on our AAV backbone.

### Institutional permissions

All animal experiments were performed under the approval of the Animal Ethics Committee of The University of Tsinghua. Animal experiments described in this manuscript should be performed in line with the permitted protocol of each institution.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Anti-GFP | CST | Cat#2955S |
| Chemicals, peptides, and recombinant proteins | | |
| PEI-MAX | Polysciences | Cat#24765 |
| PEG-8000 | Solarbio | Cat#P8260 |
| NaCl | Sigma | Cat#795429-500G |
| Phosphate-buffered saline (PBS) | Solarbio | Cat#P1010 |
| TRIS | GPC | Cat#AN077-5kg |
| Hexadimethrine bromide/polybrene | Sigma-Aldrich | Cat#H9268-5G |
| QuickExtract™ DNA Extraction Solution 1.0 | Lucigen | Cat#QE09050 |
| FBS | Gemini | Cat#900-108 |
| Penicillin-streptomycin | Gibco | Cat#15140-122 |

*(Continued on next page)*

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| DMEM | Gibco | Cat#C11995500BT |
| TRYPLE EXPRESS | Thermo | Cat#25200072 |
| Critical commercial assays | | |
| EndoFree Maxi Plasmid Kit | TIANGEN | cat # 4992438 |
| NovoStart® SYBR qPCR SuperMix Plus | Novoprotein | Cat#E096-01A |
| Experimental models: Cell lines | | |
| HEK293T | ATCC | CRL-11268 |
| Experimental models: Organisms/strains | | |
| LSL-Kras$^{G12D}$; LSL-Cas9-eGFP mice ("KCa" mice) | A gift from F. Qian (Tsinghua University) | |
| Oligonucleotides | | |
| sgTrp53 sequence | GCAGACTTTTCGCCACAGCG | |
| PCR_sgTrp53_TIDE_F | GCCCTGTGCAGTTGTGGGTCA | |
| PCR_sgTrp53_TIDE_R | GCGTCTCTATTTCCCGCCTGGAT | |
| sgCdkn2a sequence | GCAGCTCTTCTGCTCAACTA | |
| PCR_sgCdkn2a_TIDE_F | CTGGGCGTGCTTGAGCTGAAGCTATGCC | |
| PCR_sgCdkn2a_TIDE_R | CGATGTTCTACAGGAGTTTGAGTACCAGG | |
| sgSmad4 sequence | GTGAATCTCAATCCAGCACG | |
| PCR_sgSmad4_TIDE_F | TGTCTGCTAAGAGCAAGGCAGCAA | |
| PCR_sgSmad4_TIDE_R | TGCCCTCCCGTAGTTAAAGTCCA | |
| WPRE_ physical_ titer_F | GCTGTTGGGCACTGACAATT | |
| WPRE_ physical_titer_R | AAGGGACGTAGCAGAAGGAC | |
| Recombinant DNA | | |
| pHelper | A gift from Y. Li, Tsinghua University. | |
| pAAV8.RepCap | Addgene | Addgene#112864 |
| pAAV_Core (eg. AAV _sgGOI_sg3TSG_Cre) | Addgene | Cloned from Addgene #60224 and Addgene#105550 |
| Software and algorithms | | |
| GraphPad Prism 9 | GraphPad Prism Software | https://www.graphpad.com/scientific-software/prism/ |
| FlowJo™ Software V10 | BD | https://www.flowjo.com/ |

*Note:* We cloned pAAV_Core based on Addgene #60224 and Addgene #105550. In detail, Cre-WPRE-hGH-PolyA was subcloned from Addgene #105550 into the AAV-3sgRNA-EFS-based backbone (Addgene #60224) by double restriction enzyme digestion of BsiWI and EcoRV to construct pAAV_sg3TSG_Cre. Then, we used MluI to construct pAAV sgGOI_sg3TSG Cre by Gibson assembly sgGOI (gene of interest) into pAAV_sg3TSG_Cre. The final product is shown in Figure 1A. Also, it is convenient to use EcoRI and AfeI to replace Cre with other fluorescent proteins like tBFP/tRFP to trace AAV8 infected pancreatic cells.

## MATERIALS AND EQUIPMENT

| Lysis buffer | | | |
|---|---|---|---|
| Reagent | Final concentration | Stock concentration | Add to 1000 mL |
| Tris-HCl (ph8.5) | 10 mM | 1 M | 10 mL |
| NaCl | 150 mM | – | 8.775 g |
| MilliQ | – | – | Up to 1 L |

*Note:* Autoclave (121°C, 15 min) lysis buffer and allow to cool until 25°C before store in 4°C freezer.

