Protocol for AAV production

General Remarks:

This protocol is designed for the production of two AAViruses in parallel. You need in total three 10-stacks and three 1-stacks for two rounds of transfection and harvesting. Note that two 10/1-stacks are re-used in a second round of production of the same virus (mark the stacks). The third 10/1-stack is for propagation of 293T cells.

Step 1: Cell-stack expansion

Materials required:
6 confluent (~90%) 15cm dishes 293T cells
10-stack, 1-stack
Trypsin (8ml)
Medium w/o FCS 100ml or PBS
Medium 1200ml

Cells from 6 confluent (~90%) 15cm dishes are plated onto 1x 10-stack and 1x 1-stack (this corresponds to a ~1:10 splitting).

• Following trypsinization and centrifugation cells are distributed equally to 2x 550ml medium (2 medium bottles + 10%FCS + 1%P/S).

• 2x 500ml are transferred to a 10-stack; 2x 50ml are transferred to a 1-stack.
(This is generally how a cell suspension from 2 medium bottles are distributed to a 10-stack and the corresponding 1-stack (10/1-stacks) throughout the whole protocol in order to ensure, that the cell density in the 1-stack represents the 10-stack)

Step 2: Pre-transfection

Day -1/-2, a.m.

Materials required for 2x viruses:
Cells: 1x 10-stack, 1x 1-stack 80-100% confl.
2x 10-stacks, 2x 1-stacks
2x 1lt Erlenmeyer flasks
2x conical 500ml tube
220ml 10x D-PBS (Invitrogen)
2lt Braun- H_2O (DKFZ)
120ml Trypsin(0.25%)/EDTA (Invitrogen)
3.3lt medium (6 bottles+FCS, P/S)

For 2 viruses:

• Remove medium from 10-/1-stack by decanting into an Erlenmeyer flask

• Wash with 900ml/100ml D-PBS, decant into flask (minimize detachment of cells by indirectly introducing of D-PBS hitting the wall of the stack first).

• Remove D-PBS; add 100ml/10ml trypsin(0.25%)/EDTA, 37°C, 5min

• Swirl and slam the Cell-Stacks to detach cells.

• Add 500ml/50ml medium (incl. FCS) and transfer to 2x500ml conical tubes.

• Spin 500g (2000rpm), 10min, 15°C

• In the meanwhile: wash “old/used” 10-/1-stack with 900ml/100ml D-PBS

• Resuspend cells from both conical tubes into 110ml medium. For this, use 25ml from each of 4 medium bottles (for 2 10/1-stacks to be transfected for the production of 2 viruses); and 5ml from 2 medium bottles (for the third, already used and washed 10/1-stack for propagation of cells for the second round of transfection).

• Distribute the cell suspension to the according medium bottles:
4x25ml (transfection); 2x5ml (propagation)

• Fill the three 10/1-stacks with 1000ml/100ml of the corresponding cell suspensions.

Step 3: Transfection

Day 0, a.m.

Materials required for 2x viruses (2x10-stacks+2x1-stacks):
395µg dsAAV vector (for each virus construct)
994µg rep/cap plasmid (p5E18-VD2/8)
2706µg pDGΔVP
40ml H_2O (UltraPure)
36ml PEI (7,5 mM; see protocol for production of PEI)
80ml NaCl (300mM)
4x sterile bottles (min. 100ml; for mixing of transfection reagent and DNA)
2x conical 500ml tubes

- Cells should be 70-80% confluent
- Prepare DNA solution: **Mix1**

  395µg pdsAAV vector (~5.8kb)
  497µg p5E18-VD2/8 (~7.3kb) (for AAV2/8)
  1353µg pDGΔVP (~21kb)
  H_2O ad 20 ml
  + 20ml NaCl (300mM); mix
  40ml

- Prepare PEI solution: **Mix2**

  18ml PEI
  2 ml H_2O
  + 20ml NaCl (300mM); mix
  40ml

- Introduce mix 2 drobwise into mix 1 while swirling (immediate mixing avoids the generation of large DNA precipitates that are not transfectable; solution gets cloudy)
- Incubate for 10 minutes at room temperature
- Transfer 40ml of transfection mix to each of the two medium bottles/virus (including FCS and P/S)
- Remove “old” medium from the 1- and the 10-stack
- Transfer 50ml from each medium bottle to the 1-stack (100ml medium) and the remaining medium to the 10-stack (containing ~2x550ml medium).
- Change medium 24h post-transfection.

Step 4: Harvest

Day 2, p.m. (or day 3, a.m.)

