A rapid protocol for construction and production of high-capacity adenoviral vectors

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High-capacity adenoviral vectors (HC-AdVs) lacking all viral coding sequences were shown to result in long-term transgene expression and phenotypic correction in small and large animal models. It has been established that HC-AdVs show significantly reduced toxicity profiles compared with early-generation adenoviral vectors. Furthermore, with capsid-modified HC-AdV becoming available, we are just starting to understand the full potential of this vector system. However, for many researchers, the wide-scale use of HC-AdV is hampered by labor-intensive and complex production procedures. Herein, we provide a feasible and detailed protocol for efficient generation of HC-AdV. We introduce an efficient cloning strategy for the generation of recombinant HC-AdV vector genomes. Infection and amplification of the HC-AdV are performed in a spinner culture system. For purification, we routinely apply cesium chloride gradients. Finally, we describe various methods for establishing vector titers. Generation of high-titer HC-AdV can be achieved in 3 weeks.

INTRODUCTION

Adenoviral vectors

Adenoviral vectors are widely used for gene transfer into a broad variety of cell types. To date, the majority of adenovirus-based gene transfer studies in basic research and gene therapy are predicated on first- or second-generation recombinant adenoviral vectors lacking the early adenoviral gene E1 and/or E3, respectively. These vector types are easy to produce, and commercial kits are available for recombinant adenoviruses derived from the human adenovirus serotype 5 (see ref. 1). However, in transduced cells *de novo* adenoviral protein synthesis of early and late adenoviral genes still contained in the vector genome remains problematic. A major drawback of early-generation vectors are the cytotoxic effects induced in the target cell due to the production of toxic viral proteins. In addition, these proteins can result in an antigen-dependent immune response as a result of their association with the major histocompatibility complex class I on the cell surface[2].

For many research applications, adenovirus is simply used as a transfer vehicle for the introduction of foreign DNA into a target cell. To study cellular effects of introduced foreign DNA sequences, it is important to keep undesired side effects at a minimum. Owing to toxic effects of expressed adenoviral proteins, production of HC-AdV devoid of all viral coding sequences is of great interest to the research community.

High-capacity adenoviral vectors for gene delivery

Alternative terms for the HC-AdVs include 'gutless', 'gutted', 'fully-deleted' or 'helper-dependent' adenoviral vectors[3–6]. For this type of vector, the only adenoviral sequences required for genome packaging are noncoding sequences: the inverted terminal repeats (ITRs) at both ends and the packaging signal at the 5′-end. The deletion of viral sequences allows gene transfer of up to 36 kb of foreign DNA. Besides the transgene expression cassette, this viral genome may contain stuffer DNA for stabilization. This stuffer DNA may enhance transcriptional levels of transgenes or it may optimize packaging efficiencies.

Adenovirus is able to transduce a wide variety of different cell types and organs depending on the serotype used [7–10]. At this time, there are 51 known human adenoviral serotypes characterized by a respiratory, intestinal, renal, ocular or intestinal tropism dependent on the subgroup[7]. On the basis of this feature, HC-AdVs were used for therapeutic applications for monogenetic diseases, such as Duchenne muscular dystrophy[11–13], hemophilia A[14–16], glycogen storage disease type Ia (see ref. 17), hypoalphalipoproteinemia[18], hyperbilirubinemia[19], ornithine transcarbamylase deficiency[20], familial hypercholesterolemia[21], hemophilia B[22–24] as well as acquired diseases such as oxygen-induced retinopathy[25] and multiple sclerosis[26]. For these therapeutic applications, insertion of therapeutic transgenes into the HC-AdV genome and administration of these viral vectors was shown to result in long-term transgene expression and phenotypic correction in mice[11,13,16–18,20,21,24,26], rats[19,25], dogs[14,15,22,23] and nonhuman primates[27] without chronic toxicity.

Although long-term transgene expression after adenoviral gene transfer was observed in quiescent cells, vector genome copy numbers and transgene expression decrease in rapidly dividing cells[28]. This phenomenon is a result of the predominantly episomal state of recombinant adenoviral vector genomes. To maintain the transgene expression cassette contained in the adenovirus, genetic elements for persistent transgene expression were introduced into the adenoviral vector.

For somatic integration of the transgene from the adenoviral vector genome into the host genome adenovirus/retrovirus[29], adenovirus/foamy virus[30], adenovirus/Sleeping Beauty transposase[31], adenovirus/phiC31 integrase[32] and adenovirus/adeno-associated virus hybrid vectors[33,34] were created (reviewed in ref. 28). Furthermore, adenovirus was used in concert with components of the episomally maintained Epstein–Barr virus episome[35,36] or for homologous recombination and subsequent somatic integration[37,38].

In addition to *in vivo* applications, HC-AdVs were used to transduce a broad variety of cell types in culture, including neurons[39] and embryonic stem cells[37]. Additionally, capsid-modified HC-AdVs

with the adenovirus serotype 35 fiber were used to transduce cells resistant to many transfection reagents, such as human hematopoietic stem cells[40] and other hematopoietic cell lines[41].

Limitations and potential of high-capacity adenoviral vectors

Although HC-AdV lacks all viral-coding sequences, there are still limitations that need to be overcome. These include toxicity and/or immunogenicity due to the viral capsid particles themselves, and/or an immune response directed against the transgene product itself. Furthermore, the synthetic viral genome may be recognized by components of the innate immune response, such as Toll-like receptors. There is evidence that adenoviral vectors trigger the innate immune response through Toll-like receptor-dependent pathways[42,43]. Moreover, there may be a risk of low-level random integration of recombinant adenoviral vector genomes that may lead to insertional mutagenesis.

To reduce acute toxicity due to the incoming capsid particles themselves, different application methods were tested. It was shown that delivery to the cerebrospinal fluid targeting the brain[26], pseudo-hydrodynamic delivery targeting the liver[27], intramuscular injection to target muscle cells[13] and aerosol delivery using an intratracheal catheter targeting lung epithelial cells[44] make adenoviral vectors less toxic. Moreover, these approaches enabled or enhanced transduction efficiency and specificity to the respective targeted organs, and furthermore, it was shown that coating of the adenoviral capsid with polyethylene glycol makes this type of vector less immunogenic[45].

In addition, novel methodologies for cloning and manipulating adenoviral genomes derived from virtually any serotype are now available. On the basis of bacterial artificial chromosomes containing the adenoviral vector genome and methods for precise genetic manipulation, any gene can be deleted or inserted[46]. Importantly, capsid-modified vectors based on alternative adenoviral serotypes, for which the prevalence in the human population is rather low, may lower the immune response. Moreover, serotype switching will provide a broader tropism, and repetitive *in vivo* administration may be feasible.

In total, due to the broad natural tropism of adenovirus, the high potential of chimeric capsids, the possibility to generate hybrid vectors and the different application methods make this vector system very attractive for basic research, biotechnology and gene therapy.

Systems for production of high-capacity adenoviral vectors

The first system for the production of HC-AdV was described in 1996 by Parks *et al.*[6]. All protocols available at present are still based on this principle. During the production of HC-AdVs, an adenoviral helper virus lacking the early adenoviral genes E1 and E3 provides all adenoviral gene products needed for amplification, generation of viral particles and packaging in *trans*. To minimize unwanted packaging of helper virus genomes, the helper virus contains a packaging signal, which was flanked by loxP sites. During production in an adenoviral producer cell line stably expressing Cre recombinase, loxP sites are recognized and the packaging signal is excised. This leads to unpackagable helper virus genomes and HC-AdV genomes being efficiently packaged. Similar to systems based on Cre/loxP recombination, alternative amplification methodologies were based on Flp recombinase-mediated excision of the packing signal. Flp recombinase systems are available for adherent cells[47,48] and suspension cultures[49]. Furthermore, other HC-AdV systems using helper viruses based on E1/E2a-deleted[50] and E1/E2a/E3-deleted[51] adenoviral vectors were established.

Many researchers are restricted to using first- or second-generation adenoviral vectors because of the complex and sophisticated production procedure of HC-AdVs. Earlier protocols for HC-AdV production required several preamplification steps. Large-scale amplification was usually performed in many tissue-culture plates (up to sixty 150-mm dishes per HC-AdV preparation) using adherent human embryonic kidney cells[23,24,52,53].

Production and amplification of high-capacity adenoviral vectors in a producer cell line grown in suspension

To date, there are only very few laboratories, including ours, using producer cells grown in suspension for production of HC-AdV[29,54,55]. The procedure for HC-AdV production is summarized in a schematic diagram in **Figure 1**.

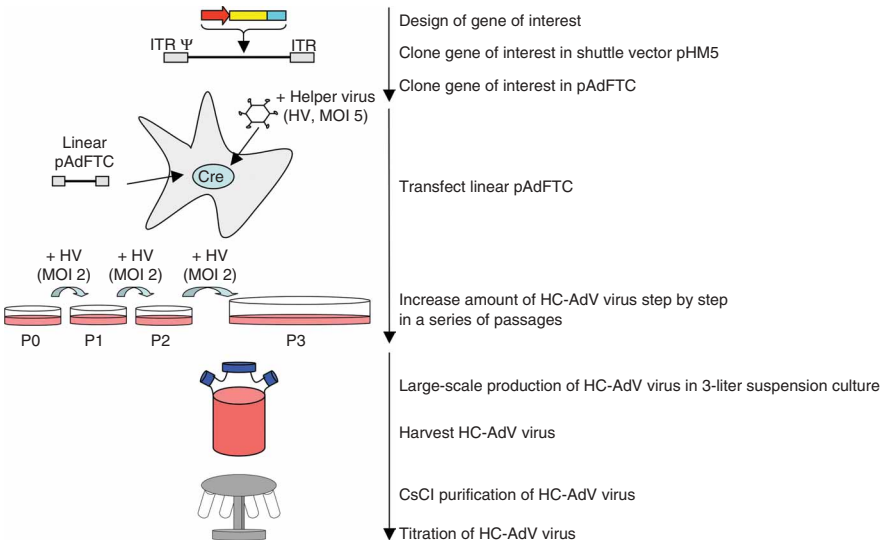

Figure 1 | Schematic diagram for high-capacity adenoviral vector (HC-AdV) production. The flowchart shows on the top the transgene expression cassette, which is inserted into the shuttle vector pHM5 (ref. 59). The red arrow represents the promoter, the yellow bar represents the gene of interest and the blue bar represents the polyadenylation signal. After cloning the complete transgene expression cassette into the HC-AdV production plasmid pAdFTC (see ref. 24) (both orientations work), the linearized construct is transfected into the cells of the HC-AdV producer cell line (116 cells (see ref. 55)), which is subsequently infected with the helper virus AdNG163R-2 (ref. 55). After three serial preamplification steps, large-scale production is performed in a 3-liter suspension culture. For purification, virus is isolated by cesium chloride gradients using ultracentrifugation. HC-AdV, high-capacity adenoviral vector; ITR, adenovirus serotype 5 inverted terminal repeat; Ψ, packaging signal; HV, helper virus; MOI, multiplicity of infection.

