AAV production and titer determination ● TIMING **7 days** including incubation

1. *Day 1 (plating)*. Plate AAV-293 cells (1.0-1.2x10^6/ml) in a 15 cm dish containing DMEM supplemented with 10% FBS and 1x pen/strep. Prepare 5 plates per construct. Place plates in an incubator set at 37°C with 5% CO_2.

▲ CRITICAL STEP Healthy host cells are so critical for virus quality that gentle and strictly proper handling are recommended.

2. *Day 2 (transfection)*. Feed AAV-293 cells with 15-20 ml of pre-warmed fresh media in a 15 cm dish 2-4 h before transfection.

▲ CRITICAL STEP Replace media gently since cells can be lifted easily.

About 30 min before transfection prepare the transfection mix.

(A) Mix DNAs in a 15 ml-tube labeled A.

Reagent	**Amount** (for 5x15cm dishes)
One of mGRASP constructs	60 μg
pHelper	56 μg
Serotype plasmid, e.g. pAAV2/1	24 μg
NaCl, 150 mM	7 ml

▲ CRITICAL STEP Choice of serotype depends on cell types of target brain region. pAAV2/1 and pAAV-DJ are successfully applied to hippocampus, cortex, and thalamus in our hands.

(B) Mix PEI in a 15 ml-tube labeled B.

Reagent	**Amount** (for 5x15cm dishes)
PEI	280 μl
NaCl, 150 mM	7 ml

(C) Vortex separately and then immediately combine mixtures A and B by pipetting mixture B into tube A.

(D) Invert mixture A+B well and incubate for 30 min at room temperature.

(E) Distribute mixture A+B drop-wise over cells (~2.9 ml/plate) and swirl well to evenly distribute the mixture.

(F) Incubate the cells for 2.5-3 days in a 37°C, 5% CO_2 incubator.

3. *Day 5 (harvest of nuclei)*. Check briefly the transfection rate under a fluorescence microscope. 50-70 % transfection rate is optimal.

▲ CRITICAL STEP A high transfection rate in healthy host cells is a prime determinant for virus quality and the final volume of concentrated virus in step 6 will be adjusted based on the transfection rate determined in this step. mCerulean fused with pre-mGRASP is quite dim under the BFP or GFP filter set of an epifluorescent microscope compared to cytosolic BFP or GFP, but is sufficiently detectable to examine the transfection rate. Illuminate only briefly to avoid photodamage.

(A) Discard media from transfected plates and rinse cells with PBS. Harvest cells with a cell scraper in 10 ml PBS per 15cm-dish and collect cells from 5x15cm-dishes in a 50 ml tube.

(B) Spin down cells by centrifugation at 300 g for 20 min at 4°C. Wash cells with PBS followed by centrifugation at 300 g for 10 min at 4°C and discard PBS.

(C) Gently resuspend the cell pellet with 5 volumes of hypotonic buffer (usually 5ml) by pipetting, and incubate on ice for 10-30 min.

(D) Add 0.11 volume of 10x restore buffer (usually ~660 μl) and mix gently by pipetting.

(E) Gently homogenize cells in a glass cell grinder for 12 strokes on ice.

▲ CRITICAL STEP Strong and harsh downstrokes will break nuclei, causing loss of packaged AAV particles.

(F) Transfer homogenized crude in a 15 ml-tube and collect the nuclei by centrifugation at 500 g for 10 min at 4°C.

■ PAUSE POINT The nuclei can be stored in a deep freezer (-80°C) until the next day or for several weeks. Cold storage is recommended to increase the efficiency of releasing packaged AAV particles from nuclei.

4. *Day 6 (CsCl gradient purification of AAV).*

(A) Thaw the frozen nuclei pellet in ice and resuspend with ice-cold 4 ml of autoclaved water and sonicate each sample for 12 pulses (1 sec pulse on and 3 sec pulse off), 50% amplitude.

(B) Incubate with 0.8-1.2 μl of Benzonase Nuclease for 30 min at 37°C to remove DNAs and RNAs from nuclei. Packaged AAV genome will be protected from Nuclease.

(C) Spin debris down by centrifugation at 3,000 g for 5 min at 4°C. Transfer the supernatant to a new 15 ml-tube and add up to 4 ml of ice-cold autoclaved water.

(D) Prepare 2.36 g CsCl per sample in a 15 ml-tube and CsCl solution by vortexing 19.5 g CsCl in 33 ml water. Thoroughly mix the supernatant with 2.36 g of CsCl by vortex.

