Aug 28, 2024

# AAV Purification Protocol with Iodixanol Gradient

DOI
**dx.doi.org/10.17504/protocols.io.ewov1n4x2gr2/v1**

Roberta Marongiu[1,2]

[1]Department of Neurological Surgery, Weill Cornell Medical College, New York, NY 10065;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA

ASAP Collaborative Rese…

Eileen Ruth Torres
Weill Cornell Medicine



**DOI: dx.doi.org/10.17504/protocols.io.ewov1n4x2gr2/v1**

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



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

**Created:** January 24, 2022

**Last Modified:** August 28, 2024

**Protocol Integer ID:** 57369

**Keywords:** ASAPCRN

**Funders Acknowledgement:**
**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 $MgCl_2$

50 ml ddH2O

Filter sterilize using 0.22 (you can use vacuum filter)

Store at $4^oC$ for a few months

**qPCR:**

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

Standards L $10^4$, L $10^5$, L$10^6$, L$10^7$

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 x 3.5 in **Beckman Coulter Catalog #**342414

OptiPrep™ Density Gradient Medium **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 $CO_2$ level to 3%.

3 Prepare transfection reagents and set aside at Room temperature : 2x Hepes Buffer Saline (HBS), sterile $H_2O$ and 2.5 M $CaCl_2$ in water.

4 Prepare DNA/$CaCl_2$ 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 $CaCl_2$ solution in sterile $H_2O$ ($CaCl_2$ 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 $CaCl_2$/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. 5m

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. 10m

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 . 35m

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**.

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 H20, then 70% alcohol, then H20 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 4Ll 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** | 15% + 1M Na | 25% | 40% | 58% |

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **ml** | Cl | | | |
| | 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 4m

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).

24 10m

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.

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 10m

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.

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.

## Tittering by RT-qPCR via standard curve method 1h 45m

30 1h 15m

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

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

32 Digest capsid: Add 10 µl 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 tittering** qPCR standards for AAV tittering.doc .