Materials required for 2x viruses:
> 2 1lt-Erlenmeyer flasks
550ml 10x D-PBS
5lt Braun-H_2O
230ml Trypsin(0.25%)/EDTA
800ml medium (incl. FCS)
4x conical 500ml tubes
Lysis buffer (150mM NaCl, 50mM TrisHCl pH8.5, sterile)

For each 10/1-Stack (virus):

- Remove medium by decanting into Erlenmeyer flasks
- Wash with 900ml/100ml D-PBS, decant into flask (if necessary spin detached cells down @ 600g, 10min, 15°C)
- Add 100ml/10ml trypsin(0.25%)/EDTA, 37°C, 5min
- Swirl and slam the Cell-stack to detach cells
- Add 350ml/40ml medium (incl. FCS) and transfer to a 500ml conical tube (390ml + 110 ml trypsin = 500 ml); (1^{st} tube).
- Add 450ml/50ml D-PBS and transfer to a 500ml conical tube (2^{nd} tube).
- Spin both tubes 600g, 10min, 15°C
- In the meantime Wash Cell-stacks with 500ml/50ml D-PBS (1^{st} round only) and add 500ml/50ml D-PBS for storage (1-2 days in 4°C room for 2^{nd} round).
- 2^{nd} tube: Discard SN, resuspend pellet in 50ml D-PBS and transfer to 50ml-Falcon
- 1^{st} tube: Transfer SN to 2^{nd} conical tube, use 50ml cell suspension from 2^{nd} Falcon to resuspend pellet and distribute to 2x 50ml-Falcons.
- Spin Falcons 2000rpm, 10min, 4°C (swing-rotor centrifuge)
- Resuspend pellets in 8ml lysis buffer and transfer one 50ml-Falcon
- Vortex and snap-freeze in N2, store at -80°C.

Repeat Step 2 to 4 for a second round of virus production reusing the 10/1-stacks for the same virus as before.

Step 5: Lysis (cont.)

Materials required for 2x viruses:
N2
Lysis buffer
Sterile MgCl2 1M
Benzonase >8000U (SIGMA)
Water bath sonicator

Starting with 2x50ml Falcons per virus containing 2x~20ml lysate from two rounds of transfection:

• Thaw in water bath at 37°C, vortex and snap-freeze in N2
• Repeat once
• Thaw, vortex, transfer into fresh 4x15ml-Falcons and spin ~3500g (6000 rpm TC centrifuge), 10min, 4°C
• Collect **SN1** (4x~10ml) into 4x15ml-Falcons and resuspend pellet in 5ml lysis buffer
• Vortex, snap-freeze, thaw at 37°C
• Repeat twice
• Transfer into fresh 4x15ml-Falcon and spin ~3500g, 10min, 4°C
• Collect **SN2** (4x~10ml) into 4x15ml-Falcons and resuspend pellet in 4ml lysis buffer
• Vortex, snap-freeze, thaw at 37°C.
• Vortex, sonicate in water bath sonicator 1min.
• Add $MgCl_2$ to 5mM and Benzonase (Sigma) to 50U/ml to **SN1+2** and to lysate (3)
• Incubate at 37°C for 30min
• Transfer lysate (3) to fresh 15ml-Falcon and spin ~3500g, 10min, 4°C
• Collect **SN3** and pool with **SN2**
• Freeze 4xSN1 (~10ml) and 4xSN2/3 (~9ml) and 4xpellets in (-80°C)

Optional: take 20µl aliquots from each fraction for possible qPCR tests (for troubleshooting in case virus yield is low at the end.)

Step 6: Gradient purification

Materials required for 2 viruses:
10x Beckman Centrifuge Tubes, Polyallomer, Quick-Seal, 39ml, 25x89
4x spacers for centrifuge tubes
UZ, 50.2Ti rotor, 3x 3 hours
126ml OptiPrep (60% iodixanol)
270µl phenol red 0.5%
MgCl2 1M, sterile
KCl 4M, sterile
NaCl 5M, sterile
120ml D-PBS 1x (Invitrogen)
Long pasteur pipettes
Lysis buffer
50ml syringes
10ml syringes
5ml syringes
filters for 50ml syringes
20G needles

For 1 virus (4 gradients):

• Prepare iodixanol dilutions while thawing lysates.

For 2 viruses prepare the according amounts (see next page) for 10 gradients for the 1st round of centrifugation and 5 gradients for the 2nd round.

• Pool each SN1 with SN2/3: 4x~19ml:

• Transfer lysates to tubes through long Pasteur pipette.

• Sequentially underlayer through Pasteur pipette (use prefilled syringes for all layers of one gradient to ensure continuous (air-bubble free) filling of the tube):

7ml 15% iodixanol dil.