(E) Transfer to a 4.9 ml-ultracentrifuge tube. Bring the sample volume to the top of the centrifuge tube with pre-made CsCl solution.

!CAUTION Balance each centrifuge tube by weighing and adjusting with CsCl solution.

(F) Ultracentrifugate at 90,000 rpm for at least 2 h and 40 min at 8 ^{o}C.

(G) Place a 18G needle through the side near the bottom of the centrifuge tube and carefully open the cap of the ultracentrifuge tube. Collect ~0.5 ml fractions (around 12 drops) in 1.5 ml-eppendorf tubes.

(H) Read the refractive index of each fraction with a Refractometer and bin the peak fractions around *fr* = 1.372 (usually 3-4 fractions, 1.370-1.374) for the second round of ultra-centrifugation.

(I) Repeat steps 5D-H.

(J) Dialyze the peak fractions in the Slide-A-Lyzer dialysis cassette against 2-3L of 5% sorbitol in PBS for at least 2 h at 4^{o}C, twice.

■ PAUSE POINT The first dialysis can be performed overnight. This is recommended for sufficiently dialyzing the CsCl.

5. *Day 7 (concentration of AAV).* Concentrate dialyzed virus by spinning down through Amicon Ultra centrifugal filter (30kDa) up to ~100 μl at 4^{o}C. Make 5-10 μl aliquots on ice and store at -80^{o}C

▲ CRITICAL STEP Final volume of concentrated virus will be adjusted with reference to the transfection rate determined in step 4. Small volume aliquots should be stored at -80^{o}C to prevent repeated freeze/thaw cycles, which significantly affect concentration and infection efficiency.

■ PAUSE POINT The frozen virus aliquots can be stored at -80^{o}C for up to one year.

6. Titer determination by quantitative PCR

(A) Prepare virus samples and serial dilutions of standard.

(i) Extract AAV single-stranded genome from 2 μl of AAV stock in step 6 and elute in 100 μl distilled water using MiniElute extract kit (Qiagen, 57704). Make serial dilutions of purified AAV genomes.

(ii) Serially dilute a standard AAV plasmid whose concentration is known; for example, 1:2, 1:10, 1:50, 1:250, and 1:1250.

(B) Take 2 μl of serial dilutions of samples and standard in the PCR reaction mixture using the WPRE primer set. Triplicate each sample for accuracy.

2x SYBR Green QPCR master Mix	12.5 μl
WPRE forward primer, 10 μM	1.25 μl
WPRE reverse primer, 10 μM	1.25 μl
Reference Dye, 10 μM	0.75 μl
Diluted AAV sample	2 μl
Water, PCR-grade	7.25 μl

(C) Run real-time PCR using optical 96 well reaction plate

cycle	Duration of cycle	Temperature
1	10 min	95 ^{o}C
35	30 sec	95 ^{o}C
	1 min *	55 ^{o}C
	1 min	72 ^{o}C
1	1 min	95 ^{o}C
	30 sec $^{\#}$	55 ^{o}C
	30 sec	95 ^{o}C

* collect data at the end of each cycle, $^{\#}$ collect data during each ramp-up phase

(D) Calculate AAV titer, genome copy (GC)/ml

For 500 base average genome size of AAV;

1 ng of AAV ssDNA = $\frac{1\times10^{-9}(g)\times Avogadro\ number}{genome\ size\ (bp)\times330}$ = 3.6x10^{8} GC

Titer (GC/ml) = Dilution Factor x AAV-ssDNA amount (ng) from qPCR x 3.6x10^{8} GC

▲ CRITICAL STEP Unpurified AAV stock can be directly used for qPCR. The titer calculation should be calibrated. The titer of mGRASP AAV should ideally be >10^{12} GC/ml. When using

Cre-independent mGRASP AAV, lower titers often result in infecting mainly glia rather than targeted neurons.

? TROUBLESHOOTING

Step	Problem	Possible reason	Solution
1-6	Low virus titer	Damaged ITR sequences in DNAs and/or Low DNA quality	Two inverted terminal repeat (ITR) sequences are important for AAV DNA replication. SmaI digestion can confirm ITR sequences are intact. Make sure to use a stable *E coli* strain and EndoFree purification.
		High cell passage number	The cell passage number affects not only transfection efficiency but also virus packaging. Despite high transfection rate, virus package rate can be reduced along with cell quality. Use low passage numbers of cells and maintain them properly.
		Loss during fractionation	Take more fractions from the first round of ultracentrifugation.