

Aug 28, 2024

AAV Purification Protocol with Iodixanol Gradient

DOI

<https://dx.doi.org/10.17504/protocols.io.ewov1n4x2gr2/v1>

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Protocol Citation: Roberta Marongiu 2024. AAV Purification Protocol with Iodixanol Gradient. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.ewov1n4x2gr2/v1>

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Protocol status: Working

We use this protocol and it's working



Created: January 24, 2022

Last Modified: September 23, 2024

Protocol Integer ID: 57369

Keywords: ASAPCRN, aav purification protocol with iodixanol gradient protocol, aav purification protocol, purification, iodixanol gradient protocol, aav

Funders Acknowledgements:

Aligning Science Across Parkinson's
Grant ID: 020608

Abstract

Protocol used in the Kaplitt and Marongiu labs to purify AAVs.

Materials

10x Gradient Buffer (GB)

10 ml Tris (pH 7.6)

30 ml 5M NaCl

10 ml 1 M MgCl₂

50 ml ddH₂O

Filter sterilize using 0.22 (you can use vacuum filter)

Store at 4°C for a few months

qPCR:

5 ul of DNA per well: samples and standards in triplicates, blank one replicate

Standards L 10⁴, L 10⁵, L10⁶, L10⁷

Primers Fw-Rev for WPRE or others that anneal on packaged DNA (10uM each or 5uM mixed primers)

If use 5uM dilution which contains both primers use a volume of 0.4 ul of the mix

Master mix for 20 ul final reaction volume

Set the standards and analyze, dilution 440000

qPCR mix		
		ul
	2x Master Mix sybr green	10
	Forward primer	0.2
	Reverse primer	0.2
	cDNA	5
	Water	4.6
	Final volume	20

Instructions to use the ultracentrifuge:

Bring the Ti70 rotor from cold room

Turn on the centrifuge

Open the door

First place the tubes in the rotor and than place the rotor in the centrifuge

Adjust speed, temperature, rotor, acceleration/deceleration

At the end: vacuum

Shut down, return rotor to the cold room.

 Polyallomer Quick-Seal Centrifuge Tubes 1 × 3.5 in **Beckman Coulter Catalog #342414**

 OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D1556 - 250mL**

 PE tubing **Warner instruments Catalog #PE50 64-0752**

Equipment

Cordless tube topper

NAME

Coulter

BRAND

PN358321

SKU



Transfection

- 1 Plate 293 cells in CellSTACK double chamber in  200 mL media (DMEM with 10% FBS and 1% Penicillin/Streptomycin) so that at the moment of transfection they reach 80% confluence.
- 2 When the cells reach 80% confluence, they are ready to be transfected. While preparing the transfection reagents, change the incubator CO₂ level to 3%.
- 3 Prepare transfection reagents and set aside at  Room temperature : 2x Hepes Buffer Saline (HBS), sterile H₂O and 2.5 M CaCl₂ in water.
- 4 Prepare DNA/CaCl₂ solution: when the cells are approximately 80% confluent, prepare total of 200-400 µg of DNA (1:1:1 molar ratio for pAAV and helper plasmids) in  10 mL CaCl₂ solution in sterile H₂O (CaCl₂ final concentration 0.25M).
- 5 Prepare the transfection mix: Place 1  10 mL of 2x HBS in a 50mL tube. Using a 5 mL pipette, bubble solution from the bottom of the tube while slowly adding the CaCl₂/DNA mix. Bubbles should appear at approximately the same rate at which the drops are released into the solution. The final volume at the end of this step will be  20 mL 1/10 of the volume of the cells plated on the cell stacks. If higher volumes of reagents are needed, the final volume can be adjusted according to this ratio.
- 6 Place a small droplet of the transfection mix on a small plate or slide and view under the microscope. The DNA precipitates should look like small particles and be visible in the center of the drop. If they look like large particles or aggregates, discard the mix and attempt to re-bubble the solution. If unsuccessful, prepare new solutions and repeat.
- 7 Add the transfection mix to  200 mL of fresh 5% DMEM (DMEM with 5% FBS and 1% penicillin/streptomycin).
- 8 Gently remove media from the cell stack and replace it with the  220 mL 5% fresh media/transfection mix. Swirl the stack gently and return it to the incubator. Incubate for  20:00:00 to  24:00:00 ; do not disturb the cells during this time.

1d 20h



- 9 Check the cells: the precipitate from the transfection mix should be visible on the cells and mostly in the areas of the stack with no cells attached. Discard the media and add  50 mL of PBS or DMEM. Gently rock the stack to wash and discard the solution. Replace with  200 mL of fresh DMEM with 5% FBS and incubate at 5% CO₂ for  48:00:00 .

2d

Harvesting

- 10 Pour the media off the cell stack into a 500 mL centrifuge bottle. Separate three  50 mL aliquots of this media in conical tubes. Add 0.5 M EDTA to each of the first two tubes, to a final concentration of 10 mM (1-2 ml).
- 10.1 i. Add the contents of one tube of media/EDTA to the cell stack and rock gently, tapping the sides of the stack to detach the cells. Return this media/EDTA back to the 50 mL conical tube.
- 10.2 ii. Add the contents of the second tube of media/EDTA to the cell stack and rock gently until the cells come off the stack. It should take no longer than  00:05:00 . Return this media/EDTA back to the 50 mL conical tube.
- 10.3 iii. Wash with the third tube of media, without EDTA. If the stack has cells wash with fresh media, HBSS or PBS.
- 10.4 iv. Use the third tube to wash the first two (EDTA) tubes – cells cling to the tubes.
- 10.5 v. Return the contents of all the conical tubes to the 500 mL centrifuge bottle and incubate for about  00:10:00 in an  On ice bucket with water/ice.
- 10.6 vi. Spin down at  4 °C at 500x g for  00:15:00 or 1000 x g for  00:10:00 . If still floating cells are present, spin extra  00:10:00 .
- 10.7 vii. Pour off the media completely and, if necessary, use a pipette to remove any remaining media drops. It is very important that all media be removed.
- 11 Resuspend pellet with  10 mL **1x GB buffer**.