**Concentrator**

| Reagent | Add to 1,000 mL |
| --- | --- |
| PEG-8000 | 400 g |
| NaCl | 70.0 g |
| 10×PBS (pH7.4) | 100 mL |
| MillQ | Up to 1 L |

*Note:* Mix with gentle stirring, heating to 60°C to help dissolve (takes about 1 h). Sterilize by filtering through 0.45µM filter. Store the solution at RT.

**Recovery solution**

| Reagent | Final concentration | Stock concentration | Add to 50 mL |
| --- | --- | --- | --- |
| Sterile 1×PBS | – | – | 49.5 mL |
| Pluronic F68 | 0.001% | 0.100% | 0.5 mL |

*Note:* Can be stored at 4°C.

## STEP-BY-STEP METHOD DETAILS
### Preparing 293T cells – Day 0

⏲ Timing: 0.5–1 h

1. Plate 3.5*$10^7$ 293T cells in a 15cm plate in DMEM containing 10% FBS (without antibiotics).

   *Note:* We passage cell in 1:3 ratio and the next day it could reach 90% confluency. Also, we perform routinely mycoplasma detection per month.

   *Note:* Cells should reach 80%–90% confluency at the time of transfection.

   *Note:* Never allow cells to reach 100% confluence, if they do, rethaw a new vial of cells to ensure a good yield of AAV8.

   *Note:* Cell state plays an important role in AAV8 production. Use 293T cell of fewer than 20 passages.

### Package AAV8 – Day 1

⏲ Timing: 0.5–1 h

2. The next day, check 293T cell state by microscope. If 293T cells have reached 80%–90% confluency, warm DMEM and 1 mg/mL PEI-MAX at room temperature for 30 min.
3. In a 15 mL tube, prepare a plasmid mixture for one 15 cm plate by adding the following amounts of plasmid into pure DMEM to give a final volume of 2 mL.

   22 µg pHelper.

   14.3 µg pAAV8.RepCap.

   13.7 µg pAAV_sgGOI_sg3TSG_Cre.

   Vortex for 15 s and centrifuge briefly.

4. In another 15 mL tube, add 0.15 mL of 1 mg/mL PEI Max to 0.85 mL pure DMEM for a final volume of 1 mL (a ratio of 3 μg PEI Max to 1 μg DNA). Mix well by vortex 15 s and centrifuge briefly.
5. Place the two 15 mL tubes at RT for 5 min and then add PEI-MAX to the plasmid mixture and pipet up and down several more times.
6. After incubating at RT for 20 min, slowly drop the transfection mixture into the dish of 293T cells and mix well by gently rocking the cell plates.

   *Note:* We add 50 μg of total DNA per 15 cm plate, maintaining a molar ratio of 1:1:1 among the three plasmids. The length of each plasmid: 11.6 kb for pHelper, 7.4kb for pAAV8.Rep-Cap, and 7.6 kb for pAAV_sgGOI_sg3TSG_Cre.

   ⚠ CRITICAL: The plasmid preparations should be of high quality, without RNA contamination and with high concentrations. We recommend use EndoFree Maxi Plasmid Kit (TIANGEN, cat #4992438) according to the manufacturer's instructions (https://en.tiangen.com/upload/file/20220509/20220509165013_17820.pdf). Usually, we isolate plasmid DNA of the highest purity from 100–200 mL of bacterial culture and could yield up to 1 mg transfection grade plasmid DNA.

### Change culture media – Day 1

⏱ Timing: 0.5–1 h

7. 12–16 h after transfection, prewarm complete DMEM media containing serum and antibiotics for 30 min at 37°C.
8. Replace the DNA-containing transfection media by gently adding the fresh media, taking care to avoid disturbing the transfected 293T cells.
9. Culture cells for another 48 h.

   ⚠ CRITICAL: If the monolayer of 293T cells is disturbed, the yield of AAV8 will decrease greatly. To prevent this, the lowest speed setting should be used for pipetting, and the pipette should be against the sidewalls of the culture dish when dispensing medium.

### Harvest AAV8– Day 3

⏱ Timing: 1.5–2.5 h

Around 72 h after transfection, the AAV is harvested. The harvest is divided into two parts, one for the culture media and one for the cells.

