Chapter 2

Construction of Capsid-Modified Adenoviruses by Recombination in Yeast and Purification by Iodixanol-Gradient

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Abstract

Adenovirus represents a valuable tool for the treatment of cancer, but tumor targeting remains a pending issue. Most common procedures to modify adenovirus genome are time-consuming due to the requirement of multiple cloning steps, and the low efficacy of the recombination process. Here, we present a new method for homologous recombination in yeast to fast construct recombinant adenoviruses. Also, an alternative procedure to purify viral stocks, based on iodixanol gradient is described. Compared to classical methods, iodixanol is nontoxic to cells, which avoids desalting to use in vitro and in vivo. Moreover, viral stocks are more viable and it can be used for large-scale purifications. Finally, a protocol for analyzing blood persistence of modified vector in vivo biodistribution is presented.

Key words: Adenovirus, Detargeting, Homologous recombination, Iodixanol, Biodistribution

1. Introduction

Many efforts in virotherapy of cancer with adenoviruses are focused on the delivery of viral particles to tumor cells. However, the hepatotropism of Ad5 following systemic administration limits its efficacy and results in toxicity.

In order to retarget adenovirus to desired cells, it is necessary to abrogate liver transduction (liver detargeting) and to expose heterologous peptides, which are specific for tumor cells (tumor targeting), on the external surface of the virus. The design of optimized vectors with modified tropism for clinical use requires knowledge and understanding of adenovirus biology and in vivo biodistribution.
Based on in vitro data, it was postulated that when virus is administered intravenously, hepatocyte entry was mediated by a direct interaction of Ad capsid with its cellular receptors CAR and integrins. However, the double ablation of CAR and integrin-binding sites failed to reduce hepatocyte transduction in vivo, suggesting the involvement of other interactions for Ad liver entry (1–3). Thereafter, it was suggested that HSPGs played a role in liver transduction through a direct interaction with KKTK motif in the fiber shaft (4), as mutation of this motif resulted in a significant reduction in liver transduction. However, subsequent studies reported that this low transduction was attributed to a defect in postinternalization steps, suggesting a blockade at the trafficking level, i.e., receptor-mediated endocytosis, capsid disassembly, endosomal escape, or nuclear translocation (5).

In 2005, Shayakhmetov et al. reported a novel pathway for liver transduction, showing that blood factors play a major role in targeting Ad to hepatocytes (6). Human coagulation factor IX and C4-binding protein was proposed to bridge the fiber knob to HSPGs and low-density lipoprotein receptor-related protein on the hepatocyte surface. A triple mutation within the fiber knob was shown to inhibit these interactions, reducing virus load in the liver by up to 50-fold, although these modifications did not completely abrogate liver transduction.

In recent years, two independent studies have described a direct interaction between vitamin K-dependent coagulation factors, predominantly factor X, and the trimeric hexon of the Ad capsid, in directing hepatocyte transduction (7, 8). Recently, Alba and collaborators identified factor X binding sites on the adenovirus hexon, and demonstrated that a single point mutation in HVR7 was enough to completely block FX-mediated transduction in vitro and in vivo (9).

In humans, adenovirus interactions with blood cells, mainly erythrocytes and platelets (10, 11), may compromise virus access to tumor cells. Therefore, capsid modifications to avoid these interactions could also increase virus bioavailability.

As long as liver detargeting is achieved, the opportunity for tumor targeting improves. In these terms, numerous strategies have been developed in an attempt to confer a novel tropism for tumor cells. Coating of targeted-polymers to adenovirus surface or the use of bi-adaptor molecules, such as diabodies and fusion proteins, have been successfully incorporated, nongenetically, by chemical conjugation (12–14). However, genetic modifications of the adenovirus genome are required in order to maintain such modifications in the virion progeny. In this sense, genetic incorporation of small peptides within various capsid locations, the use of knobless fibers fused to super-stable antibodies, affibodies or the use of pseudotyped vectors which incorporate fibers derived from other Ad serotypes, have been shown to increase infectivity of target cells (15–17).
This chapter deals with methods to modify capsid proteins, to purify recombinant adenovirus and determine biodistribution in tumor-bearing mice in order to construct truly targeted adenoviruses for cancer treatment. Commonly, Ad is constructed using homologous recombination in bacteria (18). As more versatile strategy, we present the use of yeast to construct the recombinant virus genome. Recombination only requires 40-bp homology tails at the extremes of the insert and a positive-negative selection can be applied. Iodixanol is an alternative method to CsCl to purify Ad. Compared to the commonly used CsCl (not described here), iodixanol is faster and does not require desalting the obtained virus for in vitro and in vivo applications.