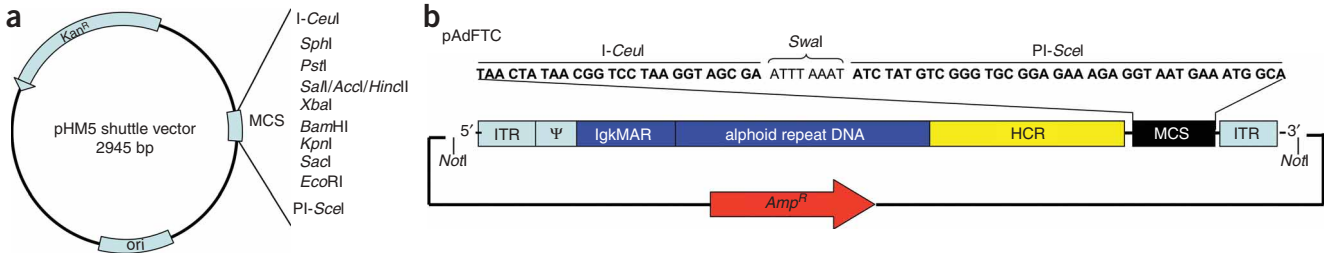

Figure 2 | Schematic maps of pHM5 and pAdFTC. (**a**) pHM5 shuttle vector[59]. All unique cloning sites are indicated. This plasmid is derived from the cloning vector pUC18 (ref. 62) and contains a kanamycin-resistance gene. Construction of pHM5 was described earlier[59]. (**b**) pAdFTC (see ref. 24) is ~31 kb in size. This plasmid contains the human adenovirus serotype 5 inverted terminal repeats (5′- and 3′-ITR), a packaging signal (Ψ), the matrix attachment region of the murine immunoglobulin κ locus (IgkMAR), alphoid repeat DNA sequences from human chromosome 17 and a liver-specific enhancer (hepatic control region, HCR). The MCS includes unique recognition sites for I-*Ceu*I, *Swa*I and PI-*Sce*I. The recognition sequences of these enzymes are indicated. The *Not*I sites are used for release of the linear HC-AdV construct (22 kb in size without insert) from the plasmid backbone (9 kb in size). pAdFTC is based on pDYAL and bears an ampicillin-resistance gene. Further information on pDYAL is provided in a previous publication[68].

In this protocol, the procedure starts with the cloning of HC-AdV production plasmid. Virus is amplified by three subsequent passaging steps and finally amplified using 3-liter suspension culture of 116 cells. HC-AdV particles are released from cells, purified by cesium chloride gradients and subsequently dialyzed. Finally, the number of viral particles is determined by measuring the absorbance at 260 nm. The number of transducing units per ml is determined by infection of adherent cells and subsequent determination of HC-AdV genomes by either quantitative real-time PCR (qPCR) or Southern blot analyses.

Cloning of high-capacity adenoviral vector production plasmids

Various HC-AdV systems for cloning of HC-AdV production plasmids are available. For efficient packaging, the total size of the DNA sequence between the 5′- and 3′-ITR, and the ITRs themselves need to exceed 27.7 kb. HC-AdVs contain optimized stuffer DNA sequences[53] or DNA of the transgene expression cassette exclusively (if the transgene expression cassette is larger than 27 kb). Alternative cloning strategies are also based on rare restriction enzymes[56,57] or homologous recombination using backbone vectors with different mammalian centromeric DNA fragments[58]. For all systems, the HC-AdV production plasmid is linearized by restriction enzyme digest and transfected into the producer cell line. Herein, we describe a simplified and efficient cloning procedure based on rare restriction enzymes for inserting the transgene expression cassette into the HC-AdV genome. The cloning strategy uses the shuttle plasmid pHM5[24,59] and the HC-AdV production plasmid pAdFTC (see ref. 24).

The shuttle plasmid pHM5 was described earlier[59] and contains a multiple cloning site (MCS) flanked by the rare restriction enzyme sites I-*Ceu*I and PI-*Sce*I and a kanamycin resistance gene. A kanamycin resistance may be advantageous for counter selection when cloning into the ampicillin-resistant HC-AdV production plasmid pAdFTC. Alternatively, any other shuttle vector in which the complete transgene expression cassette is flanked by the restriction enzyme sites I-*Ceu*I and PI-*Sce*I can be used. The cloning procedure starts with inserting the complete transgene expression cassette including promoter and polyadenylation signal into the MCS of the shuttle vector pHM5. This step is followed by releasing the transgene from the shuttle vector pHM5 by restriction enzyme digest with I-*Ceu*I and PI-*Sce*I. This DNA fragment is then ligated by the same restriction enzymes into the plasmid pAdFTC. The plasmid pAdFTC contains stuffer DNA derived from a centromere region on human chromosome 17 and a matrix attachment region from the murine immunglobuline κ locus, and the rare restriction enzyme sites I-*Ceu*I and PI-*Sce*I. One may insert up to 12 kb, but not less than 5 kb of foreign DNA into the MCS of pAdFTC. Larger insertions will exceed the optimal range for efficient packaging (maximum packaging capacity: 37.6 kb). Also, smaller insertions resulting in viral genomes less than 27.7 kb may lead to inefficient virus assembly[60,61]. To package larger foreign DNA sequences, one needs to remove stuffer DNA. For smaller inserts, stuffer DNA needs to be added. Each orientation of the insert in pAdFTC is possible. Plasmid maps and the cloning strategy of the present system are depicted in **Figures 2** and **3**.

MATERIALS

REAGENTS

- Albumin fraction V (Roth, cat. no. 8076.2)
- Calf intestinal alkaline phosphatase (CIP) (New England Biolabs, cat. no. M0290)
- Alpha-P32-dCTP (Hartmann Analytic, cat. no. SRP105) **! CAUTION** Radioactive material. When dealing with radioactive materials, appropriate safety precautions and national regulations must be followed.
- Bomix (Bode Chemie Hamburg, cat. no. 973573) **! CAUTION** Corrosive.
- Cesium chloride, CsCl (Invitrogen, cat. no. 8627.2)
- E-Toxa clean (Sigma-Aldrich, cat. no. E9029)
- I-*Ceu*I (New England Biolabs, cat. no. R0694)
- *Hind*III (New England Biolabs, cat. no. R0103)
- *Not*I (New England Biolabs, cat. no. R0189)
- PI-*Sce*I (New England Biolabs, cat. no. R0696)
- *Swa*I (New England Biolabs, cat. no. R0604)
- T4 DNA ligase (New England Biolabs, cat. no. M0202)
- RNase A, 100 mg ml^{-1}; 17,500 U (Qiagen, cat. no. 19101)
- UltraPure phenol:chloroform:isoamyl alcohol (24:24:1 vol/vol) (Invitrogen, cat. no. 15593-031) **! CAUTION** Organic solvent; work under a fume hood, use eye protection and a lab coat when using substances with chloroform. Chloroform can perforate latex gloves, therefore consider double-gloving or nitril gloves for safety.
- Ethanol (EtOH; >99.8%) (Roth, cat. no. 5054.2)
- Trypan blue (Roth, cat. no. CN76.1)
- DH10B electroporation competent cells (Invitrogen, cat. no. 18290-015)
- Superfect transfection reagent (Qiagen, cat. no. 301305)

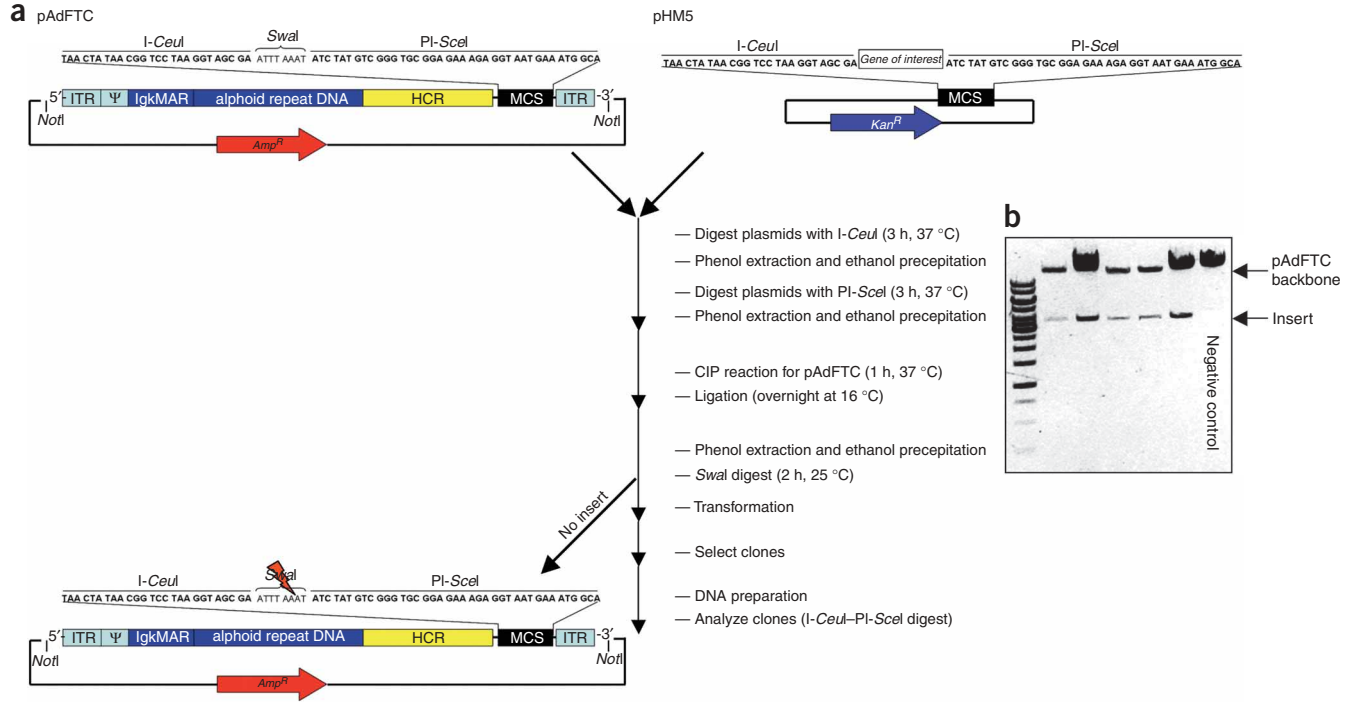

Figure 3 | Flowchart of cloning the transgene expression cassette into pAdFTC. (**a**) Step-by-step cloning procedure. For a detailed description of the single working steps, refer to Steps 1–13. In brief, digest plasmids pAdFTC and pHM5, containing the complete transgene expression cassette, with the restriction enzyme I-*Ceu*I and purify DNA by phenol–chloroform extraction and ethanol precipitation. After that, digest purified, I-*Ceu*I cutted vectors overnight with the restriction enzyme PI-*Sce*I. The digested pHM5 is loaded on a preparative agarose gel to purify the gene of interest (see also Step 6), whereas the pAdFTC is purified as described in Step 7. Set up ligation with CIP-treated pAdFTC and the transgene expression cassette overnight (see Step 8). The ligation mixture is then purified by phenol–chloroform extraction and ethanol precipitation. The *Swa*I digest of the ligation product (Step 10) is performed to prevent the growth of pAdFTC plasmids without insert. (**b**) Example analysis of clones on a 1% agarose gel. Following this cloning procedure, up to 90% of the clones are positive.

