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Version 1

AAV Purification by Iodixanol Gradient Ultracentrifugation V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol goes through AAV purification by Iodixanol gradient ultracentrifugation. To see the full abstract and additional resources, visit the [Addgene protocol page](#).

Sample Data



Figure 1: Iodixanol gradient before ultracentrifugation. The steps are clearly distinguishable thanks to the use of phenol red. Image adapted from Zolotukhin, S., et al. "Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield." *Gene therapy* 6.6 (1999): 973-985.

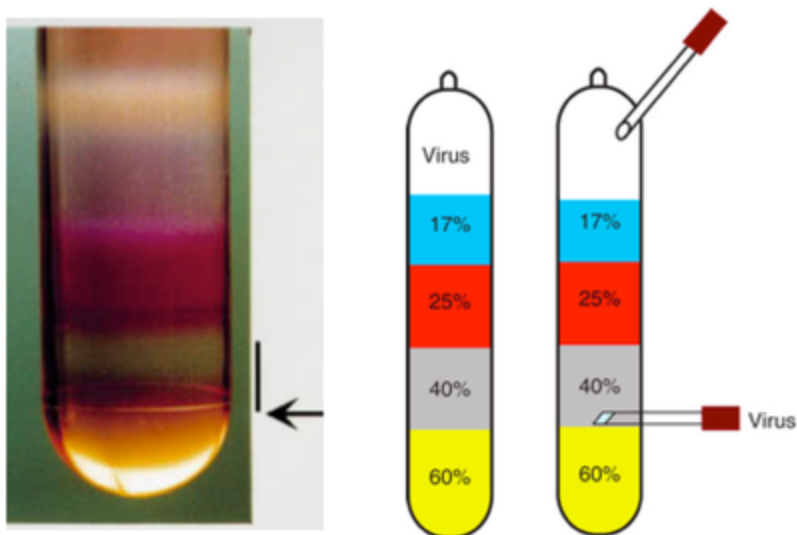


Figure 2:

Left panel: Iodixanol gradient after ultracentrifugation. The arrow indicates the 60-40% interface. The vertical black line indicates the location of the purified AAV. Left image adapted from Zolotukhin, S., et al. "Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield." *Gene therapy* 6.6 (1999): 973-985 .

Right panel: cartoon indicating the position of the needle for harvesting of the purified AAV using option #1. Right image adapted by permission from Macmillan Publishers Ltd: *Nature Protocols* Grieger, Joshua C., Vivian W. Choi, and R. Jude Samulski. "Production and characterization of adeno-associated viral vectors," copyright (2006).

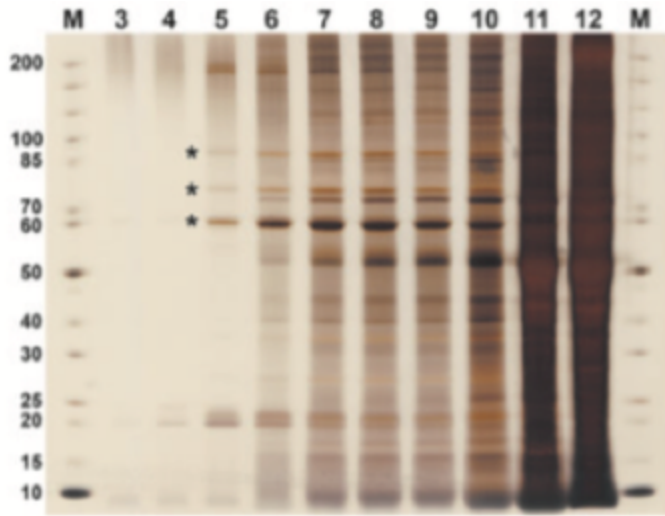


Figure 3: Example of an SDS-PAGE gel of 10 consecutive gradient fractions followed by silver stain. Note the increased number of contaminants in each fraction. * AAV capsid proteins VP1, VP2, VP3; M protein marker. Image adapted from Strobel Benjamin, Miller Felix D., Rist Wolfgang, and Lamla Thorsten. *Human Gene Therapy Methods*. August 2015, 26(4): 147-157. doi:10.1089/hgtb.2015.051.

Guidelines

Workflow Timeline

Day 1: Purify

Day 2: Buffer exchange and concentration

Note

Note, both steps could be completed in one (long) day.



Materials

Equipment

- Ultracentrifuge
- T70i rotor or equivalent

Reagents

- Opti-Prep (60% Iodixanol)
- QuickSeal tubes
- QuickSeal tube spacers
- 16 ga needle
- 18 ga needle
- 10 mL syringe
- 1x PBS pH 7.4
- 1x PBS-MK buffer
- 100X Pluronic-F68
- NaCl
- MgCl_2
- KCl
- Centrifugal filter units (MWCO 100 kDa)

Reagent Preparation

1. 1 M NaCl/PBS-MK buffer

- Dissolve  5.84 g of NaCl,  26.3 mg of MgCl_2 and  14.91 mg of KCl in 1x PBS in a final volume of  100 mL . Sterilize by passing through a 0.22- μm filter and store at  4 °C .

2. 1x PBS-MK buffer

- Dissolve  26.3 mg of MgCl_2 , and  14.91 mg of KCl in 1x PBS in a final volume of  100 mL . Sterilize by passing through a 0.22- μm filter and store at  4 °C .