10. For culture media: transfer the supernatant into a 50 mL tube and centrifuge at 800 × *g* for 10 min at 4°C then filter supernatant through 0.45 μM syringe filter into a new 50 mL tube and store at 4°C (Figure 2A).
11. For the cultured cells: harvest the cells from the 15 cm plate by directly adding 5 mL lysis buffer per plate. Gently pipet the transfected 293T cells and collect the cell suspension in a 15 mL tube.
12. Release AAV8 particles by freeze/thaw cycles: place the 15 mL tube of cell lysate in −20°C freezer (freeze) for 10 min and then thaw in a 37°C water bath for another 10 min.
13. Repeat freeze/thaw cycle two more times.
14. To remove cell debris, spin down the cell lysate at 3,000 × *g* for 10 min at 4°C (Figure 2B).
15. Carefully transfer the supernatant of cell lysate into the 50 mL tube of filtered culture media.
16. Add 1 volume of concentrator to 3 volumes of supernatant mixture (Figure 2C).
17. Make sure the lid of 50 mL tube is firmly closed and then incubate with constant rocking at least 12 h at around 60 RPM in 4°C freezer (Figure 2D).

**Figure 2. Harvesting and concentration AAV8**
(A) Collection of 293T culture media 3 days after transfection with AAV packaging plasmids.
(B) Cell pellet after three rounds of freeze/thaw cycles.
(C) Culture media and supernatant from cell pellet after adding 1/3 mixture volume of concentrator.
(D) AAV suspension after overnight rocking in the presence of concentrator.
(E) Representative photograph of AAV and cell debris pellet.
(F) Representative photograph of viral pellet after concentration.
(G) Viral pellet after resuspension.

> ⚠ CRITICAL: Gently pipet up and down several times or vortex briefly (5 s) between each freeze and thaw reaction to avoid precipitation of the solution. Use low-binding pipette tips to prevent AAV stick to tip and then reducing AAV8 titer.

**Concentration of AAV – Day 4**

⊙ Timing: 1.5–2.5 h

18. The next day, spin down the mixture in the 50 mL tube at 1,600 × $g$ for 60 min at 4°C (Figure 2E).
19. Carefully remove supernatant using electric suction apparatus, making sure not to disturb the pellet (Figure 2F).
20. Thoroughly resuspend the viral pellet into 600–1,000 μL recovery solution (Figure 2G) and transfer into a new low-binding 1.5 mL EP tube.
21. Incubate with constant rocking at around 60 RPM for at least 4 h at 4°C.
22. Centrifuge 3 min at 13,000 × $g$, 4°C to remove cellular and protein debris.
23. Transfer the clarified supernatant to a new 1.5 tube.
24. Aliquot AAV8 at 25–50 μL and store at −80°C until use. Avoid repeated freeze-thaw cycles. Troubleshooting 1.

*Note:* Addition of 0.001% Pluronic F68 can prevent AAV8 aggregation during storage in the freezer.

⚠ CRITICAL: The suspension volume of recovery solution can be adjusted based on the following principle: the lower the volume, the higher the AAV8 titer. However, do not use less than 600 μL recovery solution in order to make sure the pellet is fully resuspended.

### Physical titer determination of AAV8 – Day 5

⏱ Timing: 3–4 h

25. The next day, AAV8 titer is determined using the following steps:
26. Prepare a standard curve:
    a. Prepare pAAV_sgGOI_sg3TSG_Cre plasmid solution at 10 ng/μL to generate a standard curve.
    b. Make 7 serial dilutions, in duplicate, of your standard curve plasmid.

| Volume of 10 ng/μL stock or previous dilution (μL) | Volume of nuclease-free water (μL) | ng per μL |
|---|---|---|
| 10 of 10 ng/μL stock | 90 | $1 \times 10^{0}$ |
| 10 of $1 \times 10^{0}$ dilution | 90 | $1 \times 10^{-1}$ |
| 10 of $1 \times 10^{-1}$ dilution | 90 | $1 \times 10^{-2}$ |
| 10 of $2 \times 10^{-2}$ dilution | 90 | $1 \times 10^{-3}$ |
| 10 of $1 \times 10^{-3}$ dilution | 90 | $1 \times 10^{-4}$ |
| 10 of $1 \times 10^{-4}$ dilution | 90 | $1 \times 10^{-5}$ |
| 10 of $1 \times 10^{-5}$ dilution | 90 | $1 \times 10^{-6}$ |

27. Prepare AAV8 dilution sample:
    a. Dilute one aliquot of AAV8 from −80°C by dilute 2μL AAV8 into 198μL ddH20 (100-fold) Pipet up and down for several times.
    b. Dilute 10μL of this first dilution into 190μL ddH2O to make a 2000-fold dilution. Pipet up and down for several times and load into 384 well plate.
28. Prepare qPCR master mixture as follow:

| Reagent | Amount for one reaction | Amount for 100 reactions (1 × 384 well plate) |
|---|---|---|
| NovoStart® SYBR qPCR SuperMix Plus 2× | 5 μL | 500 μL |
| 10 μM Forward Primer | 0.2 μL | 20 μL |
| 10 μM Reverse Primer | 0.2 μL | 20 μL |
| Nuclease Free Water | 3.6 μL | 360 μL |
| Template | 1 μL | - |