- Hygromycin B, 20 mg ml^{-1}, liquid (PAA, cat. no. P02-015) **! CAUTION** Hazardous to humans and the environment. Wear a labcoat and gloves when handling this harmful agent.
- L-Glutamine, 200 mM, liquid (PAA, cat. no. M11-004)
- Fetal bovine serum (FBS) **▲ CRITICAL** Testing of different FBS batches should be performed for optimal cell growth (e.g., Sigma, cat. no. F9665, batch no. 085K3395)
- Minimal essential medium (MEM) (PAA, cat. no. E15-825)
- EX-CELL 293 serum-free medium for HEK 293 cells (Sigma, cat. no. 14571C-500ML)
- Dulbecco's minimal essential medium (PAA, cat. no. E15-843)
- OPTI-MEM I reduced serum medium (Invitrogen, cat. no. 31985)
- Trypsin-EDTA, 0.05% (wt/vol) (PAA, cat. no. L11-004)
- Trypan blue (Roth, cat. no. CN76.1)
- Dulbecco's phosphate buffer saline (DPBS) (PAA, cat. no. H15-002)
- Producer cell line 116, which is based on the human embryonic kidney cell line 293 stably expressing Cre recombinase[55]. This cell line is obtainable from Philip Ng (Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA)
- Human embryonic kidney cells, 293 cells (American Tissue Culture Collection, ATCC Cell Biology Collection)
- Helper virus AdNG163R-2 (see ref. 55). The helper virus AdNG163R-2 can be obtained from Philip Ng
- Hybond-XL Membrane (GE Healthcare, cat. no. RPN 303S)
- UltraPure glycerol (Invitrogen, cat. no. 15514-029)
- pAdFTC (see ref. 24) (plasmid obtainable from Anja Ehrhardt, Max von Pettenkofer-Institute or Mark A. Kay, Stanford University)
- pHM5 (see refs. 24,59) (plasmid obtainable from Anja Ehrhardt, Max von Pettenkofer-Institute, Mark A. Kay, Stanford University or Hiroyuki Mizuguchi, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences)
- Proteinase K (Qiagen, cat. no. 19133)
- PrimeIt II Random labeling kit (Stratagene, cat. no. 300385)
- Qiaquick nucleotide removal kit (Qiagen, cat. no. 28304)
- TaqMan universal PCR master mix (Applied Biosystems, cat. no. 4304437)
- LightCycler FastStart DNA Master^{plus} SYBR Green I (Roche, cat. no. 03515885001)
- Taq DNA Polymersae (New England Biolabs, cat. no. M0320)

EQUIPMENT

- 250-ml storage bottle, sterile (Corning, cat. no. CORN430281)
- 500-ml centrifuge tubes, sterile (Corning, cat. no. CORN431123)
- Tissue culture dishes, 60-mm and 150-mm in diameter (Falcon, cat. nos. FALC353004 and FALC353025)
- Sterile conical tubes, 15 and 50 ml (Falcon, cat. nos. FALC352070 and FALC352096)
- Dialysis tubing: Spectra/Por Membrane, MWCO: 50,000; flat width: 28 mm; diameter: 18 mm; volume/length: 2.5 ml cm^{-1}; length: 5 m or 16 feet (Spectrum Laboratories Inc./VWR)
- Dialysis closure: Spectra/Por Closures, 35 mm (Spectrum Laboratories Inc., cat. no. 132736)
- Slide-A-Lyzer dialysis cassettes (Pierce, cat. no. 66381)
- Clinical centrifuge (Hettich, Rotanta 460)
- 500-ml bucket rotor 46/46 R, 460/460 R (Hettich, art. no. HETT5624)
- Spinner flask, 3,000 ml (Bellco, cat. no. 1965-61030)
- One or five position magnetic stirrer, Bell-ennium (Bellco, cat. no. 7785-D2015)
- SW41 ultracentrifuge rotor (Beckman Coulter)
- Ultracentrifuge (Beckman Coulter)
- Ultra Clear Ultracentrifuge tubes (Beckman Coulter, cat. no. 344059)
- DNA gel apparatus and power supplies
- 37 °C bacteria incubator
- 37 °C, 5% CO_2 humidified tissue culture incubator
- 37 °C orbital shaker
- Electroporator (Bio-Rad) or similar apparatus

- Spectrophotometer
- Taqman 7500 fast real-time PCR system (Applied Biosystems)
- Light-Cycler 2.0 (Roche)
- UV Stratalinker 1800 (Stratagene)
- Phosphoimager FLA-3000 (Fujifilm)

REAGENT SETUP

Cell-culture conditions for 116 cells Growth medium: MEM (high glucose), 2 mM glutamine, 10% FBS, 40 μg ml^{-1} hygromycin B[55]. ▲ CRITICAL General culture conditions are 37 °C in a humidified atmosphere (5% CO_2). For 60- and 150-mm cell culture plates, we use 5 and 25 ml of growth medium, respectively. We observed that without antibiotics, 116 cells show better growth in tissue culture plates and in suspension in spinner culture flasks. In addition, we tested FBS from various companies and found that FBS from Sigma results in best growth of 116 cells. We routinely split these cells 1:2, 1:3, 1:4 or 1:5. It is important not to overgrow 116 cells, and we usually split at a confluence of 95%. After receiving this cell line, passage until passage 3 or 4 before long-term storage in a liquid nitrogen tank. When starting adenovirus production, these 'low passage' cells are used for transfection.

Amplification of the helper virus AdNG163R-2 Before starting the HC-AdV virus amplification protocol (Step 24), the helper virus needs to be produced[55]. This methodology is described in detail in **Box 1**.

Biosafety and containment guidance All cloning steps and cell culture work involved in helper virus and HC-AdV production and subsequent purification should be performed in accordance with BL2 guidelines.

Buffers

- Denaturation buffer: 1.5 M NaCl, 0.5 M NaOH (can be stored at room temperature (15–26 °C) for several months).
- Neutralization buffer: 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl (can be stored at room temperature for several months).
- 2× SSC: 3 M NaCl, 0.3 M sodium citrate (pH 7.0) (can be stored at room temperature for several months).
- Phosphate buffer: 1 M Na_2HPO_4, 1 M NaH_2PO_4 (pH 7.5) (can be stored at room temperature for several months).
- Church buffer: 1% BSA, 1 mM EDTA (pH 8.0), 0.5 M phosphate buffer, 7% sodium dodecyl sulfate (SDS) (prepare freshly from respective stock solutions and BSA powder).
- Proteinase K–SDS solution: proteinase K (0.5 mg ml^{-1}), 10 mM Tris-HCl (pH 7.5), 0.5% SDS, 10 mM EDTA (pH 8.0) (buffer without proteinase K can be stored at room temperature for several months. Proteinase K should be freshly added).
- HEPES-buffered saline: 140 mM NaCl, 1.5 M Na_2HPO_4, 40 mM HEPES (can be stored at 4 °C in a dry and ventilated place for several months).
- Cesium chloride solutions: these solutions are based on the following formula: 137.48–138.11/CsCl density = g CsCl + (100 − (×g CsCl)) ml H_2O. Three cesium chloride solutions are required, which are prepared using the following recipe:
  - 1.5 g cm^{-3} : 45.41 g CsCl + 54.50 ml H_2O
  - 1.35 g cm^{-3} : 35.18 g CsCl + 64.22 ml H_2O
  - 1.25 g cm^{-3} : 26.99 g CsCl + 73.01 ml H_2O
  - All three solutions should be sterile-filtered. Cesium chloride solutions can be stored for several months at 4 °C.
- Lysis buffer: 10 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 0.5% SDS (can be stored at room temperature for several months).
- Tris–EDTA (TE) buffer: 10 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0). Can be stored at room temperature for several months.

EQUIPMENT SETUP

Preparation and handling of glassware We use Bomix diluted in water to disinfect glassware. Rinse spinner flask with Bomix solution and incubate for 20 min until all the adenovirus from the previous preparation is destroyed. Rinse five times with water to remove Bomix residues. This step is followed by adding 2 liters of E-Toxa clean solution (0.5% diluted in water). Incubate for 20 min to destroy all endotoxins. Rinse spinner flask five times with water. Treat dialysis bottle by performing the same procedure. Finally, glassware needs to be autoclaved. ▲ CRITICAL Bleach can also be used to destroy remaining virus in the spinner flask. Other reagents may result in inefficient cell growth.

PROCEDURE

Cloning of HC-AdV constructs based on pAdFTC ● TIMING ~2 weeks

1| Clone the complete transgene expression cassette including promoter and polyadenylation signal into the shuttle vector pHM5 (ref. 59) (see molecular cloning manuals[62] for details on cloning techniques and protocols and **Fig. 2a**). Purify plasmid using standard kits for plasmid midi preparations according to the manufacturer's instructions or other appropriate protocols. Amplify the pHM5 plasmids with insert on kanamycin-containing LB plates or in LB medium with kanamycin.

2| Prepare a plasmid DNA mini preparation of pAdFTC (**Fig. 2b**) using alkaline lysis including phenol–chloroform extraction followed by ethanol precipitation[62] (**Box 2**).

▲ CRITICAL STEP Never vortex or vigorously pipette the large adenoviral plasmid pAdFTC. For short-term storage, keep large plasmids at 4 °C. Repeated freeze–thaw cycles should be avoided. For the cloning procedure, avoid any column purification for the plasmid pAdFTC. This will result in fragmentation of DNA and subsequently to false clones.

3| Digest 10 μg of the vector pAdFTC from Step 2 and 15 μg of the shuttle vector pHM5 containing the gene of interest from Step 1 with the restriction enzyme I-*Ceu*I (10 U) for 3 h at 37 °C. Set up digests in a total volume of 50 μl.

4| Perform a phenol–chloroform extraction and ethanol precipitation (**Box 2**).

5| Digest I-*Ceu*I-digested pAdFTC and pHM5-based shuttle vectors from Step 4 for at least 3 h or overnight with the restriction enzyme PI-*Sce*I (10 U) at 37 °C in a total volume of 50 μl. Subsequently, perform a second phenol–chloroform extraction followed by ethanol precipitation (**Box 2**). Dissolve DNA in an appropriate volume of dH_2O (~25 μl).

▲ CRITICAL STEP I-*Ceu*I and PI-*Sce*I change the mobility of DNA on an agarose gel, and therefore, a phenol–chloroform extraction and ethanol precipitation (**Box 2**) is required before running an agarose gel.

6| Separate DNA fragments of digested pHM5 plasmid containing your gene of interest (from Step 5) on a preparative agarose gel[62] and gel-purify[62] the DNA fragment to be inserted into pAdFTC (length of linearized backbone DNA fragment of pHM5 is 2.8 kb).

7| Dephosphorylate digested pAdFTC (from Step 5) with CIP (10 U) for 1 h at 37 °C in a total volume of 25 μl. Subsequently, purify DNA by phenol–chloroform extraction and ethanol precipitation (**Box 2**).

BOX 1 | AMPLIFICATION AND TITRATION OF THE HELPER VIRUS AdNG163R-2 ● TIMING ~3 WEEKS

Preamplification of the helper virus AdNG163R-2

1. Use human embryonic kidney cells (293 cells) for amplification of the helper virus AdNG163R-2 (see ref. 55). Three serial passages using 60-mm (one in total), 100-mm (two in total) and 150-mm (six in total) tissue culture dishes are required for preamplification. Start preamplification procedure with infection of a confluent (3×10^6 cells) 60-mm tissue culture dish either with purified AdNG163R-2 helper virus (5 TU per cell) or, if you received virus lysate, with 500 µl of lysate, which was treated by three freeze–thaw cycles in liquid nitrogen and a 37 °C water bath.
2. Forty-eight hours after infection, a cytopathic effect (CPE) should be observed because of helper virus replication (cells are rounded up and loosely or completely detached from the tissue culture dish).

▲ CRITICAL STEP For an effective amplification and high viral titers, CPE should be completed by 48 h. If it takes longer than 2 d for complete CPE, proceed with Step 3 of this box. After that, take complete cell–virus suspension and repeat Steps 1 and 2 described in this box.

3. Harvest cells and supernatant (= passage P1) by flushing off infected cells with the medium covering the cells from the surface of the tissue culture dish using a pipette.

■ PAUSE POINT Cell–virus suspension can be stored at −80 °C for several days before continuation of the amplification procedure.

4. For the second passage, infect 293 cells of two confluent 100-mm tissue culture dishes (9×10^6 cells per dish) with one half of the helper virus-containing cell–virus suspension, respectively (~1.5 ml; produced in Step 3 of this box). Forty-eight hours after infection, a cytopathic effect (CPE) should be observed because of helper virus replication (cells are rounded up and loosely or completely detached from the tissue culture dish). Harvest cells and supernatant (= passage 2) by flushing off infected cells as described in Step 3 described in this box.

▲ CRITICAL STEP For an effective amplification and high viral titer, CPE should be completed after 48 h. If it takes longer than 2 d for complete CPE, take complete cell–virus suspension from Step 4 and repeat Steps 1 and 2 of this box.

■ PAUSE POINT Cell–virus suspension (~20 ml) can be stored at −80 °C for several days before continuation of the amplification procedure.

