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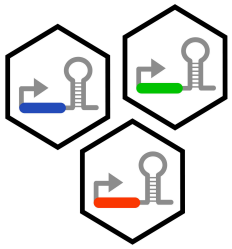
# Protocol for AAV production for in vivo CRISPR screening in the mouse brain



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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

This protocol is optimized for making large quantities of high-titer adeno-associated virus (AAV) for use in vivo, especially for CRISPRi screening applications in the mouse brain. It typically produces ~$2\times10^{13}$ to $1.2\times10^{14}$ total viral particles per 15 cm dish, at a titer of ~$4\times10^{11}$ to $2\times10^{12}$ particles/µl, when using the PHP.eB capsid (other capsids may produce different titers). This can be scaled up for larger quantities by using multiple 15 cm plates. It is based off of Negrini et al. (2020) *Curr Protoc Neurosci*, PMID: **32865885** with modifications. AAV titering is based on this protocol: **https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/** with modifications.

## Image Attribution

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## Materials

Viral Plasmids:

- 1.60 pmol (15.2 µg) AAV Helper pAdDeltaF6 plasmid (Addgene, 112867)
- 1.60 pmol (8.3 µg) AAV rep/cap plasmid, e.g. PHP.eB (Addgene, 103005) or PHP.Astro
- 1.60 pmol (X µg) cargo plasmid, e.g. hSyn1-Cre (Addgene, 105540), pAP215, pIR110, pIR111, etc. This must include AAV ITRs.

Polyethylene glycol (PEG) 8000, 40% Stock Solution in 2.5 M NaCl (Sigma Aldrich, 89510-250G-F, 250 g)

- See Appendix A

Polyethylenimine (PEI) Stock Solution (Polysciences, 23966-100, 100 mg)

- See Appendix B

Chloroform, molecular biology-grade (Sigma Aldrich, C2432)

- Use dedicated bottle specifically for AAV production

HEPES Stock Solution (50 mM HEPES, 3 mM MgCl2, pH 8.0)

- See Appendix C

DNase I enzyme, 2,000 U/ml (New England Biolabs, M0303S)

RNase A enzyme, 10 µg/µl (Thermo, EN0531)

HEK293T cells (ATCC, CRL-3216)

HEK Cell Culture Media

- 500 ml DMEM (Gibco, 11965-092)
- 50 ml FBS (e.g. VWR, Seradigm, 89510-186)
- 5 ml Pen-Strep (e.g. Gibco, 15140-122)
- 5 ml L-glutamine (e.g. Gibco, 25030-081)

Opti-MEM Medium (Gibco, 31985062)

100 kDa Amicon Ultra Centrifugal Filter Units, 0.5-ml (Millipore, UFC510024)

DPBS (Gibco, 14190-144)

Trypsin-EDTA Solution, 0.25%, 1X (Gibco 25200-056)

500 µl LoBind tubes for storing AAV (Eppendorf, 022431005)

SensiFAST SYBR Lo-ROX (Meridian Bioscience, BIO-94020) for AAV titering

Primers for AAV titering

- ITR-Fwd: 5'-GGAACCCCTAGTGATGGAGTT-3'
- ITR-Rev: 5'-CGGCCTCAGTGAGCGA-3'

## Determine required amount of plasmids

1 **Calculate required plasmid amount.** Ideal ratio of Helper:Capsid:Cargo is 1:1:1. Per 15 cm plate, you'll need 1.60 pmol per AAV you want to make:

1. Helper plasmid: pAdDeltaF6 (**Addgene, 112867**) is 15,420 bp, so 1.60 pmol = **15.2 µg**
2. Capsid plasmid: PHP.eB (**Addgene, 103005**) is 8,543 bp, so 1.60 pmol = **8.3 µg**
3. Cargo plasmid: (for example: pAP215 is 6,016 bp, so 1.60 pmol = 5.9 µg)

Use this online calculator to determine mass/molarity calculation for each cargo plasmid and adjust accordingly: https://nebiocalculator.neb.com/#!/dsdnaamt

2 **Amplify plasmids to required amounts.** Perform mini-prep/maxi-prep/etc as needed to produce required amount of DNA plasmids.

Follow manufacturer's instructions, e.g. from ZymoPURE plasmid Miniprep kit (Zymo Research, D4209) or QIAprep Spin Miniprep Kit (Qiagen, 27104). Supplied Elution Buffers from both of these kits is compatible.

## Thaw and passage HEK cells

3 **Make HEK cell culture media** (DMEM Complete):

1. DMEM, 500 ml
2. Add 50 ml FBS
3. Add 5 ml Pen/Strep
4. Add 5 ml L-Glutamine

L-Glutamine needs to be warmed at 37°C and vortexed multiple times in order to go into solution. FBS and Pen-Strep can be thawed at 4°C overnight the day before, or at RT the day-off. Keep media at 4°C for up to several weeks.

4 **Thaw HEK cells & grow as convenient.** Typically in 10 cm or 15 cm dishes.
Passage at least once before plating cells for making AAV. Passage by incubating in 0.25% Trypsin, diluting in DPBS, and spinning down 300 x g, 3.5 min, RT, and re-plating in HEK media. Don't let them grow past >80% confluency.