29. Dispense the master mix into the wells and seal plate with transparent film.
30. Centrifuge briefly in a mini centrifuge to bring the sample to the bottom of the tube.
31. Perform the following qPCR program: 98°C 10 min / 98°C 15 s / 60°C 30 s / 72°C 30 s /read plate/ repeat 39× from step 3 / melt curve
32. Analyze qPCR data by plotting standard curve and calculate the AAV8 physical titer.
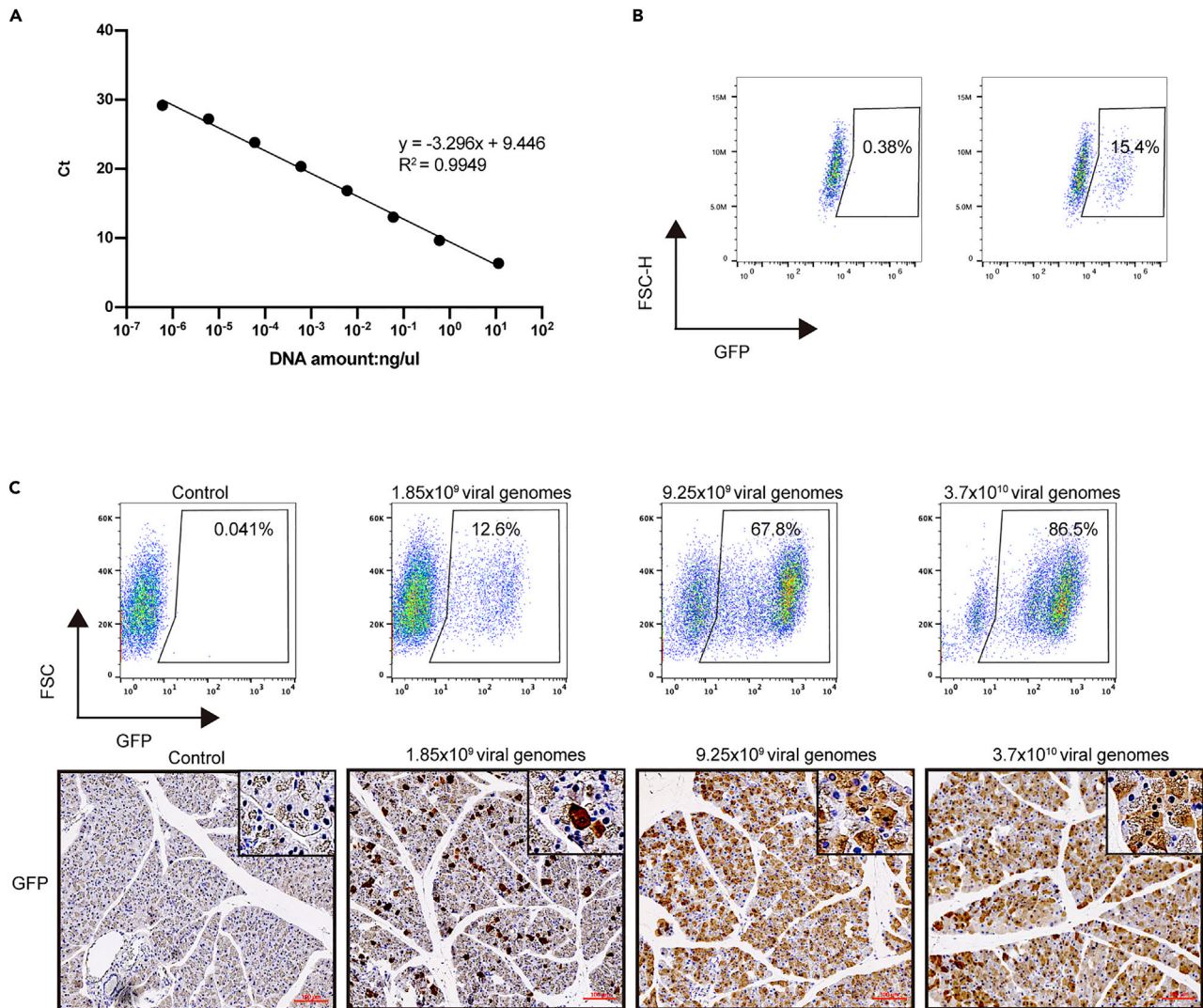
**Figure 3. AAV8 physical and biological titration**

(A) Example of valid 8-point pAAV8_sgGOI_sg3TSG_Cre plasmid standard curve.