5. For the next step, six confluent 150-mm tissue culture dishes (2×10^7 cells per dish) are needed. Make sure you further maintain 293 cells because 30 confluent 150-mm tissue culture dishes (2×10^7 cells per dish) are required for large-scale amplification (see below).
6. When cells grown in 150-mm tissue culture dishes reach 90–95% confluence, release viral particles from cells obtained in Step 4 described in this box (~20 ml) by freezing (liquid nitrogen) and thawing (37 °C water bath) four times. For the third passage, each of the six confluent 150-mm tissue culture dishes (2×10^7 cells per dish) should be infected with 3 ml of the helper virus-containing lysate.
7. Harvest cells and supernatant in three 50-ml conical tubes (~120 ml) after 48 h as described in Step 3 within this box.

▲ CRITICAL STEP To evaluate whether the helper virus preamplification was successful, a quantitative real-time PCR (qPCR) specific for helper virus can be performed (see Step 73B(i-ix)).

■ PAUSE POINT Cell–virus suspension (~120 ml) can be stored at −80 °C for several days before continuation of the procedure.

Large-scale amplification and purification of helper virus AdNG163R-2

8. Prepare 30 confluent 150-mm tissue culture dishes (2×10^7 cells per dish) with 90–95% confluence. Release viral particles from cell–virus suspension (~120 ml, obtained in Step 7 of this box) by freezing in liquid nitrogen and thawing in a 37 °C water bath three times. Each of the 30 confluent 150-mm dishes should be infected with 4 ml of the helper virus-containing lysate.
9. Harvest cells and supernatant in two 500-ml centrifuge tubes (~600 ml in total) after 48 h as described in Step 3 (see above).
10. Centrifuge for 10 min at 890*g* with a clinical centrifuge.
11. Remove and discard the remaining medium.
12. Resuspend pellets in 28 ml of DPBS by pipetting up and down 8–10 times to get a single-cell suspension.
13. Freeze resuspended virus in liquid nitrogen for 5 min. Make sure that the suspension is completely frozen. Store at −80 °C until starting purification with caesium chloride gradients and dialysis (Steps 57–72). Virus–cell suspension from one spinner culture flasks will be split on six tubes of one SW41 rotor (see Step 63).
14. For purification of the helper virus AdNG163R-2, perform CsCl gradients as described in detail in Steps 57–72.

Titration of the helper virus AdNG163R-2

15. We first determine the physical titer of the helper virus AdNG163R-2 (for details, see Step 73A).
16. To calculate the optical particle units (OPU), use the following formula:

$$\text{OPU ml}^{-1} = (\text{absorbance at 260 nm}) \times (\text{dilution factor}) \times (1.1 \times 10^{12})$$

The OPU ml^{-1} equals the physical titer, which is expressed in viral particles per ml (vp ml^{-1}). It usually ranges between 5×10^{11} and 2×10^{12} vp ml^{-1}.
17. In addition, perform a plaque-forming assay to determine the transducing units of the final vector preparation. Therefore, serially dilute the final vector preparation (1:10 dilutions) and infect 293 human embryonic kidney cells in six-well plates at a confluence of 90%.
18. Three hours later, we perform an agarose overlay and count plaque-forming units after 7–10 d as described earlier[65]. The final titer usually ranges between 4×10^7 and 8×10^7 TU μl^{-1}. The total volume is approximately 1.0–1.5 ml. Alternatively, other methods described in Step 73 can be applied.

8| Perform an analytic gel electrophoresis[62] to check whether the digests of dephosporylated pAdFTC plasmid (from Step 7) and the pHM5 shuttle vector (from Step 6) are completed and the sizes of the expected fragments are correct; digested pAdFTC runs at 31 kb and digested pHM5 backbone runs at 2.8 kb. The size of the second band excised from the pHM5 shuttle vector depends on the length of the transgene expression cassette.

BOX 2 | PHENOL–CHLOROFORM EXTRACTION OF RESTRICTION ENZYME DIGESTS FOLLOWED BY ETHANOL PRECIPITATION ● TIMING 30 MIN

1. Add dH_2O to the DNA solution up to a total volume of 100 µl.
2. Add 100 µl of phenol:chloroform:isoamyl alcohol and mix gently by inverting several times.
3. After centrifugation for 2 min at high speed (15,000*g*) in a microcentrifuge, transfer supernatant to a new tube.
4. Add 20 µl of sodium acetate (pH 5, 3 M) to supernatant and 400 µl of EtOH (>99.8%; stored at −20 °C). Mix suspension vigorously.
5. To pellet DNA, centrifuge samples for 10 min at high speed (15,000*g*) in a microcentrifuge.
6. After removal of the supernatant, add 300 µl of 70% EtOH and centrifuge for 2 min at high speed (15,000*g*).
7. Remove supernatant and air-dry DNA pellet.

▲ CRITICAL STEP Do not dry DNA pellet for a long time, as it will be harder to get DNA in solution.

8. Dissolve pellet in 10–20 µl of dH_2O.

9| Set up ligation[62] (we ligate in a total volume of 20 µl with 2- to 6-µl vector, 8- to 12-µl insert and 400 U of T4 DNA ligase). Ligate at 16 °C overnight. A schematic overview of the cloning procedure is provided in **Figure 3a**.

10| Perform a phenol–chloroform extraction (**Box 2**) and digest the ligation reaction with the restriction enzyme *Swa*I (10 U) for 2 h at 25 °C in a total volume of 20 µl. To concentrate and purify ligated DNA, perform a second phenol–chloroform extraction (**Box 2**).

11| Transform 1 µl of the *Swa*I digested ligation product by electroporation[62] into 40 µl of DH10B electrocompetent cells and spread transformation mix on ampicillin-containing LB plates (50 µg ml^{-1} ampicillin). Incubate plates for 16–24 h at 37 °C.
? TROUBLESHOOTING

12| Select 5–10 clones and prepare plasmid DNA mini preparations as described in Step 2. Digest plasmids with I-*Ceu*I and PI-*Sce*I as described in Steps 3–5. Check the size of DNA fragments by gel electrophoresis as described in Step 8. A representative gel is shown in **Figure 3b**.
? TROUBLESHOOTING

13| Amplify bacterial culture consisting of cells with the correct clone in ampicillin-containing LB (50 µg ml^{-1} ampicillin) and perform a midi-/maxi-plasmid preparation. At this point, you may use commercially available columns for plasmid purification according to the manufacturer's instructions.

■ PAUSE POINT Plasmid DNA of the HC-AdV can be stored at −20 °C for several months.

Linearize the HC-AdV production plasmid by restriction enzyme digest ● TIMING ~5 h

14| Digest 20 µg of the pAdFTC-based adenoviral production plasmid (from Step 13) in a total volume of 100 µl using the restriction enzyme *Not*I (20 U) at 37 °C for 2 h.

15| Add 90 µl of phenol:chloroform:isoamyl alcohol (24:24:1 vol/vol) and mix by inverting the tube several times.

16| Centrifuge for 2 min at high speed (15,000*g*) in a bench-top centrifuge at room temperature.

17| Carefully collect supernatant by pipetting and transfer into a new tube.

18| Repeat phenol–chloroform extraction (Steps 15–17).

19| Add 50 µl of sodium acetate (pH 5.0; 3 M) and 1 ml of precooled EtOH (>99.8% stored at −20 °C). Mix by inverting the tube several times.

20| Centrifuge samples for 10 min at high speed (15,000*g*) at room temperature. Carefully remove the supernatant by pipetting.

21| Add 500 µl of 70% EtOH to the DNA pellet obtained, invert the tube three times and centrifuge for 2 min at high speed (15,000*g*) at room temperature.

22| Remove the supernatant by pipetting and briefly air-dry the DNA pellet. Resuspend pellet in ~50 µl of TE buffer.

▲ CRITICAL STEP If you air-dry the DNA pellet too long, it will be difficult to resuspend the linearized DNA fragments.

■ PAUSE POINT Linearized DNA can be stored for several days at 4 °C or at −20 °C for several weeks.

23| Check digest by gel electrophoresis as described in Step 8. You should detect a 9-kb fragment for the plasmid backbone and, depending on the size of your transgene expression cassette, a second DNA fragment (size: 28–36 kb). Before proceeding with the protocol, make sure that you have amplified the helper virus AdNG163R-2 (see also **Box 1**).
? TROUBLESHOOTING

Transfection of 116 producer cells with the linearized HC-AdV DNA construct ● TIMING ~2 d

24| The day before transfection, seed low passage (<passage 10) 116 cells (0.8×10^6) into a 60-mm tissue culture dish. Cells should reach 50–80% confluence the next day.

25| At this point, linearized HC-AdV DNA from Step 22 can be transfected into 116 cells using Superfect transfection (option A) or calcium phosphate transfection (option B). Transfection efficiencies for large linear constructs are comparable for both transfection methods. However, reagents for calcium phosphate transfections are cheaper.

(A) Superfect transfection

(i) Mix 5 µg of *Not*I-digested DNA (from Step 22) with OPTI–MEM to a total volume of 150 µl.

(ii) Add 30 µl of Superfect reagent and mix by pipetting up and down three times.

(iii) Incubate for 5–10 min at room temperature.

(iv) Add 1 ml of 116-cell medium (MEM, 10% FBS) and mix by pipetting up and down three times.

(v) Remove the media from the 60-mm tissue culture dish (from Step 24) and immediately add the complete DNA mixture (from Step 25A(iv)).

▲ **CRITICAL STEP** Transfection using Superfect should be carried out according to the manufacturer's instructions. However, leave out the washing step with PBS because the experience shows that 116 cells change morphology and are only loosely attached to the tissue culture dish if too many media changes are performed.

(vi) Incubate for 2–3 h at standard conditions in the tissue culture incubator.

(vii) Remove the media containing the Superfect–DNA complexes and replace it with prewarmed (37 °C) complete MEM (10% FBS).

(B) Calcium phosphate transfection

(i) Prewarm 2 M $CaCl_2$ and 2× HEPES-buffered saline to room temperature and mix thoroughly. For each transfection, prepare two sterile tubes.

(ii) Mix 12 µg of *Not*I-digested DNA (from Step 22) and 37 µl of 2 M $CaCl_2$ and add sterile water to a final volume of 300 µl into tube 1. Transfer 300 µl of 2× HEPES-buffered saline into tube 2.

▲ **CRITICAL STEP** The pH of the 2× HEPES-buffered saline is very critical and should be at 7.05.

(iii) Slowly add the DNA solution from tube 1 dropwise to the HEPES-buffered saline in tube 2 while vortexing.

(iv) Incubate the combined solution at room temperature for 30 min.

(v) Vortex, then immediately add the solution dropwise to the cells (from Step 24).

(vi) Swirl the plate briefly for distribution of DNA complexes and incubate at 37 °C and 5% CO_2 until infection with helper virus (see Step 26).

(vii) Change the media (10% FBS) after 6 h.

? TROUBLESHOOTING

Infection with helper virus ● TIMING 1 h + 2 d

26| At 16–18 h post-transfection, infect cells with the helper virus AdNG163R-2 (see also **Box 1**) applying 5 transducing units (TU) per cell: carefully remove the medium and add fresh medium (MEM, 5% FBS) with the appropriate amount of helper virus (a 60-mm tissue culture dish at a confluence of 80–100% contains ~3.2×10^6 cells, and therefore one needs to add ~1.6×10^7 TU of the helper virus).

27| Make sure that the virus is equally distributed on the tissue culture dish by gently moving the dishes every 20 min during the first hour after infection.

28| Harvest cells and supernatant (= passage P0) 48 h post-infection by flushing off the cells using a pipette from the surface of the tissue culture dish using the cell culture medium of this plate. Freeze at −80 °C.

▲ **CRITICAL STEP** Two days after infection, a cytopathic effect (CPE) should be observed because of virus replication (cells are rounded up and loosely or completely detached from the tissue culture dish). For an efficient amplification, CPE is observable 48 h post-infection.