2. Materials

2.1. Adenovirus Construction by Homologous Recombination in Yeast

Reagents (when not specified can be purchased at Sigma):

1. Polyethylene glycol 3350 (50%, w/v): Dissolve 50 g of PEG 3350 in 30 ml of H₂O in a 150-ml beaker on a stirring hot plate. Cool down the solution to room temperature; fill volume up to 100 ml, mix thoroughly by inversion and autoclave. Store, securely capped, at RT. Evaporation of water from the solution will increase the concentration of PEG and severely reduce the yield of transformants.

2. 10× AA Solution: 5.7 g Brent supplement mixture-His–Leu–Try–Ura + 0.5 g Leucine (Do not add for LEU-) + 0.2 g tryptophan + 0.1 g histidine + 0.1 g uracil (Do not add for URA-). Add ddH₂O up to 500 ml and autoclave 15 min. Store at 4°C.

3. 1.0 M lithium acetate: Dissolve 5.1 g of lithium acetate dihydrate in 50 ml of H₂O, sterilize by autoclaving, and store at RT.

4. Transformation mix: 240 µl of PEG (50%, w/v) + 36 µl 1.0 M lithium acetate + 10 µl Boiled SS-Carrier DNA (10 mg/ml) + 74 µl [(vector)+(insert)] DNA plus H₂O (Milli-Q, autoclaved).

5. YPDA++ (Yeast extract/peptone/dextrose/adenine rich medium): 5 g yeast extract + 10 g bacto-peptone. Add ddH₂O up to 450 ml and autoclave 20 min. Then, add 50 ml 20% glucose; 20 ml 0.5% adenine (previously filtered through 22 µm filter). For YPDA++ plates, add 15 g bacto agar before autoclave.

6. SC (basic medium): 3.35 g yeast nitrogen base [YNB w/o AA, with ammonium sulfate (Difco)]. Add ddH₂O up to 400 ml and autoclave. Then, add 50 ml of 20% glucose and 50 ml of 10× AA solution (URA- or LEU-, or URA/LEU-). For SC plates, add 15 g of bacto agar before autoclave.
7. SC plates with 5-fluoroorotic acid (5-FOA):

(a) In a beaker, mix: 0.63 g BSM–His–Leu–Try–Ura + 0.04 g uracil (plasmids that grow in FOA plates have lost the URA gene) + 0.02 g Tryp + 0.01 g His + 0.05 g leucine (do not add if the plasmid has CAL) + 0.5 g FOA + 3.5 g [YNB w/o AA, with ammonium sulfate (Difco)] + 10 g glucose/dextrose. Add ddH₂O up to 250 ml. Stir and heat on a stir plate to dissolve. Try to keep temperature below 45°C. It may take a while to dissolve 5-FOA. Filter-sterilize when dissolved and still warm.

(b) Mix: 10 g bacto agar + 250 ml ddH₂O. Autoclave and cool down to 45°C. Add filtered mixture (a) and mix thoroughly in an independent flask. Pour in plates (protect from light).

8. Yeast miniprep mix: 2% Triton-X100, 1% SDS, 0.1 M NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA.

9. 425–600 μm glass beads.

2.2. Purification of Viral Prestocks by Banding on Iodixanol

1. Dulbecco’s modified Eagle’s medium (DMEM) (Gibco/BRL) supplemented with 10 or 5% fetal bovine serum (FBS; Hyclone).

2. HEK-293 cells or other adenovirus packaging cell lines.

3. Tetrachloroethylene (Fisher Scientific).

4. Ultracentrifuge: Beckman Coulter Optima L90K o L100XP and rotor SW40Ti (Beckman Coulter). Polyallomer centrifuge tubes for SW40Ti rotor (Beckman Coulter).

5. Stock solutions:
   - Sol A: 60% iodixanol (if it is not sterile we filter sterilize).
   - Sol B: 10× PBS containing 10 mM MgCl₂ and 25 mM KCl (10× PBS-MK).
   - Sol C: PBS containing 1 mM MgCl₂ and 2.5 mM KCl (PBS-MK).
   - Sol D: 2 M NaCl in PBS-MK.