5ml 25% iodix. dil.

4ml 40% iodix. dil.

4ml 60% iodix. dil.

• Top up and balance pair-wise with lysis buffer so that ~1ml air remains on top

• Seal tube nozzle with heat and place spacers

• Load ultracentrifuge in 50.2Ti rotor and set up vacuum

• Spin at 50krpm, 2.5h, 10°C

• Insert a 20G needle on top of tube and into the lysate layer

• Insert a 5ml syringe with 20G needle into the 60% layer and collect max. 3.5ml from the 40% layer from underneath. Avoid collecting from the 25% layer

• Separate aliquots as required (20µl), pool suspensions from 4 gradients per virus (~14ml; can be stored at -80°C).

Iodixanol dilution	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
15%	7ml	21ml	35ml	70ml	84ml
25%	5ml	15ml	25ml	50ml	60ml
40%	4ml	12ml	20ml	40ml	48ml
60%	4ml	12ml	20ml	40ml	48ml

15% iodix. dil.	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
OptiPrep	1.75ml	5.25ml	8.75ml	17.5ml	21ml
PBS-MK-NaCl	5.25ml	15.75ml	26.25ml	52.5ml	63ml
Final	7ml	21ml	35ml	70ml	84ml

25% iodix. dil.	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
OptiPrep	2.08ml	6.24ml	10.4ml	20.8ml	25ml
PBS-MK	2.92ml	8.76ml	14.6ml	29.2ml	35ml
Phenol red 0.5%	12.5µl	37.5µl	62.5µl	125µl	150µl
Final	5ml	15ml	25ml	50ml	60ml

40% iodix. dil.	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
OptiPrep	2.67ml	8ml	13.35ml	26.7ml	32ml
PBS-MK	1.33ml	4ml	6.65ml	13.3ml	16ml
Final	4ml	12ml	20ml	40ml	48ml

60% iodix. dil.	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
OptiPrep	4ml	12ml	20ml	40ml	48ml
Phenol red 0.5%	10µl	30µl	50µl	100µl	120µl
Final	4ml	12ml	20ml	40ml	48ml

PBS-MK*	Final	1 gradient	3 gradients	5 gradients	10 gradients	12 gradients
D-PBS 1x	1x	6ml	18ml	24ml	45ml	55ml
MgCl2 1M	1mM	6µl	18µl	24µl	45µl	55µl
KCl 4M	2.5mM	3.75µl		15µl	28µl	34.37µl

* sterilize by filtration

PBS-MK-NaCl*	Final	1 grad.	3 grad.	5 grad.	10 grad.	12 grad.
D-PBS 1x	~0.8x	5.6ml	16.8ml	23.2ml	44ml	52.8ml
MgCl2 1M	1mM	7µl	21µl	29µl	55µl	66µl
KCl 4M	2.5mM	4.37µl	13.11µl	18.12µl	34µl	41.25µl
NaCl 5M	1M	1.4ml	4.2ml	5.8ml	11ml	13.2ml

* sterilize by filtration

2nd round gradients

• Use 1-2 gradients per virus
• Add lysis buffer to suspensions up to 25ml total volume and transfer to a Beckman tube
• Sequentially underlayer with:
5ml 25% iodix. dil.
4ml 40% iodix. dil.
4ml 60% iodix. dil.
• Top up and balance pair-wise with lysis buffer so that ~1ml air remains on top
• Seal tube nozzle with heat (Kleinschmidt) and place spacers
• Load ultracentrifuge in 50.2Ti rotor and set up vacuum
• Spin at 50krpm, 2.5h, 10°C
• Insert a 20G needle on top of tube and into the lysate layer
• Insert a 5ml syringe with 20G needle into the 60% layer and collect max. 3.5ml from the 40% layer from underneath. Avoid collecting from the 25% layer
• Separate aliquots and proceed to dialysis or freeze at -80°C

Dialysis and concentration

Materials required for 2 viruses:
Slide-A-Lyzer-kit 10 KD 3-12 mL (Perbio)
300ml 10x D-PBS
3ltr Braun-H_2O

• Dialyse in slide a lycer (12ml extra strength) following instructions in the kit:

• Dialyse against 1l D-PBS each for

3h at 4°C

over night at 4°C

3h at 4°C

• Transfer into 15 ml Falcon tubes.

• Transfer gradually into Vivaspin tubes for concentration; spin at 2000-4000rpm for 3-5 min until the final volume is about 500-1000µl; piped up and down while loading new volume on the column to remove virus from the filter walls.

• Take a 5µl aliquot for titration.

• Make 200-250µl aliquots and freeze at -80°C.