■ **PAUSE POINT** Lysate can be stored at −80 °C for at least several days before continuation of the amplification procedure.

Viral preamplification steps using adherent 116 cells ● TIMING ~12 d

29| Three serial passaging steps are required (passages P1–P3) as described in the following steps. Make sure that tissue culture dishes with 116 cells are available for Steps 31, 33 and 36. We use one-third (~1 ml) of the lysate from passage P0 (Step 28) to infect one 60-mm dish of 116 cells at a confluence of 90–95%. To break up cells and release the virus from passage P0 (Step 28), freeze (in liquid nitrogen) and thaw (in 37 °C water bath) several times. If the lysate from passage P0 was stored at −80 °C, conduct three freeze–thaw cycles. If freshly harvested virus from passage P0 is used, perform four freeze–thaw cycles.

▲ **CRITICAL STEP** It is not necessary to spin down and remove cellular debris after freeze–thaw cycles.

? TROUBLESHOOTING

30| Mix fresh media (MEM, 5% FBS) with one-third of the lysate from Step 29 to a final volume of 3 ml and add the helper virus; at this stage, coinfect with the helper virus applying 2 TU per cell (a 60-mm tissue culture dish at a confluence of 80–100% contains $\sim 3.2 \times 10^6$ cells, and therefore one needs to add $\sim 6.4 \times 10^6$ TU of the helper virus).
? TROUBLESHOOTING

31| Remove the medium from a 60-mm dish with 116 cells (90–95% confluence; from Step 29) and infect with the viral mixture (from Step 30).
? TROUBLESHOOTING

32| Harvest cells and supernatant 48 h after infection by completely detaching cells from the tissue culture dish and thorough aspiration of the cell–medium suspension, and freeze at −80 °C. This viral suspension is called passage P1.
■ **PAUSE POINT** Cell–virus suspension from passage P1 can be stored at −80 °C for several days before continuing.
? TROUBLESHOOTING

33| Use one-third of the lysate from passage P1 (from Step 32) to infect a 60-mm dish of 116 cells at a confluence of 90–95% by repeating Steps 29–32 to obtain passage P2.
■ **PAUSE POINT** Cell–virus suspension from passage P2 can be stored at −80 °C for several days before continuing.
? TROUBLESHOOTING

34| Use two-thirds of the cell–virus suspension from passage P2 (obtained at Step 33) to infect a 150-mm dish of 116 cells at a confluence of 90–95%. To break up cells and release the virus from passage P2 (Step 33), freeze (in liquid nitrogen) and thaw (in 37 °C water bath) several times (see also Step 29).
? TROUBLESHOOTING

35| Mix fresh media (MEM, 5% FBS) with two-thirds of the lysate from Step 34 to a final volume of 20 ml and add the helper virus. Coinfect with the helper virus using 2 TU per cell (a 150-mm tissue culture dish at a confluence of 80–100% contains $\sim 2 \times 10^7$ cells, and therefore one needs to add $\sim 4 \times 10^7$ TU of the helper virus).
? TROUBLESHOOTING

36| Remove the medium from a 150-mm dish of 116 cells (90–95% confluence) and infect with the viral mixture.
? TROUBLESHOOTING

37| Harvest cells and supernatant 48 h after infection as described in Step 32 and freeze at −80 °C. This cell–virus suspension is called passage P3.
▲ **CRITICAL STEP** Some vectors may not amplify sufficiently until finishing passage P3, and they may require additional passages. At this point, primary amplification of HC-AdV containing a beta-galactosidase or green fluorescent protein (GFP) coding sequence can easily be evaluated using beta-galactosidase or GFP readout systems, respectively. As not every HC-AdV contains a marker gene, one may alternatively monitor the status of viral amplification by infection of HeLa cells with aliquots of the viral lysates (passages P0–P3) and subsequent Southern blot analysis or qPCR (see also Step 73).
■ **PAUSE POINT** Cell–virus suspension from passage P3 can be stored at −80 °C for several days before continuation of the amplification procedure.
? TROUBLESHOOTING

Amplification of 116 cells in suspension using a spinner culture system ● TIMING 5 d

38| Add 900 ml of prewarmed (37 °C) fresh MEM (10% FBS) with hygromycin B (100 μg ml^{-1}) into the spinner culture flask.
? TROUBLESHOOTING

39| Remove medium from individual 150-mm tissue culture dishes with 116 cells and flush off cells with 10 ml of fresh media (MEM, 10% FBS with 100 μg ml^{-1} hygromycin B). Adherent 116 cells are easy to detach. No trypsin is required. In fact, trypsin may negatively affect growth of suspension cells. Immediately after adding the media, pipette up and down several times to get a homogeneous cell suspension. Subsequently, transfer cells directly into the spinner flask, which contains already 900 ml of fresh medium (MEM, 10% FBS) with hygromycin B (100 μg ml^{-1}).
▲ **CRITICAL STEP** One needs ten 150-mm tissue culture dishes with 116 cells at a confluence of 95–100% to set up a 3-liter suspension culture in a spinner flask (**Fig. 4a**). Detach cells from each single 150-mm dish one after the other. If you wait too long after removing the media and adding the fresh media, cells will become harder to detach.
? TROUBLESHOOTING

40| Incubate spinner flask with the final volume of 1 liter on a magnetic stirrer in a tissue culture incubator for 24 h at 37 °C and 5% CO_2. During the amplification procedure, the magnetic stirrer is adjusted to 70 r.p.m. Rotation avoids attachment of cells to glass surfaces and optimal growth.
? TROUBLESHOOTING

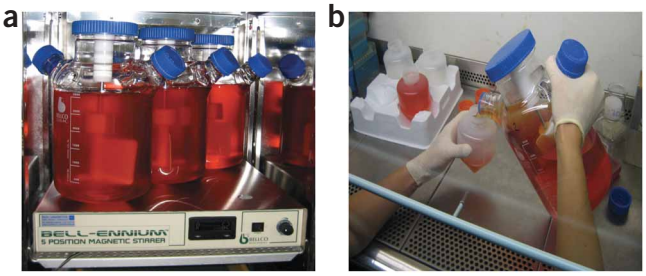

Figure 4 | Amplification of high-capacity adenoviral vectors (HC-AdV) in spinner flasks. (**a**) Producer cells, 116 cells (see ref. 55), grow in suspension and as adherent cells. Cells are amplified in 3 liters of medium using spinner flasks. The Bell-Ennium 5 position magnetic stirrer allows amplification of cells in five 3-liter spinner culture flasks simultaneously. (**b**) Ninety-six hours after setting up the spinner, culture cells are harvested for infection with the HC-AdV and coinfection with the helper virus. Forty-eight hours post-infection cells are harvested using 500-ml centrifuge tubes. Infected cells are resuspended in 28 ml of DPBS. This suspension is used for purification using CsCl gradients.

41| 24 h after setting up the spinner culture, add 500 ml of fresh media (MEM, 10% FBS) supplemented with hygromycin B (100 μg ml^{-1}).
? TROUBLESHOOTING

42| *Optional*: monitor cell growth in spinner culture flask. Each day you may remove an aliquot (1 ml), perform a Trypan blue staining and count cells to monitor cell viability (see **Box 3**). The density of cells usually ranges between 2×10^5 and 5×10^5 cells per ml.
? TROUBLESHOOTING

43| 48 h after setting up the spinner culture, add 500 ml of fresh media (10% FBS) with hygromycin B (100 μg ml^{-1}).
? TROUBLESHOOTING

44| 72 h after setting up the spinner culture, add 1,000 ml of media (10% FBS) supplemented with hygromycin B, resulting in a total volume of 3-liter cell suspension. After 24 h, continue with the infection procedure (Step 46).
? TROUBLESHOOTING

45| *Optional*: one day before infection, we plate an aliquot (2 ml) of 116 cells grown in suspension in a 60-mm tissue culture dish. About 6 h later, these cells should be adherent, and the next day the dish should have a confluence of approximately 30–50%. With this procedure, one can examine whether cells in the spinner culture are viable and have grown to sufficient amounts.
? TROUBLESHOOTING

Infection of 116 cells grown in suspension and HC-AdV amplification ● TIMING ~2 d

46| Harvest 116 suspension cells from Step 44 (we use 500-ml centrifuge tubes). Centrifuge for 10 min at 500*g* in a clinical centrifuge at room temperature (**Fig. 4b**). The supernatant can be discarded. Keep the emptied spinner culture flask under the tissue culture hood.

47| Resuspend all the cells in MEM with 5% FBS (no hygromycin) by pipetting up and down about 8–10 times to get a single-cell suspension. The amount of fresh resuspension medium depends on whether one uses the lysate from Step 37 (130-ml resuspension medium) or a purified viral stock from Step 72 (150-ml resuspension medium). The total volume of the initial infection should be 150 ml.

48| *Infection of 116 cells grown in suspension*: you may use the virus/cell suspension from Step 37 or purified virus from Step 72.

(A) Infection of 116 suspension cells with lysate (primary amplification) ● TIMING ~1.5 h

(i) To break up cells and to release the virus from cell–virus suspension of passage P3 (Step 37), perform three freeze (in liquid nitrogen) and thaw (in 37 °C water bath) cycles. If the freshly harvested virus from passage P3 is used, perform four freeze–thaw cycles.

BOX 3 | COUNTING OF EUKARYOTIC CELLS TO MONITOR CELL VIABILITY AND CELL DENSITY ● TIMING 10 MIN

1. Aseptically, take 0.1 ml of suspension cells and add 0.1 ml of Trypan blue solution (0.4%, wt/vol).
2. Incubate for 5 min at room temperature and subsequently deliver them to a hemacytometer.
▲ CRITICAL STEP Before counting the cells, vortex cell supension to obtain single-cell solution because cells in suspension partially grow in cell aggregates.
3. Count a total of at least 100 cells and determine the percentage of cells, which are not viable and therefore blue. There should be only a limited percentage of blue cells present (<15%).
4. To ascertain the density of the cells per ml, count the cells in four squares of a standard hemacytometer and calculate the average amount of cells. The cell density is determined by the following formula: cell ml^{-1} = average of counted cells $\times 2 \times 10^4$ ml^{-1}.

(ii) Transfer cells from Step 47 to a 250-ml storage bottle and put in a sterile magnetic stir bar.
(iii) Coinfect cells with the virus within the lysate from Step A(i) and the helper virus (2 TU per cell). Assuming a density of 3×10^5 cells per ml, the total amount of cells in a 3-liter spinner flask is 9×10^8 cells. Thus, one needs to add 1.8×10^9 TU of the helper virus AdNG163R-2.

(B) Infection of 116 suspension cells with purified virus stock (reamplification of HC-AdV) ● **TIMING** ~**20 min**

(i) Transfer cells from Step 47 to a 250-ml storage bottle and put in a sterile magnetic stir bar.
(ii) Infect cells with 100 viral particles per cell (vp, physical titer as determined in Step 73 (option A) by measuring the absorbance at 260 nm) of formerly purified HC-AdV (Step 72) and coinfect with the helper virus (2 TU per cell). Assuming a density of 3×10^5 cells per ml, the total amount of cells in a 3-liter spinner flask is 9×10^8 cells. Thus, one needs to add ~9×10^{10} vp of the purified HC-AdV and 1.8×10^9 TU of the helper virus AdNG163R-2.

49| Stir the 250-ml storage bottle containing the cell–virus mixture in the tissue culture incubator set at 37 °C and 5% CO_2 for 2 h at 60 r.p.m. on a magnetic stirrer. Make sure that the storage bottle is not completely closed.

50| Transfer the total volume of 150 ml from the storage bottle into the spinner culture flask and add 1,850 ml of prewarmed (37 °C) and fresh media (MEM + 5% FBS, no hygromycin B, 2 liters in total).