(B) Example of *in vitro* biological titration assay. ASFs were transduced with 2.5 μL AAV_sgRosa_sg3TSG Cre in 0.5 mL complete media, resulting in approximately 15.4% GFP-positive ASFs after 72 h transfection (right panel). Left panel is control.

(C) Example of *in vivo* biological titration assay. We set four dilutions of AAV_sgRosa_sg3TSG Cre and injected IP into LSL-Cas9$^{eGFP}$ mice. Upper panels show flow cytometry of GFP in the pancreas 7 days post infection. Histology analysis of GFP is shown in lower panels. Control is mice without AAV infection. Scale bar = 100 μm.

An example is shown in Figure 3A and Table 1. Troubleshooting 2 and 3.

> *Note:* qPCR primers target WPRE, sequences shown in key resources table.

⚠ CRITICAL: Do not forget to include a no-template control (NTC = master mix + water). The dilution should be pretested to determine a proper dilution within the qPCR standard curve. In the case of virus produced using AAV_sgGOI_sg3TSG_Cre, we dilute to 2000-fold. Please note that different pAAV_Core constructs can produce varying amounts of virus, depending on a variety of factors including genome size. The appropriate dilution should be determined empirically for each new construct packaged.

**Table 1. Example of AAV physical titer calculation**

Viral genomes weight of pAAV_Cre_sgGOI_sg3TSG per mL:

$$1.5379858 * \frac{10^3}{10^{-9}} = 1.5379858 * 10^{-6} \text{ (g)}$$

Molecular weight of pAAV _Cre_sgGOI_sg3TSG:

$$7577 * 660 = 5.00082 * 10^6 \text{ (g /mol)}$$

Copied number of viral genomes of pAAV_Cre_sgGOI_sg3TSG per mL:

$$N = n * NA = \frac{m}{M} * NA = \frac{1.5379858 * 10^{-6}}{5.00082 * 10^6} * 6.02 * 10^{23} = 1.85 * 10^{11} \text{ (vg)}$$

Note:
Size in bp of pAAV_Cre_sgGOI_sg3TSG: 7,577 bp.

According to the qPCR result, the Ct values of pAAV_sgGOI_sg3TSG_Cre with dilution of 2000 are 19.72, 19.77 and 19.64, so the concentration of pAAV_Cre_sgGOI_sg3TSG is 1.5379858 ng/mL. Based on this, we calculate AAV physical titer as below:

**Transduction test**

Titer AAV using qPCR is a very sensitive method, but ITR recombination could cause AAV8 inactivation which cannot be distinguished by qPCR. Consequently, we recommend performing transduction assays to test the infectivity of the AAV8 prior to performing experiments.

According to experimental needs, you could choose from two alternative transduction assays:

1) The easiest way to test whether the AAV8 produced is functional is to directly infect adult skin fibroblasts (ASF) isolated freshly from mouse ear or tail.[17]

2) Most applications of AAV8-mediated pancreatic tumorigenesis require specific infection rates. For example, when comparing the effects of a GOI knockout to a control (sgGOI vs sgRosa), for the results to be interpretable it is essential to achieve comparable infection rates for the two AAVs. We have found that the most reliable way to achieve this is to first titer the virus by testing infection of the pancreas *in vivo*. However, this method is time-consuming and increases the number of animals used.

**Alternative 1: In vitro biological titration assay – Day 5–7 (optional)**

⏱ Timing: 5–7 days

33. Isolate ASF from Rosa-26-LSL-Cas9-eGFP mice.[18]
34. Plate 15,000 ASF per well in a 24 well plate.
35. Immediately after plating, thaw one aliquot of the AAV viral stock at −80°C on ice.
36. Dilute AAV in complete media plus polybrene (1:1000, 4 mg/mL stock). In general, a dilution range from 100-fold to 2000-fold is acceptable, although this will depend on viral titer.
37. Add 500 μL of virus-containing media to 24 well.
38. At 24 h post-transfection, remove virus-containing media and replace with pre-warmed complete media.
39. 72 h later, analyze GFP expression level of infected cells using flow cytometry.
40. Calculate the titer, transduction units (TU)/mL:

$$\text{Transduction Units (TU)} \Big/ \text{mL} = \frac{(\text{\# of cells at Transduction}) \times \text{Dilution Factor})}{(\text{Transduction Volume in mL})}$$

A sample AAV8 *in vitro* biological titer is shown in Figure 3B. Troubleshooting 4.

