User Manual

Adeno-X™ Maxi Purification Kit User Manual

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Note: The viral supernatants produced by transfecting Adeno-X 293 cells with recombinant Adeno-X Viral DNA could, depending on your DNA insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant adenovirus. **The user is strongly advised not to create adenoviruses capable of expressing known oncogenes**.

Appropriate NIH, regional, and institutional guidelines apply, as well as guidelines specific to other countries. NIH guidelines require that adenoviral production and transduction be performed in a Biosafety Level 2 facility. Section IV in this User Manual contains a brief description of Biosafety Level 2 as well as other general information and precautions.

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I. Introduction & Protocol Overview

The **Adeno-X Maxi Purification Kit** is a complete chromatography-based system for purifying and concentrating recombinant adenovirus. It provides a superior alternative to cesium chloride (CsCl) density gradient centrifugation. Although centrifugation in CsCl is an extremely effective method for purifying adenovirus, it is also time-consuming, technically demanding, and toxic (Graham & Prevec, 1991). Furthermore, the procedure is restrictive in that it is not easily scaled up or down. The Adeno-X Maxi Purification Kit, on the other hand, can be scaled up or down without difficulty. The kit also allows you to purify a maximal amount of recombinant adenovirus directly from the cell pellet. You simply wait until the cytopathic effect (CPE) is complete—when the viral titer is highest—harvest the cells and purify the virus. The Adeno-X Purification Kit is not only faster than CsCl methods, it is also easier, safer, and just as effective.

A chromatographic method

The Adeno-X Maxi Purification Kit lets you purify adenovirus chromatographically, using an adsorbent filter membrane that selectively binds adenoviral particles based on their distinctive surface-associated properties (Figure 1). The membrane is housed in a small, single-use cartridge that fits securely on a disposable Luer-Lok syringe. For added convenience, the syringe-filter assembly comes completely pre-assembled and ready to use (Figure 2). This apparatus includes a one-way valve that makes it easier to load and wash the filter. The virus-containing medium is simply drawn into the syringe through the one-way valve and pushed through the purification filter cartridge. Here, the adenoviral particles are trapped and effectively removed from the solution. Once bound, the viral particles can be eluted with a small volume of 1X Elution Buffer (provided).

The purification protocol

The procedure is simple. After the cytopathic effect is complete, the cells are harvested, lysed, and centrifuged. The resulting supernatant is then treated with Benzonase Nuclease to digest nucleic acids, and cleared (clarified) through a syringe-tip pre-filter. Next, a syringe is used to push the virus-containing extract through the purification cartridge, where the adenoviral particles bind to the filter. The bound particles are then eluted with a small volume of buffer. The entire protocol, from harvest to purification, takes just 1-1.5 hours to complete (Figure 3). Aliquots of the purified, high-titer adenoviral stock can be stored in Elution Buffer at –70°C. Please note: All of these steps must be conducted under sterile conditions in a Biosafety Level 2 certified hood.

I. Introduction & Protocol Overview *continued*

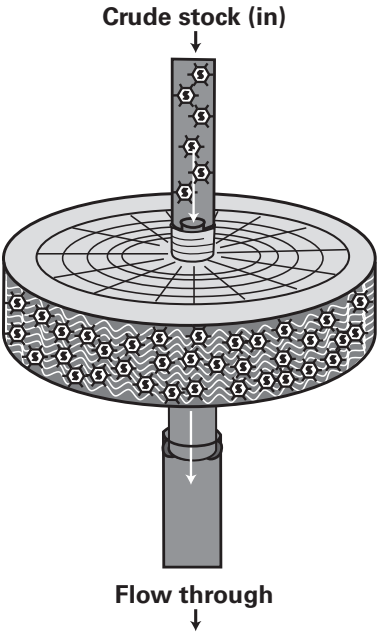

Figure 1. Principle of Adeno-X virus purification. The Adeno-X Purification Filter consists of several layers that selectively bind adenoviral particles as they pass through. The filter is enclosed in a small cylindrical cartridge that resembles a radial flow column, complete with entrance and exit ports. The Maxi purification cartridge is 3.5 cm wide and 1 cm thick.

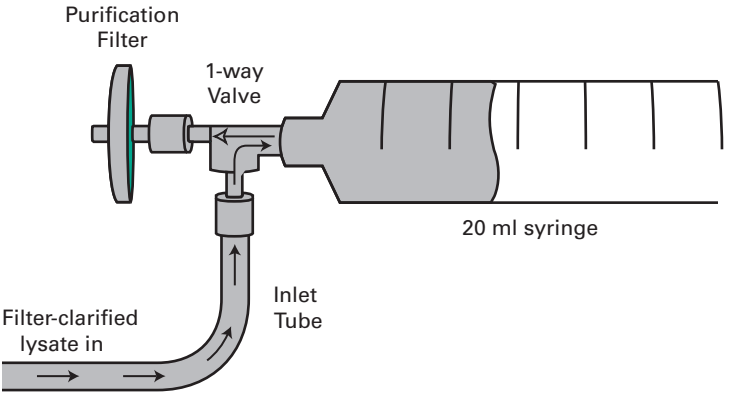

Figure 2. The Adeno-X Maxi Purification Assembly. The apparatus comes pre-assembled.

II. List of Components

Store Benzonase Nuclease at –20°C. Store all other components at room temperature.

The **Adeno-X Maxi Purification Kits** (Cat. Nos. 631532 & 631533) contain sufficient reagents for 2 and 6 Maxi purifications, respectively.