51| Incubate for 48 h in the tissue culture incubator at 37 °C and 5% CO_2. Stir at 70 r.p.m.

52| Harvest cells by centrifugation for 10 min at 890*g* at room temperature (we use 500-ml centrifuge tubes and spin them in a clinical centrifuge).

53| Remove the remaining medium.

54| Resuspend pellets (from 2 liters) in 28 ml of DPBS by pipetting up and down 8–10 times to get single-cell suspensions.

55| Freeze resuspended cell–virus suspension in liquid nitrogen for 5 min. Make sure that the suspension is completely frozen. Store at −80 °C until starting the purification using cesium chloride gradients (Steps 57–60). Virus–cell suspension from one spinner culture flask will be split in six tubes (Step 63) of one SW41 rotor.

56| *Optional*: to monitor HC-AdV amplification, one may keep an aliquot for further investigation. When establishing the protocol, each large-scale amplification step of an HC-AdV containing a marker gene coding sequence (beta-galactosidase or GFP) may be monitored by removing an aliquot (**Fig. 5**). As not every HC-AdV contains a marker gene, one may alternatively monitor viral amplification by infection of HeLa cells with collected aliquots and subsequent qPCR or Southern blot analysis (see also Step 73B,D).

■ **PAUSE POINT** Frozen virus–cell suspension can be stored at −80 °C for several weeks until purification.

Purification of HC-AdV ● **TIMING 2 d**

57| Before starting the purification procedure, prepare cesium chloride solutions (see REAGENT SETUP).

58| To prepare viral lysate from Step 55 (frozen cell–virus suspension resuspended in DPBS) for cesium chloride gradients, thaw cell–virus suspension in a 37 °C water bath.

59| Shake the tube intermittently to avoid local heating, which could reduce viral titer.

60| Freeze in liquid nitrogen for 5 min. Make sure that the solution is completely frozen.

61| Repeat freeze–thaw cycle two times (Steps 58–60) to disrupt cellular

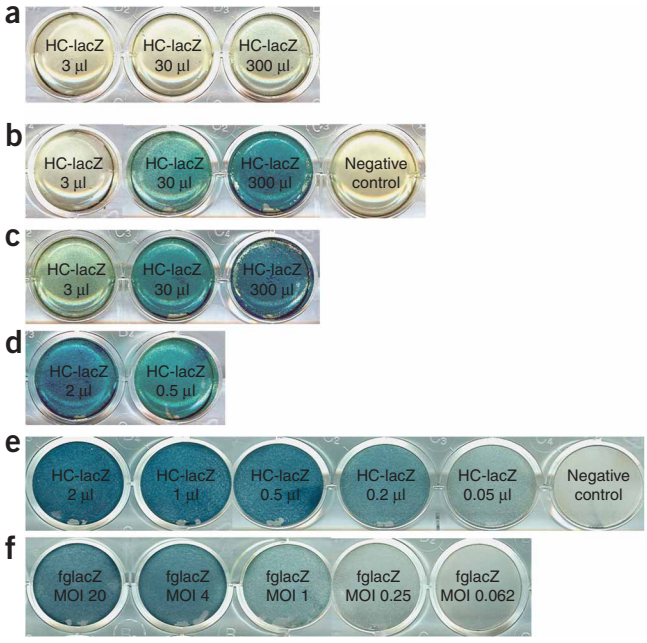

Figure 5 | Monitoring adenoviral amplification steps during large-scale production. In this example, the amplified HC-AdV expresses beta-galactosidase under the control of a cytomegalovirus promoter[55]. HeLa cells were seeded in a 12-well plate. At 90% confluence, cells were infected with an aliquot of the viral progeny of each amplification stage. Twenty-four hours post-infection, cells were stained for beta-galactosidase expression. Infection volumes are indicated. (**a**) Twenty-four hours after infection of the 3-liter suspension, culture with HC-AdV and helper virus (Step 51). (**b**) Forty-eight hours after infection of the suspension culture (Step 51). (**c**) Harvested cell suspension resuspended in 28 ml of DPBS (Step 54). (**d**) Purified virus after CsCl gradient und dialysis (Step 72). (**e**) Reamplified virus from CsCl-purified stock (Step 72). (**f**) Standard curve. HeLa cells were infected with defined multiplicities of infection (MOIs) using a first-generation virus containing the same beta-galactosidase expression cassette (fglacZ) as the analyzed HC-AdV (**Fig. 5a–e**).

membranes and to release viral particles from cells. In total, four freezing and thawing steps are required (freezing step for storage included).

62| After the last thawing step, centrifuge at 500*g* for 8 min at room temperature and collect the supernatant containing the HC-AdV. Collect the supernatant in a 50-ml conical tube. The pellet containing cell debris can be discarded.
■ **PAUSE POINT** The collected supernatant can be stored for several hours at 4 °C.

63| During the freeze–thaw cycles (Steps 58–60), prepare cesium chloride (CsCl) step gradients in six UltraClear centrifuge tubes; carefully and slowly pipette CsCl solutions into UltraClear centrifuge tubes in the following order: 0.5 ml of 1.5 g cm^{-3} CsCl solution, 3 ml of 1.35 g cm^{-3} CsCl solution and 3.5 ml of 1.25 g cm^{-3} CsCl solution (**Fig. 6a**).
▲ **CRITICAL STEP** To avoid disruption of the phase interface, reduce the distance between the pipette and solution to a minimum. Disruption of phases leads to more diffuse viral bands (see Step 66).

64| Carefully overlay ~4.5 ml of cleared vector supernatant from Step 62 on top of 1.25 g ml^{-1} CsCl layer. To avoid imbalance, add ~0.5 ml of DPBS on top of cleared vector supernatant until reaching the top of the tube.

65| Centrifuge the gradients in an ultracentrifuge at 12 °C for 1.5 h at 226,000*g* (35,000 r.p.m.) to separate packed viral particles from unpacked particles and remained cellular fragments.
▲ **CRITICAL STEP** Set slow acceleration and deceleration to avoid disruption of the gradient.

66| Take out the UltraClear centrifuge tubes with tweezers. You will see a diffuse band of cell debris as a top layer. Below you will find two white bands. The lower band equals the HC-AdV, and the upper band contains empty particles. When the physical titer (see Step 73A) of the upper band is determined by measuring the absorbance at 260 nm, the values indicate the presence of protein but absence of DNA. The gradient after centrifugation is schematically shown in **Figure 6a**.
? TROUBLESHOOTING

67| Collect the lower bands of each gradient (about 1 ml each tube) after carefully removing the layers of cell debris and empty particles with a 1-ml pipette. Collect and transfer virus with a clean pipette tip into a sterile 50-ml conical tube.
? TROUBLESHOOTING

68| Add 1.35 g ml^{-1} CsCl solution up to a volume of 24 ml. Mix carefully by pipetting up and down. Transfer 12 ml to two UltraClear centrifuge tubes, respectively, and fill them with 1.35 g ml^{-1} CsCl solution to the top.
▲ **CRITICAL STEP** Never centrifuge more than ~3 ml of virus (from Step 67) in one tube because this will result in a diffuse virus band.
? TROUBLESHOOTING

69| Centrifuge overnight (18–20 h) at 226,000*g* (35,000 r.p.m.) at 12 °C in an ultracentrifuge (slow acceleration and deceleration).
? TROUBLESHOOTING

70| Retrieve the HC-AdV (see also **Fig. 6**). You may see two bands of which the lower band should be collected as described in Step 67. The upper band equals residues of empty particles.
? TROUBLESHOOTING

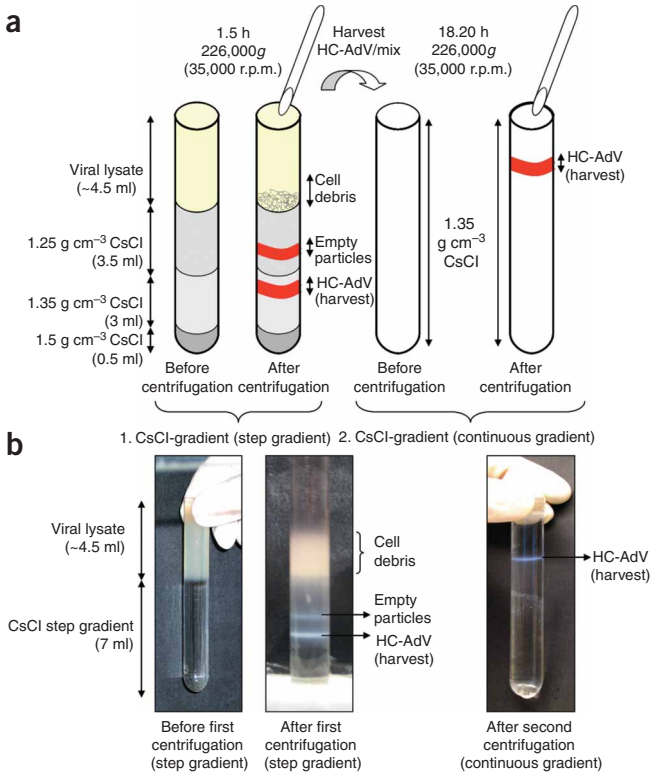

Figure 6 | Flowchart of CsCl purification of HC-AdV. For both centrifugation steps, centrifuge tubes are shown before and after centrifugation. The cesium chloride steps are indicated as well as the respective centrifugation parameters. (**a**) Schematic diagram of CsCl gradients. After three freeze–thaw cycles, the viral lysate is carefully loaded onto the top of the CsCl gradient. After centrifugation at 226,000*g* (35,000 r.p.m., 1.5 h, 12 °C), the band containing the HC-AdV is harvested from the top with a 1-ml pipette (around 1 ml per centrifuge tube). Empty particles (without viral genome) concentrate in the 1.25 g cm^{-3} CsCl phase, whereas complete HC vector particles concentrate in the 1.35 g cm^{-3} CsCl phase. In the continuous CsCl gradients, the HC-AdV particles concentrate in the upper part of the tube. Note that the density of empty particles that are eventually present in the preparation is lower than the density of packed particles. Therefore, empty particles are concentrating above the complete particles. Be sure to harvest the complete particles in a volume as small as possible to concentrate them. (**b**) Photographs of the collection tubes before (left picture) and after the step gradient centrifugation (middle picture) and after the continuous gradient centrifugation (right picture). The particle bands (empty and complete) appear white with a light blue shadow. After the first centrifugation, the collected virus is mixed with 1.35 g cm^{-3} CsCl solution and centrifuged at 226,000*g* (35,000 r.p.m.) overnight at 12 °C. The HC-AdV is visible in the upper part of the tube after second centrifugation (right picture), collected and subsequently dialyzed.

Dialysis of virus for buffer exchange ● TIMING **2 d**

71| One can either dialyze collected virus from Step 70 in purchased ready-to-use dialysis cassettes (option A) or dialysis tubing closed with dialysis closures (option B). The latter option may be cheaper in the long-term run.

(A) Dialysis cassettes

(i) Fill dialysis cassettes according to the manufacturer's instructions.
▲ CRITICAL STEP Avoid any air bubbles in the dialysis cassette.

(ii) Dialyze at 4 °C in 500-ml dialysis buffer (10 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM MgCl2 in deionized H_2O) with slow stirring. Exchange dialysis buffer twice after 60 min and 120 min.

(iii) Third dialysis step should be performed overnight in 500 ml of dialysis buffer at 4 °C with slow stirring.

(iv) Retrieve virus from dialysis cassette.

(B) Dialysis tubing

(i) Cut off dialysis tubing (a strip of about 8 cm in length).

(ii) Wash dialysis tubing three times with sterile and deionized H_2O.

(iii) Close dialysis tubing on one side with a dialysis closure.

(iv) Transfer collected virus into dialysis tubing using a 1-ml pipette.

(v) Close dialysis tubing on the other side with a dialysis closure. Before closure, make sure that all air bubbles are removed.