## Day 0: Plate HEK cells

30m

5 **Seed plates with cells.** Seed HEK cells at 15 million cells per 15 cm dish the day before (= ~70-80% confluency the next day). Scale number of plates as necessary to create final amount of AAV.

## Day 1: Transduce cells

45m

6 In **5 ml Opti-MEM**, mix the following purified plasmids:

- 1.60 pmol AAV Helper plasmid (= 15.2 µg)
- 1.60 pmol AAV capsid plasmid (= 8.3 µg)
- 1.60 pmol cargo plasmid (calculate depending on cargo size, see Step 1)

7 Add **100 µl PEI** into **5 ml Opti-MEM/plasmid mixture**
DO NOT add PEI directly to pure plasmid mixture, plasmids must be diluted in Opti-MEM first

8 Incubate **10 mins** at Room temperature

9 Change media in 15 cm plate of HEK cells with **25 ml fresh HEK media**

10 Gently drip **5 ml Opti-MEM/plasmid/PEI solution** over HEK cells distributing through the 15 cm dish and swirl gently

11 Incubate Overnight

## Day 2: Change media to Opti-MEM

12 Remove full media from plate and replace it with **25 ml Opti-MEM** (fresh)
Ideally, pre-warm Opti-MEM to 37 °C before use.

15m

13 Wait **60-72 hours** after Opti-MEM media change
We have also done 48h, but 60-72h improves titer substantially

3d

## Day 5 (or Days 5-6): Virus purification

3h

14 ***Note:*** This step can be done in either 1 or 2 days, depending on experimental convenience. We often opt for the 2 day method as we suspect it increases yield slightly.

If doing 1 day option, pre-cool large TC centrifuge and 1.5 ml tube centrifuge to 4 °C

If doing 2 day option, do this tomorrow instead.

15 **Remove all cells and media from plates.** Add to 50 ml conical tube by re-suspending until cells dissociate
- Use serological pipette to vigorously triturate cells (most AAV is inside the cells not the media)
- Use cell scraper if necessary

16 Add **chloroform** (1:10 final volume, should be around 3 ml) and shake *vigorously* for 30sec
- Use serological pipette to measure volume. Precision is important with this step.

17 Centrifuge at **3,000 x g, 5 min,** Room temperature

18 Transfer supernatant (aqueous phase, red, on top) to fresh 50 ml tubes

19 Add **PEG stock solution** (1 part PEG to 4 parts supernatant). *Be as precise as possible.*
- Again, use serological pipette to accurately measure volume.

20 Incubate **1-24 hours at** 4 °C

We typically incubate in a 4 °C fridge Overnight

However, if short on time, you can incubate for as little as 1 hour On ice instead.

21 Centrifuge **3,000 x g, 30 min,** 4 °C

22 Gently pour off supernatant and place tubes upside-down on paper towels to **dry for 10 min**, dabbing to dry. *Note:* Remaining procedures may be done outside of the TC hood, depending on downstream application.

23 Make DNase/RNase Solution by mixing (1 ml per virus):

1. **1 ml HEPES Solution** (See Appendix C)
2. **10 µl DNase I**
3. **1 µl RNase A**

24 Resuspend pellet in **1 ml DNase/RNase Solution**
Vortex for 5 min (or less time is ok too)

25 Incubate **20 mins at** 37 °C

This will digest away un-packaged RNA and DNA.

26 Split resuspended solution into two 1.5 ml tubes
Should be ~500-750 µl each tube

27 Add **chloroform** 1:1 proportion (v:v) to each tube. *Be as precise as possible.*
Use chloroform bottle specifically for making AAV.

28 Vortex for **10 sec**

29 Centrifuge **16,000 x g, 5 min,** Room temperature

30 Carefully collect aqueous phase (on top) and add to fresh 1.5 ml tube.
Make sure volume is exactly 500µl.

31 **Repeat** chloroform purification step once again by adding chloroform 1:1 (v/v) (should be 500 µl) to these tubes, vortexing 10 sec, and centrifuging again **16,000 x g, 5 mins,**
Room temperature

32 Carefully collect aqueous phase (on top) and pool into fresh 1.5 ml tubes
*Optional:* keep the lid open for a little while to evaporate any trace chloroform out

33 Add **≤ 400 µl of sample** to an Amicon ultracentrifugal filter column
Label filter unit, but no need to label tube

34 Centrifuge **14,000 x g, 3 min,** Room temperature
Discard flow-through
Keep using same discard tube until last step

35 **Repeat** until you've loaded all the sample into the filter column (usually 2-3 times)

36 Add **400 µl 1X DPBS**, pipette carefully 3-4x to mix solution without touching pipette to inside surface of filter column

37 Centrifuge **14,000 x g, 3 min,** Room temperature
Discard flow-through

38 **Repeat** this DPBS wash **twice**, for 3 washes total

Spin for 3 mins each time

39 Place column in new 2.0 ml tube, flip filter column upside-down and centrifuge **1,000 x g, 2 min,** Room temperature to recover AAV

40 Transfer AAV to 500 µl Eppendorf LoBind tube for long-term storage at 4 °C
Expected final volume ~50 µl.
Store at 4 °C for up to ~6 months. **Do not freeze.**

41 For CRISPR screen library viruses, take 1 µl of virus, dilute 1:100 in nuclease-free water, and freeze at -80C for later sequencing to get starting sgRNA distribution (VERY important for survival screens).