5m

10m

35m



- 12 **Freeze** -80 °C for at least 01:00:00 if purifying on the same day (or until purification day). 1h
- 13 **Thaw in** 42 °C ° water bath.
- 14 **Sonicate:** Wash sonication probe with H2O, then 70% alcohol, then H2O again and wipe with a clean kimwipe. Lyse with a sonication probe: output control – 3, duty cycle % – 30. Sonicate for 6 to 10 beats. The probe should be ~1 cm from the tube bottom.
- 15 **Freeze in** -80 °C for 01:00:00 . Thaw in 42 °C water bath – Freeze/thaw like this 3x. (Can do last freeze O/N, and proceed to benzonase step next day). 1h
- 16 Bring sample to 37 °C , add **CaCl2** (use 4uL of 2.5M stock CaCl2) and then treat with **Benzonase** (2uL in 10mL) at 37 °C for 01:00:00 -swirl every 15 minutes. 1h
- 16.1 **CaCl2** (2.5M for transfection) – 2500x, so use 4LI in 10mL.
- 16.2 **Benzonase** (25 KU stock), need 500 units, so add 2uL to 10 mL.
- 17 Centrifuge at 3000 x g for 00:15:00 at 4 °C . 15m
- 18 Transfer supernatant to new tube, store at 4 °C Overnight (or continue on). 15m
- 19 Prepare optiprep gradients – Mix, store at 4 °C .
- 20 Prepare gradients: **12-13 mL AAV, 6 mL 15%, 8 mL 25%, 8 mL 40%, 5 mL 58%**

	A	B	C	D	E
	To make >100 ml	15% + 1M NaCl	25%	40%	58%
	Optiprep (60%)	40 ml	46.7	64	96.67
	10x GB	16 ml	11.2	9.6	3.33
	5M NaCl	32 ml	-	-	-
	ddH2O	72 ml	54	22.4	-
	Phenol Red (5mg/ul)	-	280 ul	-	240ul
	TOTAL VOL:	160 ml	112 ml	96 ml	100 ml

	A	B	C	D	E
	To make ~25 ml	15% + 1M NaCl	25 %	40 %	58%
	Optiprep (60%)	5 ml	11.68	16	24.17
	10x GB	2 ml	2.8	2.4	.83
	5M NaCl	4 ml	-	-	-
	ddH2O	9 ml	13.5	5.6	-
	Phenol Red (5mg/ul)	-	70 ul	-	60 ul
	TOTAL VOL:	20 ml	28 ml	24 ml	25 ml

- 21 Centrifuge in ti70 rotor at 360,000g (70,000rpm) for 01:10:00 , 18 °C , use acceleration and deceleration protocol #9.

1h 10m



22 Collect virus fraction:

22.1 i. Puncture tube at 58/40 interface with 18G needle attached to 10ml syringe.

22.2 ii. Collect about  2 mL with bevel up and 2 ml with bevel down. AVOID PROTEIN BAND AT 40/25 INTERFACE.

23

Equilibrate the Millipore® membrane in a 15 mL conical tube with  4 mL PBS-Mg: Add solution to the membrane and centrifuge at 2000 x rpm in TC room until all liquid has passed through (about  00:02:00). Check after  00:02:00 if the liquid is going through at same speed in different columns. If there is one there is washing faster discard and take a new one (that means the column could be damaged).

4m

24

Discard the PBS-Mg from bottom of the conical tube and load the virus solution. Centrifuge at the same speed until only  1 mL remains above the filter (about  00:10:00). Add  3 mL PBS-Mg and pipette up and down with a P1000 pipette without touching the membrane. Spin down as before until 1 mL remains. Repeat five times. During the last wash, spin down and leave only 300-500 µL above the membrane in which to recover the virus.

10m

25

Wash down the sides of the membrane by pipetting this volume. Transfer to a 1.5 mL Eppendorf tube. Wash twice with 100-200 µl PBS-Mg while scratching back and forth over the membrane with the pipette tip.

26

Do a quick spin on the bench top centrifuge before sonication to have all the pieces of membrane on the bottom. Sonicate 6 bursts/beats (output control 1.5-2, duty cycle % - 30) to break up viral aggregates. Centrifuge at 5000 x g for  00:10:00 at  4 °C . Transfer supernatant to a new tube.

10m

27

Equilibrate a 0.22µm filter with 500 µl PBS-Mg. Aspirate the volume with a 18 1/2 GA needle

28

Using the same needle and syringe, aspirate the virus.



29 Gently pass the virus through the filter. Collect into a 1.5 mL Eppendorf tube.

Titering by RT-qPCR via standard curve method

1h 45m

30 Prepare DNA probe: 2 μ l virus, 1 μ l 10x DNase 1 buffer with MgCl_2 , 1 μ l DNase 1, and 6 μ l ddH_2O in a PCR tube. Incubate at 37 °C for 00:15:00 to 01:00:00 .

1h 15m

31 Inactivate DNase: Add 2 μ l EDTA (25 mM stock solution) to the tube and incubate at 70 °C for 00:10:00 .

10m

32 Digest capsid: Add 10 μ l 2 M NaOH and incubate at 56 °C for 00:20:00 . Then dilute immediately 1:100 in 10 mM Tris pH 8.0.

20m

33 Standards: prepare standards as described in dedicated protocol.

Preparation of qPCR standards for AAV titering

qPCR standards for AAV titering.doc .