15,000 ASFs were transduced with 2.5 μL AAV_sgRosa_sg3TSG Cre in 0.5 mL complete media, resulting in approximately 15.4% GFP-positive ASFs after 72 h transfection, so we calculate AAV8 *in vitro* biological titer as below:

$$\text{Transduction Units} = \frac{15000 * 15.4\% * 200}{0.5} = 9.24 * 10^5 \text{ TU}\Big/\text{mL}.$$

⚠ CRITICAL: For accurate titer, the infection rate should be lower than 20% 72 h post transduction. Therefore, infecting with several dilutions is recommended. The adult skin fibroblasts should be in a good state and growing exponentially to obtain optimal AAV infection.

**Alternative 2: In vivo biological titration assay – Day 5–13 (optional)**

⏱ Timing: 5–13 days

41. Thaw one aliquot of the AAV viral stock at −80°C on ice.
42. According to the previous qPCR result, dilute the AAV stock with recovery solution to three dilutions: $1.85*10^9$ viral genomes, $9.25*10^9$ viral genomes and $3.7*10^{10}$ viral genomes per mice in 200 μL volume.
43. Gently pipette up and down the virus several times.
44. Weigh mice and inject AAV intraperitoneally.
45. Seven days post AAV injection, euthanize mice and collect the pancreas tissue.
46. Isolate pancreas into single cells[19] to detect the GFP expression of pancreas by flow cytometry. Troubleshooting 5.

An example of flow and GFP IHC data is shown in Figure 3C.

⚠ CRITICAL: When titer Cre-expressing AAV (For example, AAV_sgGOI_sg3TSG_Cre), it is necessary to use a mouse strain harboring Cre-inducible fluorescence to estimate infection rate. In the case described here, we use Rosa-26-LSL-Cas9-eGFP mice. Mice aged 6–10 weeks are optimal for AAV injection.

**Gene editing efficiency assay (optional)**

⏱ Timing: 2 days

47. At the appropriate time point (for *in vitro*, 3 days post AAV8 infected, for *in vivo*, 7 days post AAV IP into mice), use fluorescence activated cell sorting (FACS) to sort transfected pancreatic cells (in the case of LSL-Cas9-eGFP mice infected with pAAV_sgGOI_sg3TSG_Cre, GFP-positive cells are sorted).
48. Extract genomic DNA using QuickExtract™ DNA Extraction Solution 1.0 (1 μL Quick Extract buffer per 1,000 cells).
49. Design PCR primers and perform PCR according to TIDE website's instructions.[20]
50. Purify PCR product from DNA gel and send for Sanger sequencing.
51. Load Sample/control chromatogram into TIDE (http://shinyapps.datacurators.nl/tide/) and calculate the percentage of indels.
52. Example TIDE results of *in vitro* & *in vivo* biological titration assay is shown in Table2.

*Note:* sgRNA sequence and TIDE primer is shown in key resources table.

**Table 2. Example of Gene editing efficiency assay result use sorted AAV8 infected cell**

| sgRNA | sgRNA cutting efficiency by TIDE | | |
|---|---|---|---|
| | *Trp53* | *Cdkn2a* | *Smad4* |
| *in vitro* biological titration assay | 88.30% | 87.80% | 88.80% |
| *in vivo* biological titration assay | 94.20% | 82.40% | 22.40% |