6. Solutions for iodixanol gradient (prepare from stock solutions):
   - Sol 1 (54% iodixanol in PBS-MK): 13.5 ml Sol A + 1.5 ml sol B.
   - Sol 2 (40% iodixanol in PBS-MK): 4 ml Sol 1 + 1.4 ml Sol C.
   - Sol 3 (25% iodixanol in PBS-MK): 2.5 ml Sol 1 + 2.9 ml Sol C.
   - Sol 4 (15% iodixanol in 1 M NaCl PBS-MK): 1.5 ml Sol 1 + 2.7 ml Sol D + 1.2 ml Sol C.

7. PBS 1× Ca⁺⁺/Mg⁺⁺ (Gibco), Glycerol anhydrous (Fluka).
3. Methods

3.1. Adenovirus Construction by Homologous Recombination in Yeast

Homologous recombination in yeast confers several advantages compared to homologous recombination in bacteria, i.e., the high efficiency of the process and the requirement of shorter regions of homology (40 bp). The adenovirus plasmids should contain three elements for the correct selection and amplification in yeast: a centromere “CEN,” an autonomously replicating sequence “ARS” and a yeast selection gene (URA or LEU that allows the yeast to grow in media without uracil or leucine, respectively). The fragments containing the centromere, ARS, and uracil or leucine, are referred as CAU or CAL, respectively. Such fragment can be amplified by PCR from the plasmid pRS416 for the URA selection and from pRS425 for the LEU selection (Stratagene). The primers to amplify this fragment should contain 15 nt at their 3’ ends corresponding to the beginning and end (complementary sequence) of the CAU or CAL fragment, and 40 nt tails at their 5’ ends that are homologous to the site targeted in the adenovirus plasmid (see Note 1).

Once the CAU/CAL fragment is inserted on the Ad plasmid (CAL is always recommended as it allows positive–negative selection in the future), two different strategies are followed depending on the presence of restriction sites: cut-repair or URA-positive–negative selection (Fig. 1). If one or two restriction sites are available in the pAdCAU or pAdCAL plasmids (vector), it can be linearized and recombined with a DNA sequence (insert) that shares at least 40 nt of homology at both sides of the cut. With the recombination, the plasmid is circularized again and yeast can grow in URA- or LEU-deficient plates.

If no restriction sites are available, URA fragment can be inserted at position to be modified without the need to cut it (URA fragment can be amplified from pRS416 using primers with tails of 40 nt homologous to flanking sequences of position to be modified). A positive selection (incorporation of URA) using uracil and leucine-deficient plates will select recombinant clones. Then, the URA gene is replaced for the desired fragment using a negative selection (loss of URA) in FOA and leucine-deficient plates (see Note 2). Only those plasmids which have lost the URA gene will grow (5-FOA gives a toxic compound in the presence of URA which will not let these yeast grow). Once yeast colonies are obtained, the DNA from yeast plasmid (low copy) is isolated and transformed to bacteria (high copy) in order to analyze it. A similar system has been published by Hokanson et al. (19).

There are different types of inserts: the CAU or CAL to adapt a regular bacterial plasmid to grow in yeast, the URA insert to use positive–negative selection in a plasmid containing LEU in its
backbone, and a regular DNA insert obtained by restriction or PCR to repair (recircularize) a linearized yeast plasmid. The common requirement is that the 5' and 3' ends (a minimum of 40 bp) of the insert are homologous to a region in the receptor vector that will be replaced.

1. Primer design: Oligos to amplify CAL (CAU uses the same oligos, but the template for PCR is pRS416 instead of pRS425): Forward: 40 bp homology upstream of targeted site + ACCT GGGTCCTTTTCATCAC. Reverse: 40 bp homology downstream of targeted site (reverse orientation) + CATCTGTGCG GTATTTCACA.

   Oligos to amplify URA (from pRS416): Forward: 40 bp homology upstream of targeted site + TCAATTCATCATTTT. Reverse: 40 bp homology downstream of targeted site (reverse orientation) + GTAATACTGATATAA. Examples to introduce URA fragment into the fiber, penton base, or hexon in order to introduce detargeting punctual mutations are given.