3. 0.001% Pluronic-F68 (formulation buffer)

- Add  5 μL of 100X Pluronic F-68 to  49.95 mL of PBS. Store at  4 °C for up to one month, or aliquot and store at  -80 °C for up to one year.



Safety warnings

! See SDS (Safety Data Sheet) for safety warnings and hazards.

Procedure

- 1 Preparation and loading of the iodixanol gradient:
 - 15% iodixanol step: mix  4.5 mL of 60% iodixanol and  13.5 mL of 1 M NaCl/PBS-MK buffer
 - 25% iodixanol step: mix  5 mL of 60% iodixanol and  7 mL of 1x PBS-MK buffer and 30 µL of phenol red
 - 40% iodixanol step: mix  6.7 mL of 60% iodixanol and  3.3 mL of 1x PBS-MK buffer
 - 60% iodixanol step: mix  10 mL of 60% iodixanol and  45 µL of phenol red
- 2 Overlay each solution into a QuickSeal tube in the order below using a  10 mL syringe and an  18 g needle, taking care to avoid bubbles (Figure 1 sample data in abstract section).
 1.  5 mL of 60% iodixanol step
 2.  5 mL of 40% iodixanol step
 3.  6 mL of 25% iodixanol step
 4.  8 mL of 15% iodixanol step
- 3 Carefully add up to  5 mL of clarified supernatant on top of the gradient. Use 1x PBS (or cell lysis buffer) to top off the tube.
- 4 Seal the QuickSeal tubes.
- 5 Centrifuge at 350,000 g for  01:30:00 in a T70i rotor at  10 °C .

Note

Pro-Tip

If you need more time, you can alternatively centrifuge for  02:00:00 at 200,000 g at  18 °C .

- 6 Carefully take the QuickSeal tubes out of the rotor and place them in a stable rack. ****Make sure not to disturb the gradient!****

7 Collect Fractions

Option #1

- Prepare a row of roughly 20 open 1.5 mL microcentrifuge tubes in a rack. These will be used to collect fractions from the gradient. You can collect fewer fractions once you have a good idea when the virus elutes from the gradients.
- In a biosafety cabinet, carefully puncture the QuickSeal tube at the interface of the 60% and 40% gradient (see Figure 2, in abstract section) with an 18 g needle.
- Place the first microcentrifuge tube under the needle's opening to collect the fractions.
- Puncture the top of the QuickSeal tube with a 16 ga needle and start collecting  0.5 mL to  1 mL fractions per tube.
- Avoid the proteinaceous material near the 40-25% interface.

Note

Pro-Tip

As soon as the top of the tube is punctured, AAV-containing iodixanol solution will flow from the needle set at the 40-60% interface. Make sure that the microcentrifuge tubes are well positioned to collect the solution or you will lose a significant amount of your virus. When the first fraction is collected, move the rack to the next empty tube to collect the next fraction. The flow will be rapid at first and will progressively slow down.

Option #2

- Puncture the QuickSeal tube at the bottom using an 18 g needle.
- Immediately start collecting  .5 mL to  1 mL fractions in microcentrifuge tubes.
- Repeat for each QuickSeal tube.
- The first  8 mL collected corresponds to the 60% phase and can be discarded.
- The fractions obtained from the 40% phase contain the purified AAV.

Option #3

- Puncture the QuickSeal tube slightly below the 60-40% interface with an 18 g needle attached to a  10 mL syringe.
- The bevel of the needle should be up, facing the 40% step.
- Collect up to  5 mL per tube, taking care to avoid collecting the proteinaceous material at the 40-25% interface.

- Repeat for each QuickSeal tube.
- 8 (Optional) For first time users, it is a good idea to assay each fraction by silver stain to determine purity. Only the cleanest fractions should be kept and pooled for dialysis.
- 9 Pool clean fractions.

10 Concentration and buffer exchange:

Stocks of Pluronic F68:

- Pluronic F68, 10% solution
- A: 0.1% in PBS:  49.5 mL PBS +  500 µL Pluronic F68
- B: 0.01% in PBS:  45 mL PBS +  5 mL A
- C: 0.001% in PBS:  45 mL PBS +  5 mL B + 200 mM NaCl

Procedure:

1. Cover the filter membrane with  15 mL of 0.1% Pluronic F68 PBS and incubate for  00:10:00 at room temperature
2. Take out the 0.1% Pluronic F68 PBS and add  15 mL of 0.01% Pluronic F68 PBS.
3. Spin at 3000 RPM for  00:05:00 at  4 °C
4. Discard the flow through and add  15 mL of 0.001% Pluronic F68 + 200mM NaCl PBS.
5. Spin at RPM rpm for  00:05:00 at  4 °C .
6. Discard the flow through and add your sample.
7. Spin at 3500 RPM for  00:08:00 at  4 °C , discard the flow through.
8. Add more sample and spin 3500 rpm for  00:04:00 at  4 °C , discard the flow through. **Repeat this step as needed.**

Note

- Please note that iodixanol is not easily removed. After each spin, add more formulation buffer and sample and make sure to pipet back and forth a few times to mix the iodixanol that has settled at the bottom of the column.
- We recommend concentrating to a minimum of 500 µL. If the concentrate volume is less than 500 µL, bring up the volume with formulation buffer.

9. Use a P1000 to the bottom of the filter and pipette up/down and wash off the walls of the filter to recover as much virus as possible.



- 11 Store at  4 °C for short term (2 weeks), or aliquot and store at  -80 °C for long term.