## Titering AAV via qPCR

1h 30m

42 **Create qPCR Standards.** Mini-prep your plasmid cargo and establish a stock at 2 × $10^9$ molecules/µl. This will be used to generate a standard curve. Perform Qubit dsDNA High-Sensitivity assay (Invitrogen, Q32851) to accurately determine concentration of plasmid stock (do not use NanoDrop).

Make a 10-fold dilution series in a PCR strip tube $2\times10^9$, $2\times10^8$, $2\times10^7$, $2\times10^6$, $2\times10^5$, $2\times10^4$, $2\times10^3$, and blank. Can make multiple strips and freeze aliquots to make it easier for next time

43 **Dilute AAV.** Generally a 1:10,000 times dilution works best. Can also try 1:100,000. Do this in a PCR strip tube and dilute with UltraPure water.

44 **Set up qPCR reactions**. We use SensiFAST SYBR Lo-ROX kit (Meridian Bioscience, BIO-94005), following manufacturer's instructions. Do 10 µl reactions in duplicate or triplicate. AAV ITR primers come from Aurnhammer et al (2012), PMID: 22428977.

| | A | B | C |
|---|---|---|---|
| | | 1 rxn (10 µl) | Scaled up: |
| | 2X SYBR Lo-ROX | 5 µl | |
| | ITR-Fwd (10 µM) | 0.4 µl | |
| | ITR-Rev (10 µM) | 0.4 µl | |

| | A | B | C |
|---|---|---|---|
| | AAV (1:10k dilution) or DNA standards | 4.2 µl | |

45 **Run qPCR.** Use Lo-ROX, 2-cycle. We use a C1000 Touch Thermal Cycler with CF96 Real-Time System (Biorad). 1h

46 **qPCR Analysis**

1. Plot standard curve, remember you're loading 4.2 µl of $2\times10^X$ molecules (= $8.4 \times 10^X$ molecules per run), so plot those values against the average Cq for that standard and do a linear regression
2. Using equation generated from standard curve, determine # of molecules that corresponds to average Cq values for your diluted AAVs
3. Convert that to titer in copies/µl by multiplying it by 4.2 µl and dividing that by 10,000 (or 100,000 if that's how much you diluted it). This should generate a readout in copies per µl.

Can use attached spreadsheet:

AAV Titering template.xlsx 18.9KB

47 ***Alternative:*** Instead of qPCR, can use digital PCR (dPCR) to titer AAV. This will read out absolute quantification, versus relative concentration to a standard. This is likely more accurate, but requires access to a dPCR machine. We recommend QIAcuity EvaGreen PCR Kit (Qiagen, 250111) on 8.5k partition nanoplates (Qiagen, 250011) on QIAcuity One instrument, following manufacturer's instructions.

## Appendix A: Making PEG stock solution (40% PEG in 2.5 M NaCl)

48 Weigh out the following and add to 50 ml tube:

1. 20 g PEG powder
2. 7.3 g NaCl powder

49 Fill 50 ml tube to 50 ml mark using nuclease-free $H_2O$.

50 Microwave ~20 sec on high until powder dissolves. Loosen cap slightly so it doesn't explode.

51 Cape tube and shake on rocker for 30+ min at room temp, letting tube roll around to mix well.

52 Store at 4°C, or cool fully to 4°C on ice before use (if making fresh for same-day use).

## Appendix B: Making PEI stock solution

53 Dissolve PEI (e.g., Polysciences, cat. no. 23966-1) by swirling using sterile water heated to 80°C and using ~90% volume required to reach a final concentration of 1 g/L (1 µg/µl). Let cool to room temperature.

54 Adjust pH to 7.0 with HCl.

55 Add sterile water to a final concentration of 1 µg/µl. Filter using a 0.22-µm membrane filter, and divide into aliquots. Store at −20°C for up to 1 year.

56 When ready to use, thaw aliquot in a 37°C water bath until precipitates have fully dissolved.

57 Store thawed aliquot at 4°C for up to 1 month. Redissolve precipitates by incubating at 37°C before use, if necessary.

## Appendix C: Making HEPES stock solution

58 HEPES Stock Solution: 50 mM HEPES, 3 mM MgCl2, pH 8.0

1. 2.5 ml 1 M HEPES in water
2. pH to 8.0 using NaOH
3. 150 µl 1 M $MgCl_2$ in water
4. Fill to 50 ml using water

## Protocol references

Negrini M, Wang G, Heuer A, Björklund T, Davidsson M. AAV Production Everywhere: A Simple, Fast, and Reliable Protocol for In-house AAV Vector Production Based on Chloroform Extraction. *Curr Protoc Neurosci*. 2020 Sep;93(1):e103. doi: 10.1002/cpns.103. PMID: 32865885.

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