![Diagram of adenovirus construction by homologous recombination in yeast.](image)

Fig. 1. Adenovirus construction by homologous recombination in yeast. Main steps involved in the generation of recombinant adenoviruses using homologous recombination in yeast. Centromere-autonomously replicating sequences – Leucine gene (CAL) renders a bacterial plasmid competent for yeast growth. The adenovirus genomic plasmid with CAL can be used to insert a DNA of interest either via positive–negative selection with the URA gene (a) or via cut-repair when a unique restriction site is available at the targeted site (b).
Table 1

Primers used for positive URA-selection to construct tropism-modified adenoviruses

<table>
<thead>
<tr>
<th>Protein modified</th>
<th>Phenotype (ablation)</th>
<th>Primer orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber (knob)</td>
<td>CAR</td>
<td>Forward</td>
<td>GAAAATGGGAGTGTCACTAAACAATTCCCTCCTCCTGGACCCCTGAA TCAATTCATCATTTCAGCTGTGCCTTCAGCATGATATTAATTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGGCTGTGCTTCAGTAAATCTCCATTCTTCAAGTTCCA GTAATAACTGATATAA</td>
</tr>
<tr>
<td>Fiber (shaft)</td>
<td>Heparan sulfates</td>
<td>Forward</td>
<td>CCTTACCTCCAAAATGTAACCACCTGTGAGCC CACCTCCTCAATTCATCATTTCAGCTGTGAGGGGTGCAGATATTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TAACTGTGAGGGGTGCAGATATTTTCCAGGTGTATGTTTGA GTAATAACTGATATAA</td>
</tr>
<tr>
<td>Penton base</td>
<td>Integrin</td>
<td>Forward</td>
<td>GGCAATGCAGCCGGTGGAGGACATGAACGATCATGCCCATCAGCTCATTCTCCAGCGCCCGGATGTGGCAAGAGTGTGAATAACTGATATAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TAACTGTGAGGGGTGCAGATATTTTCCAGGTGTATGTTTGA GTAATAACTGATATAA</td>
</tr>
<tr>
<td>Hexon</td>
<td>Coagulation factor X</td>
<td>Forward</td>
<td>TGGATGGGAAAAAGATGCTACAGAATTTCAGATAAAAATTCATCATTCTTCTTCTCTTATGTTTGA GTAATAACTGATATAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TTAGATTGATTTCCATCCATGCAAATTATTTTCCCAA CTCTTATGTAATAACTGATATAA</td>
</tr>
</tbody>
</table>

Example of primers to insert URA at different sites of capsid proteins to ablate the interaction with the Ad receptors. These primers can be used to amplify URA gene by PCR from the template pRS416. Each primer contains a fragment of homology with the extremes of URA gene (15 b-underlined) and a fragment of homology with each site of insertion in the fiber, penton base, or hexon protein (40 b-tail). All sequences are given from 5’ to 3’. After recombination, recombinant colonies that have incorporate URA fragment will be selected in URA- and LEU-deficient plates.

in Table 1. Once URA fragment is inserted, it needs to be replaced by the insert (amplified by PCR or restriction) containing such modifications (Table 2).

In addition to these punctual mutations, larger modifications, such as pseudotyping, affibodies, or knobless fibers, can also be constructed with targeting aims using this strategy.

2. PCR mix: 0.5 μl template DNA (20 ng) (e.g., pRS416); 25 μl Ex-Taq 2x; 1 μl 20 μM oligo F; 1 μl 20 μM oligo R; 22.5 μl Milli-Q-autoclaved H₂O.

3. PCR program (PCR fragment 1 kb): 1 min at 95°C, 30× (30 s at 95°C – 30 s at 55°C – 2 min 30 s at 72°C), 5 min at 72°C.

4. After the PCR, digest the template plasmid adding 10 U of DpnI (only cuts the methylated DNA template, not the PCR product) to the PCR and incubate 2 h at 37°C. Then, use phenol–chloroform extraction and clean with gel purification, ethanol precipitation, and resuspend in ddH₂O.
Protocols for yeast transformation have been adapted from Gietz and Woods (20, 21).

Day 1

1. In a 50-ml Falcon, inoculate 5 ml of YPDA++ (yeast extract, peptone, dextrose, adenine rich medium) with one colony of yeast or 10^7 ml of yeast glycerol stock (strain YPH857).