## EXPECTED OUTCOMES

The above protocol allows the packaging of a wide variety of engineered AAV8 virus particles that can be used in a variety of manners. We have successfully used Cre-expressing AAV in short-term experiments to assess the effect of knockout of specific transcription factors on PDA development.[1] To accomplish this, after infection by viruses expressing control sgRNAs or sgRNAs targeting specific transcription factors, mice were treated with caerulein to promote inflammation and tumor initiation.[21] In the case of mice infected with control AAV, within 20 days of caerulein treatment, we observed widespread neoplasia, and the appearance of areas of invasive carcinoma (Figure 4B). In contrast, mice infected with AAV that expressed sgRNAs targeting *Klf5* displayed little abnormality (Figure 4B). The fate of infected cells was tracked using IHC for GFP (Figure 4B), while effective knockout of tumor suppressors was confirmed using TIDE (as described above). As expected, AAV8-mediated introduction of sgRNAs targeting tumor suppressors to the pancreas of LSL-Cas9 mice significantly reduced their survival compared to mice in which AAV8 delivered only control sgRNAs (Figure 4C).
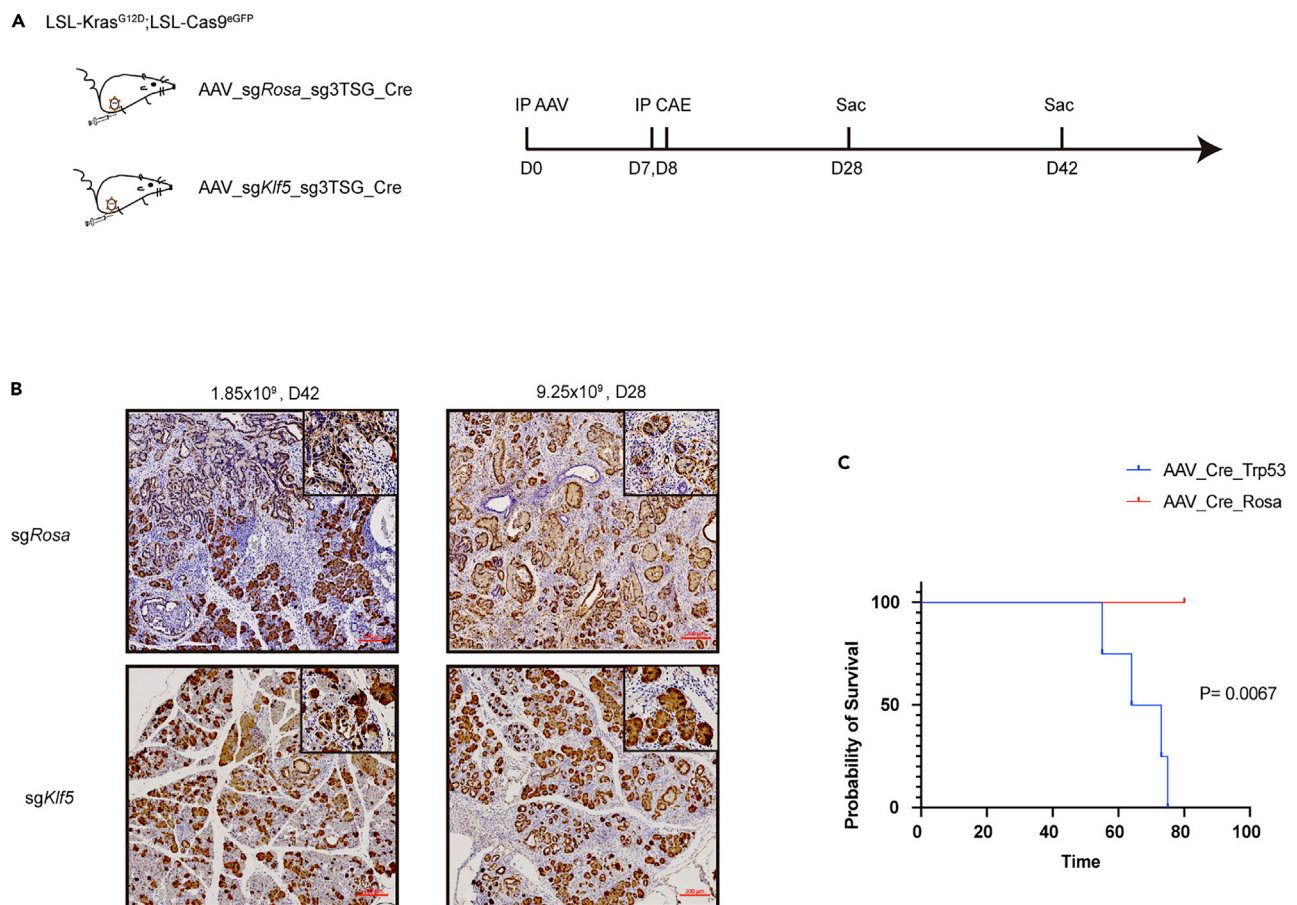


**Figure 4. Examples of mouse pancreatic cancer formation assay using AAV8**

(A) Experimental scheme for PDAC induction using AAV. LSL-Kras$^{G12D}$; LSL-Cas9-eGFP mice ("KCa" mice) were injected with AAV_sg3TSG_Cre expressing either a control sgRNA (targeting the Rosa locus) or an sgRNA targeting Klf5. Mice were then treated with caerulein for consecutive two days to induce inflammation (Morris et al.[21]). Mice infected with a high titer of AAV (9.25*10$^9$ viral genomes) were sacrificed at day 28 and mice infected with a lower titer (1.85*10$^9$ viral genomes) were sacrificed at day 42.

