Production of Adenoviral Vectors for Application in Test Animals

by A. Untergasser (contact address and download at www.untergasser.de/lab)
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This protocol explains how to produce adenoviral vectors in an easy way. **Adenoviral vectors can be harmful!** Take care of the security issues and read more about it then only this protocol. I don't stress safety issues and basic things in this protocol, I expect that you know how to work with viruses and with tissue culture.

The stocks are sufficient to be used in tissue culture, but if you need to infect test animals they are not clean enough, not enough concentrated, not suitable for injection in any animal and therefore need further purification.

The following method is useful to produce clean and highly concentrated adenoviral vectors which are optimal for injection in animals.

Large Scale Production

Prepare 9 big 175 cm² flasks with 293A cells that they are 80% confluent on the day of infection, preferably a Tuesday.
Every flask is infected with a MOI of 5-10 which corresponds to 1 - 2 x 10^8 IU. This is done best by filling 250 ml of medium in a bottle and adding 1 - 2 x 10^9 IU to it, mix well and exchange the medium in the flasks with this mix. Work as precise as possible, otherwise the cells don't detach after 48 hours.

Harvest the 293A Cells

After 48 - 60 hours beat the cells of the plastic. Use as much force as necessary even if the hand hurts and the plastic cracks. Do not discard the medium.

This cell suspension is spinned down at 2000 RPM for 5 min. Discard the supernatant and keep the pellet. Up to 90% of all the adenoviral vectors are still in the cells. They can be released by repeated freezing and thawing cycles. Unfortunately, freezing and thawing also destroys the vectors, so we only do three cycles to get an optimum of active particles. Therefore we freeze them at -80 °C or in liquid nitrogen and thaw them in a 37 °C waterbath. This we repeat two times (so in total it was done three times).

Spin the suspension for 5 min at 4000 RPM. The adenoviral vectors are now in the suspension. Discard the pellet and transfer the supernatant into a new tube. Spin again for 10 min at 4000 RPM. Discard the pellet and transfer the supernatant into a new tube. If the supernatant is not transparent and clear, spin again for 10 min at 4000 RPM and keep the supernatant.

Purification by Ultra-Centrifugation

Required solutions:
500 ml - 10 mM Tris-Cl at pH 8,0
100 ml - 1.2 CsCl-solution (26.8 g CsCl + 92 ml 10 mM Tris-Cl at pH 8.0)
100 ml - 1.4 CsCl-solution (53.0 g CsCl + 87 ml 10 mM Tris-Cl at pH 8.0)

Required: Ultra-Centrifuge with SW28-rotor or similar.

First centrifugation: Fill two tubes with 8 ml of the 1.4 CsCl-solution. Then add slowly 6 ml of the 1.2 CsCl solution on top. Be careful and keep an eye on what you are doing. The best is to drop it in the middle to avoid turbulences.

Now load carefully the 10 ml of the cleared supernatant of the previous step.
Add carefully ca. 8 ml 10 mM Tris-Cl to fill the tube 4 - 5 mm below the edge.
Last, add 2 ml mineral oil to fill the tube 3 - 4 mm below the edge.

Important: Tubes must be always!! filled 3 - 4 mm below the edge, otherwise they are not stable and collapse during centrifugation. The tubes are filled in the buckets, screwed close and weighted. Buckets opposite of each other can differ maximally 0.1 g! Not all buckets have to be filled, but all have to be hanged in the rotor.

We centrifuge by 100 000 x G (23 000 RPM in SW28) for 1.5 hours without break. For the complete run the centrifuge will take around three hours. Then you should see ca. 1 cm above the bottom of each tube a white-bluish adeno-band. You can suck it out with a syringe piercing trough the wall. Alternatively you can pipet it off from the top. The adeno-solution of both tubes is pooled and diluted at least with a equal volume of 10 mM Tris-Cl. Otherwise it is of too high density to be loaded on the second gradient.

Important: The second band on top contains empty defect particles and should be discarded!
Second centrifugation: Fill two tubes with 8 ml of the 1.4 CsCl-solution. Then add slowly 6 ml of the 1.2 CsCl solution on top. Same as before.

Now load carefully the diluted virus solution of the previous step on only one tube, load the other with an equal amount of 10 mM Tris-Cl.
Add carefully 10 mM Tris-Cl until the tube is filled 4 - 5 mm below the edge.
Last, add 2 ml mineral oil to fill the tube 3 - 4 mm below the edge.

Important: Tubes must be always!! filled 3 - 4 mm below the edge, otherwise they are not stable and collapse during centrifugation. The tubes are filled in the buckets, screwed close and weighted as before.

We centrifuge by 100 000 x G (23 000 RPM in SW28) for 1.5 hours without break. Then you should see ca. 1 cm above the bottom of each tube a white-bluish adeno-band. You can suck it out with a syringe piercing trough the wall. Alternatively you can pipet it off from the top. The adeno-solution of both tubes is pooled and not diluted!

Dialysis of the adenoviral vectors

At the moment the stocks contain lots of CsCl which is very toxic for the animals. It has to be removed by dialysis.

Required solutions:
3 liter - dialysis-buffer (10 mM Tris-Cl at pH 8.0; 2 mM $MgCl_2$; 4 % w/v Sucrose)

First we need to water the dialysis slides in 800 ml dialysis-buffer for 30 sec. Then we inject the adenoviral suspension into the slide. We let it stir in the suspension for one hour, then we replace the buffer for fresh and let stir for 2 hours. Then we replace the buffer a last time and stir for 3 more hours. After in total 6 hours of dialysis the solution is sucked out of the slides, aliquoted and frozen at -80 °C. Then one small aliquot is defrosted and titrated.

This is a clean virus stock. If you need to know the ratio of unfunctional particles to infectious particles you have to measure the adenoviral particles by dotblot or qPCR.

Materials needed:

ViraPower Adenoviral Expression System (# K4930-00 or K4940-00) by Invitrogen
Slide-A-Lyzer 10kDa irradiated 3-12 ml (# 66453) by PIERCE or PERBIO

Known Issues:

- Adenoviruses have a size limit in what they can package into their capsid. A maximum of 10% more than the wildtype genome size fits in, then things get tricky. Take care that your construct is within this size limits.
- The method of purification is robust. Adenovirus will only precipitate if you pool the content of more than 50 big flasks in one single tube. If you stick to the protocol, you are far from that.
- Adenoviral vectors are sensitive to the pH and tend to precipitate. For this reason you should only use the dialysis-buffer as described which can contain up to 10^{13} IU/ml and 0.9% NaCl to dilute and for injection in animals. Do not use PBS, the viruses precipitate at 10^{11} IU/ml and it inactivates some of the particles. It is not suitable!
- Too many freeze/thaw-cycles damage the adenoviral particles. Aliquot you stocks and store them at -80°C. Glycerol is not required.

References and Comments:

This protocol was developed in U. Protzers Laboratory based on the instructions provided with the gateway vectors and the AdEasy protocol. I have done it many times and optimized it along the way. I guess my protocol gives a better overview, but have a look at the provided protocol from gateway for details and instructions were to buy the things. With this protocol I produced adenoviral vectors without any problems.

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