2. Incubate O/N in a rotary shaker at 30°C.

Day 2

1. Dilute O/N growth 1/10 in YPDA++ to measure OD 600 (use YPDA++ as blank, 1 OD 600 = 1.5 × 10^7 cells/ml). Calculate dilution to prepare 50 ml of prewarmed YPDA++ at OD 600 = 0.15 (0.15 OD 600 = 2.25 × 10^6 cells/ml).

2. Incubate the flask on a rotary shaker at 30°C and until exponential growth is achieved (OD 600 = 0.4–0.9, approximately 5 h).

3. Transfer the 50 ml to Falcon and spin down at 3,000 × g for 5 min, room temperature.

4. Decant supernatant and wash pellet with 25 ml ddH2O. Spin again (3,000 × g, 5 min).

### Table 2

<table>
<thead>
<tr>
<th>Modification</th>
<th>Protein modified</th>
<th>Phenotype (ablation)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y477A</td>
<td>Fiber</td>
<td>CAR</td>
<td>AAATGGAGTGCTACTAAACAATTCCTCTGGACCCTGGAGATCTTACTGAAGCAGCAGCTTA</td>
</tr>
<tr>
<td>GATK</td>
<td>Fiber</td>
<td>Heparan sulfates</td>
<td>CTTTACCTCCCAATGTAAACCCTGAGCCCTTGAGCACTGAGGAGATCTTACTGAAGCAGCAGCTTA</td>
</tr>
<tr>
<td>RAE</td>
<td>Penton base</td>
<td>Integrins</td>
<td>GGCGATGACGGCGGCTGAGCAGCAGCAGCTGAGGAGATCTTACTGAAGCAGCAGCTTA</td>
</tr>
<tr>
<td>E451Q</td>
<td>Hexon</td>
<td>Coagulation Factor X</td>
<td>TGGATGGAGGAGAAGATGCTACAGAATTTCCAGATGATAAATACAGATAAGGATTTTGAGGCAATGGAAA</td>
</tr>
</tbody>
</table>

Once URA is inserted at fiber, penton base, and hexon sites to be modified, it should be replaced with these inserts (obtained by PCR or restriction cut) containing the corresponding mutations using a negative selection. After recombination, recombinant colonies that have lost URA gene will be selected in FOA- and LEU-deficient plates.

3.1.2. Homologous Recombination in Yeast: Preparing Competent Yeast and Cotransformation with Vector and Insert

Once URA is inserted at fiber, penton base, and hexon sites to be modified, it should be replaced with these inserts (obtained by PCR or restriction cut) containing the corresponding mutations using a negative selection. After recombination, recombinant colonies that have lost URA gene will be selected in FOA- and LEU-deficient plates.
5. Decant supernatant and resuspend the cells in 1 ml of ddH$_2$O.

6. Boil salmon sperm or Herring DNA (10 mg/ml) in a boiling water bath (use 10 µl for each transformation) for 5 min and then keep on ice while harvesting the cells.

7. Transfer the yeast suspension to an Eppendorf, centrifuge for 30 s at 6,500 × $g$ in a microcentrifuge, and discard the supernatant.

8. Add ddH$_2$O to a final volume of 1 ml and vortex-mix vigorously to resuspend the cells.

9. Pipette 100 µl samples (10$^8$ cells) into 1.5-ml microcentrifuge tubes, one for each transformation, centrifuge at 6,500 × $g$ at RT for 30 s, and remove the supernatant.

10. Make up the transformation mix: 240 µl PEG (50%, w/v) + 36 µl 1.0 M lithium acetate + 10 µl boiled SS-Carrier DNA (10 mg/ml) + 74 µl plasmid DNA and insert plus H$_2$O (Milli-Q, autoclaved).

11. Add 360 µl of transformation mix to each transformation tube and resuspend the cells by mixing vigorously (vortex).

12. Incubate the tubes in a 42°C water bath for 40 min.

13. Microcentrifuge at 6,500 × $g$ at room temperature for 30 s and remove the supernatant with the micropipette.

14. Pipette 1.0 ml of H$_2$O (Milli-Q, autoclaved) into each tube, stir the pellet with a micropipette tip and vortex vigorously.

15. Plate appropriate dilutions of the cell suspension onto SC-URA or SC-LEU plates. Spread gently (few movements). Use plates without URA or LEU or both according to the presence of URA, LEU, or both genes, respectively, in the vector or the insert.