(vi) Dialyze in 1,000 ml of dialysis buffer (10 mM Tris-HCl (pH 7.5), 10% glycerol and 1 mM MgCl2 in deionized H_2O) at 4 °C with slow stirring for 2 h.

(vii) Remove dialysis buffer and dialyze overnight in 2,000 ml of dialysis buffer at 4 °C with slow stirring. Alternatively, one can use sucrose buffer for dialysis. This sucrose-based dialysis buffer (140 mM NaCl, 5 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4 and 730 mM (= 5%) sucrose (pH 7.8)) was shown to avoid aggregation of viral particles. For the whole procedure, please refer to the protocol in ref. 63.

(viii) Collect virus using a 1-ml pipette.

72| Make multiple aliquots (25–50 µl or larger) and store at −80 °C. To titer the virus preparation, additionally make three aliquots of 25 µl. After storage of the HC-AdV at −80 °C, minimize the numbers of freeze–thaws because this may result in the reduction of infectious units[64]. Storage or shipping of adenoviral vectors on dry ice will negatively impact the activity of the virus due to pH changes after exposure to CO_2 (see refs. 45,65).

■ PAUSE POINT Virus can be stored at −80 °C.

Characterization and titration of final vector preparations ● TIMING **1–5 d**

73| There are numerous ways to titrate the HC-AdV preparations and to determine the helper virus contamination: measuring the physical titer of final HC-AdV preparations (option A), characterization of HC-AdV preparations and helper virus contamination levels by qPCR (option B), measuring infectious units of the HC-AdV in the final vector preparation by qPCR (option C), quantification of infectious units by Southern blot analysis (option D) and physical and infectious titer standardized by adenovirus reference material (option E). The method of choice, however costly, is the titer determination by qPCR (option B). We routinely perform options A, C and D.

? TROUBLESHOOTING

(A) Measuring the physical titer of final HC-AdV preparations by optical density ● TIMING **1 h**

(i) Dilute 25 µl of the final vector preparation from Step 72 with 475 µl of dilution buffer.

(ii) Gently shake for 20 min at room temperature. Centrifuge at room temperature in a microcentrifuge at 15,000*g* for 2 min. Subsequently, measure the absorbance of the supernatant at 260 nm (A260).

(iii) Use the following formula to calculate the optical particle units (OPU): OPU ml^{-1} = (absorbance at 260 nm) × (dilution factor) × (1.1×10^{12}) × (36)/(size of HC-AdV in kb).

(iv) As the A260 values are usually low, we measure four times using 100 µl of the diluted virus.

(v) The OPU equals the number of viral particles, and the final concentration is expressed in viral particles per ml (vp ml^{-1}).

(B) Measuring the physical titer of final HC-AdV preparations and helper virus contamination levels by qPCR ● TIMING **4 h**

(i) Incubate 20 µl of purified virus from Step 72 for 2 h with 200 µl of proteinase K–SDS solution at 56 °C.

(ii) Precipitate viral DNA by adding 20 µl of 3 M sodium acetate (pH 5) and 600 µl of precooled EtOH (>99.8%; stored at −20 °C).

(iii) Centrifuge for 8 min at full speed (15,000*g*) at room temperature in a microcentrifuge and discard the supernatant by pipetting.

(iv) Add 600 µl of 70% ethanol and mix gently by inverting the tube several times. After centrifugation at 15,000*g* at room temperature for 5 min, remove the supernatant by pipetting.

(v) Air-dry pellet briefly and resuspend in 25 µl of sterilized dH_2O. Use 5 µl for qPCRs.
■ PAUSE POINT DNA can be stored at −20 °C for up to 1 month. However, for optimal PCR results, it is recommended to use DNA as fresh as possible.

(vi) For precise determination of the total number of HC-AdV and contaminating helper virus genomes present by qPCR, generate three serial dilutions (10^{-3}, 10^{-4} and 10^{-5}) of purified HC-AdV DNA obtained in Step B(v).

(vii) For generation of a standard curve for qPCR, 10^2–10^8 genome copies of a purified wild-type adenovirus serotype 5 preparation or any first-generation virus with known physical titer can be assayed. To prepare adenoviral DNA, perform Step B(i–v). Alternatively, a standard plasmid with the appropriate adenoviral sequence (L3) (see ref. 66) or the gene of interest contained in the HC-AdV can be used. However, the following steps describe the determination of the physical titer using wild-type adenovirus serotype 5.

(viii) To determine the physical titer of HC-AdV in final vector preparations, we perform qPCR using the LightCycler Systems (Applied Biosystems). The following primers can be used for every HC-AdV: Ad5ITRfw1-24 5′-CAT CAT CAA TAA TAT ACC TTA TTT-3′ (400 nM) and 436Rev 5′-ACG CCA CTT TGA CCC GGA ACG-3′ (400 nM). These primers amplify the ITR/packaging signal region with the LightCycler FastStart DNAMaster^{Plus} SYBR Green I kit. The program is set as follows: Preincubation at 95 °C for 10 min, amplification in 45 cycles at 95 °C for 10 s, 55 °C for 5 s and 72 °C for 30 s. On the basis of the standard curve (Step B(vii)), the total number of adenoviral genomes in your final vector preparation can be calculated. The amount of helper virus as determined in Step B(ix) needs to be subtracted.

(ix) qPCR for helper virus contamination is carried out by the Taqman 7500 Fast Real-Time PCR System (Applied Biosystems) amplifying a 235-bp area of the adenoviral late gene 3 (L3). Using the oligonucleotides L3 forward 5′-AGA AGC TTA GCA TCC GTT ACT CGA GTT GG-3′ (400 nM) and L3 reverse 5′-ATA AGC TTG CAT GTT GGT ATG CAG GAT GG-3′ (400 nM) together with an L3-specific probe 5′-Fam-CCA CCC GTG TGT ACC TGG TGG ACA-Tamra-3′ (300 nM) (Ella Biotech), the PCR is performed with the following program: AmpErase UNG reaction at 50 °C for 2 min, preincubation/activation at 95 °C for 10 min, amplification and data collection during 40 cycles (95 °C for 15 s and 60 °C for 1 min). Universal PCR Mastermix was used for Taqman qPCR. On the basis of the standard curve (Step B(vii)), the total number of helper virus genomes in your final vector preparation can be calculated.

(x) Owing to the back-recombination of E1 sequences contained in the genome of the 116 producer cell line, it is important to rule out whether replication-competent adenovirus is present in the HC-AdV preparation and the helper virus stock[66]. Purified virus can be analyzed by qPCR using the following primers, which specifically detect E1: E1-Adenovirus Forward 5′-GGG TGA GGA GTT TGT GTT AGA TTA TG-3′ (400 nM); E1-Adenovirus Reverse 5′-TCC TCC GGT GAT AAT GACAAG A-3′ (400 nM); E1-Adenovirus probe TET-5-AGC ACC CCG GGC ACG GTT G-3-TAMRA (300 nM).

▲ **CRITICAL STEP** These oligonucleotides will also detect chromosomal DNA from 293 cells in which the left arm of the adenoviral DNA, including E1, is stably integrated. Thus, these primer sets should be used only for final vector preparations.

(C) Measuring total particles and infectious units of the HC-AdV in the final vector preparation by qPCR ● TIMING 4 d

(i) Seed 293 cells in four 60-mm tissue culture dishes. For infection, cells should reach 90% confluence.

(ii) To determine infectious units, infect cells of one tissue culture dish with 0.4 µl of CsCl-purified virus and a second tissue culture dish with 2 µl of CsCl-purified virus from Step 72. Harvest cells after 3 h with trypsin. For trypsinisation, remove medium, add enough trypsin to cover the whole plate (15 ml) and incubate at 37 °C and 5% CO_2 for 5 min. Flush off the cells with the trypsin covering the plate using a pipette. Pellet the cells by centrifugation at 890*g* for 3 min at room temperature. Resuspend pellet in 200 µl of DPBS. Wash the cells thoroughly with DPBS to remove free (noninfective) vector particles by centrifugation at 890*g* for 3 min at room temperature. Discard the supernatant and resuspend pellet in 200 µl of DPBS.

▲ **CRITICAL STEP** At least two different volumes of CsCl-purified virus should be used for infection used after qPCR and to be within the range of the standard curve (for generation of a standard curve, see also Step B(vii)). For determination of the infectious titer, it is crucial to remove all noninfective viral particles from the cell surfaces and the medium by trypsin treatment and thorough washing.

(iii) For determination of the number of total particles (physical titer), harvest 293 noninfected cells from Step C(i) without trypsin by flushing off the cells with the medium covering the cells using a pipette. Pellet the cells by centrifugation at 890*g* for 3 min at room temperature. Resuspend pellet in 200 µl of DPBS. Add 0.4 µl of CsCl-purified HC-AdV preparation directly to the pelleted cells.

(iv) Repeat Step C(iii) with the last tissue culture dish from Step C(i), but infect harvested cells with 2 µl of CsCl-purified HC-AdV.

(v) Isolate genomic DNA from cells in Steps C(ii–iv) (for a detailed protocol, see **Box 4**).

■ **PAUSE POINT** Genomic DNA can be stored at −20 °C for up to 1 month. However, for optimal PCR results, it is recommended to use DNA as fresh as possible.

(vi) To determine total particles and infectious particles, analyze serial dilutions of genomic DNA of infected 293 cells from Step C(ii), Step C(iii) and Step C(iv) by qPCR using specific primer (see Step B(viii)) for the detection of the adenoviral ITR (for qPCR setup, see Steps B(vi–ix)).

(D) Quantification of infectious units by Southern blot analysis ● TIMING 5 d

(i) Seed HeLa cells in six 60-mm tissue culture dishes (0.8×10^6 cells per dish).

(ii) The next day, infect four dishes with multiplicity of infection (MOI) of 33, 11, 3.7 and 1.2 of a first-generation adenoviral vector for which the viral titer was determined by a plaque-forming assay (see **Box 1** (C)).

(iii) Infect one tissue culture dish from Step D(i) with 0.4 µl and the last tissue culture dish with 2 µl of the CsCl-purified HC-AdV from Step 72. Incubate all six dishes for 3 h in a tissue culture incubator (37 °C; 5% CO_2).

BOX 4 | ISOLATION OF GENOMIC DNA FROM CELL PELLETS ● TIMING 2 D

1. Resuspend cell pellet in 200 µl of DPBS and vortex for 3 s.
2. Add 200 µl of proteinase K–SDS solution.
3. Vortex suspension for 3 s and incubate 12–16 h at 55 °C while gently shaking.
4. Add 2 µl of RNAse A and incubate for 30 min at 37 °C.
5. To perform a phenol–chloroform extraction, add 350 µl of phenol:chloroform:isoamyl alcohol.
6. After centrifugation for 2 min at high speed (15,000*g*) in a microcentrifuge, transfer the supernatant to a new tube. Repeat phenol–chloroform extraction as described above (Steps 5 and 6 described in this box).
7. Add 50 µl of sodium acetate (pH 5, 3 M) to the supernatant and 1 ml of precooled EtOH (>99.8%; stored at −20 °C). Mix suspension vigorously.
8. To pellet genomic DNA, centrifuge samples for 10 min at high speed (15,000*g*).
9. After removal of the supernatant, add 500 µl of 70% EtOH and centrifuge for 2 min at high speed (15,000*g*). Remove the supernatant and add 500 µl of 70% EtOH.
10. Shake for ~30 min at room temperature.
11. After centrifugation at high speed (15,000*g*) for 2 min, remove the supernatant and air-dry DNA pellet.

▲ CRITICAL STEP Do not dry DNA pellet for a long time, as it will be harder to get genomic DNA in solution.

12. Resuspend the DNA pellet in 120 µl of TE buffer and shake the samples. If you encounter difficulties in getting the DNA into solution, incubate for ~1 h at 37 °C while shaking. If the DNA solution still appears viscous, shake the samples for several hours at 37 °C, or you may incubate samples at 55 °C for ~1 h.