(B) GFP Immunohistochemistry in pancreases of mice treated as described in (A). Result indicates that Klf5 knockout restrains pancreatic cancer development. Scale bar = 200 μm.

(C) Kaplan-meier curve of KCa mice injected IP with pAAV_sgRosa_Cre and pAAV_sgTrp53_Cre respectively. In this case, mice were not treated with caerulein. Each group contained 4 mice. Log rank (Mantel Cox) test.

Although AAV serotype 8 can infect mouse pancreas tissue efficiently, when using IP injection to deliver the virus, it is also able to infect other organs such as lung, skin, and liver. To prevent mutant *Kras* and Cas9 activation and genome editing in other tissues, pancreatic-specific Cre alleles (such as Pdx1-Cre or Ptf1a-Cre) or pancreas-specific delivery methods such as retrograde bile duct injection[14] can be used to ensure pancreas-specific gene disruption. We routinely use pAAV_sgGOI_sgTSG_RFP to infect mice harboring Pdx1-Cre; LSL-Kras$^{G12D}$; LSL-Cas9-eGFP. When care is taken to ensure equal initial AAV infection, this approach can be used for survival studies (Figure 4C). For these experiments, we typically do not inject mice with caerulein, and simply await spontaneous tumor formation.

To achieve pancreas-specific transgene expression, pancreas-specific Cre alleles can be combined with Cre-inducible reverse tetracycline-controlled transactivator (rtTA), allowing pancreas-specific, doxycycline-inducible transgene expression. Alternatively, the transgene can be cloned within a FLEX cassette[22] in the pAAV_Core vector to allow Cre-inducible expression within the pancreas.

## LIMITATIONS

The purity of AAV8 is not well-determined. Because AAV is only crudely purified, there is potential for contaminants resulting in immune responses, although we have seen no evidence for this in any organ examined.

An additional limitation is that the packaging limit for AAV8 is 4.7kb, smaller than that of many commonly used viruses. This imposes limits on the size of cDNAs that can be expressed in the pancreas using AAV8.

Unlike other viruses commonly used for transgene expression such as lentivirus, AAV does not actively integrate into the genome. Instead, AAV can integrate at sites of DNA DSBs induced by CRISPR at efficiencies of nearly 50%.[7] While failure to integrate is not an issue for genome editing, which presumably occurs relatively soon after infection, it may present problems if infected cells are being tracked by expression of a fluorescent protein, or if AAV8 is being used to experimentally deliver a transgene. This is because it is expected that as cells proliferate, AAV8 will be lost by clones that fail to integrate the virus. In practice, we have found high variability in the proportion of AAV8-induced tumor cells that retain AAV long-term.

## TROUBLESHOOTING

### Problem 1
AAV8 have precipitation in the bottom after −80°C freeze (related to step 24).

### Potential solution
Usually, we would gentle peptide the AAV8 and avoid bubble after freeze and then IP mice.

### Problem 2
Physical titer is not correlated with biological titer (related to step 32).

### Potential solution
Physical titer is based on the amount of genome contained in the AAV8 capsid, but ITR recombination could cause AAV8 inactivation which cannot be distinguished by qPCR. In this case, establishing the biological titer is more useful than the real-time PCR-based method. For first-time users of this protocol, we recommend performing a direct biological titer assay. After becoming familiar with this protocol, the qPCR assay can be used to predict AAV biological titer.

### Problem 3
AAV8 biological titer result is low (related to step 32).

### Potential solution

AAV8 is very sticky so use low-binding tip and tube during this protocol.

### Problem 4

The infection rate is improper (higher than 30%) to predict/calculate MOI or biological titer in transduction assay (related to step 40).

### Potential solution

Several dilutions are recommended for transduction assay. For Cre-based AAV8. It is better to perform qPCR first and dilution AAV8 range from 100-fold to 2000-fold.

### Problem 5

There are variations between experimental mice *in vivo* transduction assay (related to step 46).

### Potential solution

To have a clear view about AAV8 biological titration, use no less than 3 mice per group in *in vivo* transduction assay.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Charles J. David, cdavid@mail.tsinghua.edu.cn.

### Materials availability

Plasmids used in this study not available on Addgene are available upon request.

### Data and code availability

No datasets or code was generated or analyzed during this study.

## AUTHOR CONTRIBUTIONS

M.Z. performed most experiments, prepared the figures, and drafted the manuscript. J.M. performed the survival curve assay. C.J.D. provided scientific guidance, allocated funding, and revised and finalized the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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