16. Incubate at 30°C for 2–3 days until yeast colonies appear. There should be more colonies in the plates with (vector) + (insert), than in the (vector) and (insert)-alone controls.

1. Seed 2 ml of liquid SC-URA (or SC-LEU) O/N at 30°C with the desired colony.

2. Transfer 1.5 ml to Eppendorf tube. Centrifuge at maximum speed 5 s in a microcentrifuge. Discard supernatant and resuspend the yeast pellet in residual liquid.

3. Add 400 µl of Yeast Miniprep mix (2% Triton X-100, 1% SDS, 0.1 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and mix.


5. Add 0.3 g glass beads. Close Eppendorf with parafilm.

6. Vortex for 2 min at 4°C.

7. Centrifuge at maximum speed 5 min.
8. Take 300 μl of supernatant and add 600 μl of EtOH + 2% NaAc. Invert and leave at −80 or −20°C to increase DNA precipitation.

9. Centrifuge at maximum speed 20 min at room temperature.

10. Resuspend in 25 μl of H2O or TE (H2O is better when planning to transform this DNA by electroporation).

11. Use 2 μl for electrocompetent bacteria (e.g., DH5α strain) transformation. Next day, pick up colonies and purify the plasmid DNA by classical bacterial Miniprep.

12. Identify positive recombinants and check their genomic structure by a battery of informative restriction enzymes.

An E1-deletion renders adenovirus incapable of replicating by itself, so a permissive cell line like HEK-293, that provide the E1 genes, should be used to generate and amplify recombinant viruses reaching high titers up to 10^12 vp/ml.

When a complete cytopathic effect is observed between 48–72 h after the infection on HEK-293 monolayers, up to 90% of adenoviral vectors remain intracellularly. The classical procedure to release the virus from the cells to the supernatant is based on repeated freezing and thawing cycles (up to three times). However, this procedure may destroy the virions if the sample is frosted and thawed too many times, and it would reduce the titer of the stock. Here, we describe an alternative method as efficacious as freezing and thawing in releasing the virus from the cell, but less time-consuming.

Virus purification is essential in order to remove defective particles, remove cell debris and media components and concentrate the virus to a level suitable for injection.

Classical methods to purify adenovirus are based on density gradient centrifugation with cesium chloride. However, the concentrations used to band the virus are hyperosmotic and the exchange of buffer of virus preparation is required. A new method based on iodixanol overcomes this problem, as is nontoxic to cells and can be administered in vivo ([22]). Furthermore, other advantages as an increase of functional to physical particles ratio and the possibility of a large-scale purification pose this method as the election for virus purification.

3.2. Purification of Viral Prestocks by Banding on Iodixanol

3.2.1. Virus Amplification

1. Amplify the cells. Typically, ten 15-cm-diameter plates of 293 cells yield approximately 4 × 10^12 vp in total (30,000 vp/cell).

2. Infect cells with either 300 vp/cell or do a 1/8–1/10 dilution from a cell extract (usually, cell extracts contain 1 × 10^9 TU/ml and a cell extract from a 15-cm-diameter plate can be used to infect 8–10 plates of 15 cm diameter).
3. Leave the cells in the incubator for 48–72 h. Check the cytopathic effect 48 h postinfection. Make sure to harvest the cells when these are rounded but not completely detached. If you wait too long, most of the virus will be in the supernatant, and this virus is lost during the purification process.

4. Harvest the cells and the medium by pipetting the cells and centrifuge the 50-ml Falcon tubes at $3,000 \times g$ during 5 min.

5. Keep 40 ml of the supernatant and discard the rest by aspiration. Resuspend the cells from each Falcon tube carefully and join the cell suspensions into one Falcon tube. Leave approximately 5 ml of supernatant and transfer the cell suspension to a new 15-ml Falcon tube.

1. Add an equal volume of tetrachloroethylene.

2. Shake vigorously until a single phase is formed. This lysles the cells, releasing the virus.

3. Centrifuge for 20 min at $3,000 \times g$ to separate organic and aqueous phases. Lower layer corresponds to tetrachloroethylene, cell debris remains in the interphase and upper layer corresponds to medium containing the virus (see Note 3).

4. Pipette off upper layer and transfer to a new tube. Store at 4°C.

1. Lay the following amounts of each solution carefully into each Beckman ultracentrifuge tubes to form the step gradient: 0.5 ml Sol 1 + 1.8 ml Sol 2 + 1.8 ml Sol 3 + 1.8 ml Sol 4.