(iv) Harvest cells by trypsin treatment (see Step C(ii)) and centrifuge them at 10,600*g* with a microcentrifuge for 2 min at 4 °C. Freeze cell pellets at −20 °C.

■ PAUSE POINT Cell pellets can be stored at −20 °C for several weeks.

(v) Thaw cell pellets and isolate genomic DNA (see **Box 4**). Digest 15 µg of DNA with *Hin*dIII to generate DNA fragments[62]. For specific detection of your gene of interest, you may also use other restriction enzymes.

(vi) For Southern blot analysis[62], prepare a 0.8% agarose gel[62] and load purified and digested genomic DNA from Step D(v). Perfom gel electrophoresis to separate DNA fragments.

(vii) Treat the gel with 200 ml of 0.125 N HCl for 30 min and subsequently with 200 ml of denaturation buffer (30 min). Transfer DNA from agarose gel to an alkaline membrane by capillary transfer in denaturation buffer (24 h)[62].

(viii) After transfer, incubate the membrane in neutralization buffer for 1 min at room temperature and cross-link three times by UV light irradiation at a dose of 1.5 J cm^{-2} (using UV Stratalinker 1800) to fix DNA to the membrane[62].

■ PAUSE POINT Cross-linked membrane can be stored at room temperature at a dry place for several weeks.

(ix) To generate a probe for Southern blot hybridization, which can be used for any HC-AdV preparation, PCR-amplify the 5′-ITR and the packaging signal for hybridization using the following primers: Ad5ITRfw1-24 5′-CAT CAT CAA TAA TAT ACC TTA TTT-3′ and 436Rev 5′-ACG CCA CTT TGA CCC GGA ACG-3′. PCR conditions: 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, 35 cycles using Taq DNA Polymerase from New England Biolabs.

(x) Incubate the membrane in 2× SSC (25 ml) for 1 min at room temperature and subsequently prehybridize for 2 h at 62 °C in church buffer.

(xi) Label the probe generated in Step D(ix) with radioactive nucleotide derivate [α^{32}P]dCTP or [α-^{32}P]dATP using the PrimeIt II Random labeling kit according to the manufacturer's instructions.

(xii) Hybridize blots with [α-^{32}P]dCTP- or [α-^{32}P]dATP-labeled probe from Step D(ix) diluted in church buffer for 16–24 h at 62 °C (see ref. 62).

(xiii) Detect probe binding using Phosphoimager FLA-3000. Compare intensities of signals from samples (from infected cells from Step D(iii)) and from the standard samples (from infected cells from Step D(ii)) to estimate infectious titer. An example of a Southern blot analysis is shown in **Figure 7**.

(E) Physical and infectious titer standardized by adenovirus reference material ● TIMING 4 d

(i) For accurate measurements and production of clinical-grade HC-AdV, the adenoviral reference material (ARM) may be used in combination with Step A, B and D. The ARM is distributed by ATCC (cat. no. VR-1516) and is fully characterized regarding particle concentration and the amount of infectious units per volume. The advantage of this method is that adenoviral preparations produced in different laboratories can be directly compared also with respect to future clinical trials. This method is described in detail elsewhere[67].

(ii) To determine the physical and infectious titer of the HC-AdV, the adenovirus reference material might be used instead of the first-generation vector (see Step B(vii) and Step D(ii)).

(iii) Comparison of the intensities of bands specific for ARM or HC-AdV DNA indicates the relative infectivity. This method was described in more detail earlier[67].

TIMING

The whole procedure takes ~5 weeks. An flow diagram overview is provided in **Figure 1**. However, preparations including amplification of the helper virus and the amplification of 116 cells are necessary and need to be completed during cloning and before starting the generation and amplification of HC-AdV. The following list will give an overview for the duration of the most important steps. Detailed timing is provided in the step-by-step procedure.

Box 1, amplification of the helper virus AdNG163R-2: ~2 weeks

Steps 1–13, cloning of the production plasmid of HC-AdV : ~2 weeks

Steps 14–23, linearization of HC-AdV production plasmid for transfection: ~5 h

Steps 24–37, transfection of linearized HC-AdV production plasmid and preamplification of HC-AdV in adherent 116 cells: ~14 d

Steps 38–45, amplification of 116 cells in suspension culture for infection with HC-AdV and helper virus AdNG163R-2: 5 d

Steps 46–50, infection of 116 cells in suspension with HC-AdV and helper virus AdNG163R-2: 3 h

Step 51, large-scale amplification of HC-AdV in spinner flask: 2 d

Steps 52–56, harvesting infected cells from spinner flask: 30 min

Steps 57–72, purification of HC-AdV: 3 d total

Step 73, Characterization and titration of final vector preparations: 1 h to 5 d, depending on the method of choice

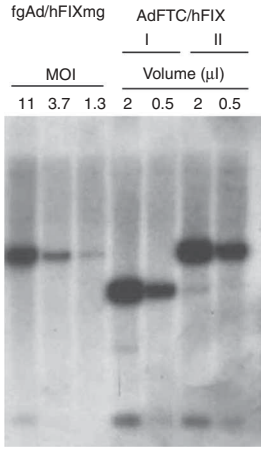

Figure 7 | Transducing units in final vector preparations as determined by Southern blot analysis. HeLa cells were seeded in 60-mm tissue culture dishes and transduced with the respective volumes of the final vector preparations. A standard curve was generated by infection with defined multiplicities of infection (MOIs) of cells with a first-generation adenoviral vector containing in part identical DNA sequences. Three hours postinfection, cells were harvested, genomic DNA isolated and samples were subjected to Southern blot analyses. To create a standard curve, the first-generation adenovirus fgAd/hFIXmg[24], which expresses the human coagulation factor IX (hFIX), was used for infection of HeLa cells. The Southern blot analysis shown here was performed to analyze two different HC-AdV preparations (I and II) with two different FIX expression cassettes.

? TROUBLESHOOTING

Too many or no colonies after transformation into DH10B cells (Step 11)

If you observe too many colonies on ampicillin-containing LB plates, either the I-*Ceu*I digest or the PI-*Sce*I digest of the vector pAdFTC or the *Swa*I digest of the ligation reaction was not complete.

If you have a low number of colonies or no colonies, check the competency of your competent DH10B cells. In addition, check the quality of DNA used for transformation and transformation conditions.

No insert detected in the plasmid pAdFTC derivate after ligation and transformantion (Step 12)

If you have difficulties cloning your gene of interest from the shuttle vector pHM5 into the adenoviral plasmid pAdFTC, make sure that all your materials for ligation (insert and vector) are freshly prepared on the same day and/or increase the amount of insert when setting up the ligation reaction. Alternatively, do not gel-purify the insert (Step 6), but purify I-*Ceu*I- and PI-*Sce*I-digested pHM5 shuttle plasmid by phenol–chloroform extraction followed by ethanol precipitation. Use this DNA for the ligation reaction (pHM5 contains a kanamycin and pAdFTC, an ampicillin resistance gene; therefore, religated pHM5 shuttle plasmid are unable to grow (Step 11)).

Incomplete release of the bacterial backbone from the HC-Ad production plasmid detected by agarose gel electrophoresis (Step 23)

Check the performance of your *Not*I enzyme and extend the time of *Not*I digestion and/or use more units of *Not*I. Make sure that precipitated DNA is resuspended completely.

Transfection efficiency of linearized vector is low (Steps 25A and B)

Check all reagents used for transfection. Make sure that the quality of DNA transfected into 116 cells is optimal. For quality control, transfect a plasmid expressing a marker gene (e.g., GFP or beta-galactosidase). Transfection efficiencies should range from 40% to 80%.

Insufficient viral preamplification steps using adherent 116 cells are not sufficient (Steps 29–37)

Some vectors may not be amplified sufficiently after finishing P3, and therefore, they may require additional passages. One may also increase the volume of the lysate used for infection: instead of one-third, one may use two-thirds of the cell–virus suspension for an additional amplification step (repeat Steps 34–37).

When establishing the protocol, it is best to start with amplifying an HC-AdV expressing a marker gene. If a marker gene is integrated in your HC-AdV genome (e.g., GFP or beta-galactosidase), amplification can be easily monitored at each step during pre- and large-scale amplification.

If you encounter difficulties amplifying HC-AdVs without a marker gene after establishing the protocol, simultaneously amplify an HC-AdV containing any marker gene. At each step, check amplification of the HC-AdV with the marker gene similar to the experiment depicted in **Figure 5**.

Make sure that your HC-AdV production plasmid contains all important components for virus amplification by restriction enzyme digest. To confirm that all DNA sequences essential for viral amplification are correct, you may also analyze by DNA sequencing whether the adenoviral 5′-ITR and packaging signal (using primer pDYAL21180 5′-ATG GCC TGG GCA TGG ACC GCA-3′) as well as the 3′-ITR (using primer pDYAL3858 5′-CGT GTG AGA TGG ACA TCC AGT-3′) are complete and not mutated.

Check the quality of the helper virus AdNG163R-2. Make sure by performing a plaque-forming assay (see **Box 1**) that infectious titer is not reduced.

The 116 production cell line must be free of mycoplasma, as the presence of mycoplasma contamination in the helper cell line may dramatically lower the adenovirus yields.

No or inefficient amplification of 116 cells grown in suspension (Steps 38–45)

Check for contamination, viability and growth (see **Box 3**) of 116 cells on each day after setting up the spinner flask (Step 38). Make sure that FBS was added to the tissue culture medium, no antibiotics were added and that the magnetic stirrer works properly. To compensate low growth rates, incubate for 48 h instead of 24 h after adding 1 liter of media in Step 44.
The 116 production cell line must be free of mycoplasma, as the presence of mycoplasma contamination in the helper cell lines may dramatically lower the adenovirus yields.

Only thin or no band visible in CsCl step gradient (Steps 66–70)

If the band visible in the CsCl step gradients consisting of packed viral particles (lower band) is thin and the titer of the CsCl-purified HC-AdV preparation as determined in Step 73 is low, the pre- and large-scale amplification was not optimal. Reamplify purified virus in 3-liter suspension culture of 116 cells (Step 38–56) using purified, low-titer HC-AdV preparation for infection (Step 48(B)). After reamplification, the band is usually much stronger.

If there was no band visible for packed viral particles after the CsCl step gradient, check virus amplification after each amplification step. You may monitor viral amplification during preamplification in adherent 116 cells and in 116 cells grown in suspension by removing an aliquot at each step (see also **Fig. 5**).

The quality of the linearized adenoviral genome for transfection (Step 23) and transfection efficiencies are also critical for achieving high final vector titers during preamplification. In addition, check the quality of the helper virus preparation by plaque-forming assay (see **Box 1**).

Titer in final vector preparation is low or no HC-AdV is detected after titration (Step 73)

Check all reagents and methods used for measuring the viral titer and repeat the procedures. If you obtain the same result with two different methods (titer of final vector preparation is low), the pre- and large-scale amplification was not optimal. Reamplify purified virus in 3-liter suspension culture of 116 cells (Step 48B). After reamplification, the band is usually much stronger.

Other reasons for low-titer HC-AdV preparations could be problems occuring during dialysis procedure in general. Therefore, repeat large-scale amplification and collect an aliquot after the second ultracentrifuge purification step (Step 70). Check if your HC-AdV is present before starting dialysis using one detection method (Step 73).

ANTICIPATED RESULTS

Average yields for an HC-AdV preparation from a 3-liter suspension culture are 1–2 ml, with an infectious titer of 5×10^6 to 5×10^7 TU μl^{-1} or, if determined as physical titer, 5×10^7 to 2×10^8 vp μl^{-1}. The contamination with helper-virus ranges between 0.02% and 0.2% (see refs. 55,67). Notably, when using the cell–virus suspension from Step 37 for initial large-scale amplification, the titer might vary dependent on preamplification steps.

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