2. Add 6.5 ml of the cell extract onto each tube.

3. Ultracentrifuge at $155,000 \times g$ at 10°C for 1 h. Adenovirus will appear as a bluish white band.

4. Remove and discard the upper portion by suction.

5. Carefully remove the band with a pipette and collect into a 50-ml Falcon tube and place it on ice. Try to take the virus (bluish band) in volume as small as possible to reduce virus dilution (1.5–2.5 ml).

6. Aliquot the virus and store at −80°C (see Note 4).

After capsid modification, the behavior of adenoviruses injected systemically can be altered. To analyze these changes, it is important to distinguish between two terms that are sometimes confusing: biodistribution and transduction. The former, refers to the virus fate once it is administered and depends on anatomical features like fenestration size, and the interaction with blood cells and proteins. On the other hand, transduction includes two additional steps, gene transfer (dependent on the capsid) and expression (dependent on gene promoter).
The decrease in liver transduction due to detargeting mutations sometimes is reflected in a higher viremia.

Methods to evaluate virus biodistribution (e.g., RT-PCR) and transduction (e.g., luciferase expression) are extensively explained elsewhere, so here we focus on a method to determine viremia and blood persistence after systemic administration of adenovirus.

1. Prepare the virus at the desired concentration in 150 μl of PBS at room temperature. For most vectors and mouse strains, the lethal dose 50 is around $5 \times 10^{10} - 10^{11}$ vp/mouse.

2. Load the virus in a 0.5- to 1-ml syringe, insert a 28-G needle, and remove the air from the syringe.

3. Immobilize the animal in a small chamber. For 25–35 g animals, a 50-ml Falcon tube with V-shaped cut next to the cap as a tail exit and a hole at the bottom to allow breathing can be used.

4. Localize the tail veins at the sides of the tail and clean the tail with ethanol. The warmth of a lamp will dilate the vein and would help to better visualize it.

5. Inject the needle with the syringe as horizontal as possible. A site at three-quarters the length of the tail toward the tip is a good point to start because, in case of misinjection, it is possible to use the same vein closer to the animal body. The needle should enter 2–3 mm into the vein.

6. Start the injection slowly. If a resistance is noted or the injection site becomes white, it indicates that the solution is not entering the vein.

7. Upon retraction of the needle, the injection site should bleed if the injection is correct. Apply a pressure for 30 s to stop bleeding.

3.3.2. Determination of Viremia and Blood Persistence

1. At different times, postinjection (1’, 5’, 10’, 15’, 20’, 30’, 60’, 4 h, and 24 h), bleed the animal from the tail, cutting it 0.5 cm from the tip. Control the bleeding by applying light pressure on the tail and collect one drop of blood into a heparinized tube.

2. Centrifuge 5 s to bring the blood to the bottom of the microcentrifuge tube. Keep the tube on ice until all time points have been collected.

3. Spin down the cells briefly and take an aliquot of plasma to assay the virus concentration.

4. Viral particles can be measured by RT-PCR and functional particles by antihexon staining can also be analyzed depending on vector modification.
4. Notes

1. As the only plasmid that can grow in yeast is the recombination product, it is not necessary to open or linearize the adenovirus genomic plasmid for the recombination. However, if a site with a unique enzyme is available, then such a site should be targeted. That is, the 40-bp flanking regions of the CAU or CAL fragments should fall upstream and downstream of this site. Then, linearization of the adenovirus genomic plasmid with this unique enzyme increases the rate of homologous recombination.

2. Alternatively to the negative selection step the URA gene can be flanked by unique restriction sites and cut it after the positive selection to proceed as in a cut-repair protocol.

3. Usually, 20 min of centrifugation is sufficient. Upper layer sometimes remains slightly turbid (centrifugation time can be increased if upper layer is still very turbid).

4. Virus eluted in iodixanol can be frozen at −80°C and preserves the virus well, but iodixanol does not allow quantification of physical titer by OD_{260}. In order to solve this either, the virus can be maintained in iodixanol and calculate the physical titer with another method (quantitative PCR, protein quantification, etc.) or the virus can be passed through sephadex G100 column and eluted in PBS+/+5% glycerol.

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References


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Methods and Protocols
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