	Cat. No. 631532	Cat. No. 631533	
•	15 ml	2x15 ml	1X Equilibration Buffer
•	15 ml	2x15 ml	1X Dilution Buffer
•	60 ml	2x60 ml	1X Wash Buffer
•	12 ml	2x12 ml	1X Elution Buffer
•	40 µl	40 µl	Benzonase Nuclease (25 U/µl)
•	2	6	Syringe-tip Pre-filter
•	2	6	Adeno-X Maxi Purification Assembly
•	2	6	Syringe (5 ml)
•	2	6	Syringe (20 ml)

III. Additional Materials Required

The following materials are required but not supplied.

- **1X Formulation Buffer**
  2.5% glycerol (w/v), 25 mM NaCl, and 20 mM Tris-HCl, pH 8.0 (GTS buffer; Hoganson, *et al.,* 2002).
- **Tissue culture plates and flasks**
  (e.g. 10 or 15 cm plates, T75 or T175 flasks, or roller bottles)
- **Centrifuge**
  (Swinging-bucket and fixed-angle rotors compatible with 15 ml, 50 ml, and if needed, 100 ml centrifuge tubes)
- **Sterile 50 ml centrifuge tubes**
- **Optional:** For adenoviral stock titration, we recommend using Clontech's **Adeno-X Rapid Titer Kit** (632250), **Adeno-X qPCR Titration Kit** (Cat. No. 632252), or **Adeno-X GoStix™** (Cat. No. 632270).
- **Adeno-X 293 Cells** (Cat. No. 632271)
- **Optional:** If you plan to perform buffer exchange from elution buffer into formulation buffer, you may use PD10 Desalting Columns (GE Healthcare), Millipore Centriprep YM-50 Centrifugal Filter Unit (50,000 NMWL) or dialysis using Slide-A-Lyzer Cassette MW-10K MWCO (Pierce) according to the manufacturer's instructions.

IV. Safety & Handling of Adenoviruses

The protocols in this User Manual require the production, handling, and storage of infectious adenovirus. It is imperative to fully understand the potential hazards of and necessary precautions for the laboratory use of adenoviruses.

The National Institute of Health and Center for Disease Control have designated adenoviruses as Level 2 biological agents. This distinction requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The viruses packaged by transfecting Adeno-X 293 cells with the adenoviral-based vectors described here are capable of infecting human cells. These viral supernatants could, depending on your gene insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

For these reasons, due caution must be exercised in the production and handling of any recombinant adenovirus. **The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2, see the following reference:

- *Biosafety in Microbiological and Biomedical Laboratories*, (BMBL), 5th Edition (December 2009) U.S. Department of Health and Human Services, CDC, NIH.
  (Available at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm)

Biosafety Level 2:
The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete*. Details of the practices, safety equipment, and facilities required for Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with adenoviruses.

- Practices:
  - perform work in a limited access area
  - post biohazard warning signs
  - avoid generating aerosols
  - decontaminate potentially infectious wastes before disposal
  - take precautions with sharps (e.g., syringes, blades)
- Safety equipment:
  - biological safety cabinet, preferably Class II (i.e., a laminar flow hood with microfilter [HEPA filter] that prevents release of aerosols)
  - protective laboratory coats, face protection, double gloves
- Facilities:
  - autoclave for decontamination of wastes
  - unrecirculated exhaust air
  - chemical disinfectants available for spills

V. Adenovirus Purification Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. Test the Titer of the Adenoviral Stock

1. Determine the optimal amount of viral stock needed to infect your cells. To do this, test the viral titer in a small scale cytopathic effect assay, using culture conditions that closely approximate those that will be used for the actual adenoviral prep.
   a. Seed low passage Adeno-X 293 cells (Cat. No. 632271) on a 12-well tissue culture dish at a density of $1x10^5$ cells/cm^2, using 0.28 ml/cm^2 medium. At 1 ml/well, this is equivalent to ~$3.57x10^5$ cells/well.
   b. Place the cells at 37°C and 5% CO_2 while you dilute the adenovirus.
   c. Infect the cells with a range of adenoviral concentrations. If you know the titer of your stock, aim for a multiplicity of infection (M.O.I.) of 1-2. If you don't know the titer, infect the cells with a range of dilutions, starting with 1 µl of stock and serially diluting 3X from there.

      Note: We recommend using our **Adeno-X Rapid Titer Kit** (Cat. No. 632250) to quickly and easily titrate your viral stock.
   d. Incubate the cells at 37°C and 5% CO_2 until the cytopathic effect is complete; this should take approximately 3-5 days.

      Notes:

      - The cytopathic effect (CPE) refers to the morphological changes that the cells undergo after infection. Infected cells typically remain intact, but round up and may detach from the dish individually or in "grape-like" clusters that float in the medium. **Optimally, the purification assay should be performed when 50% of the cells are detached, and the remainder are attached but rounded**.
      - **Adeno-X GoStix** (Cat. No. 632270) can be used to monitor the culture prior to cytopathic effect (CPE) formation.

2. Use the results from the test titer to determine the optimal amount of virus to use in the actual purification prep. The full scale prep requires seeding several 150 mm cell culture dishes with $1.46x10^7$ cells/dish. Therefore, if 0.1 µl of adenoviral stock optimally infected the $3.57x10^5$ cells used to seed the test titer, you would need 4 µl of virus for each 150 mm dish in the actual prep.

V. Adenovirus Purification Protocol *continued*

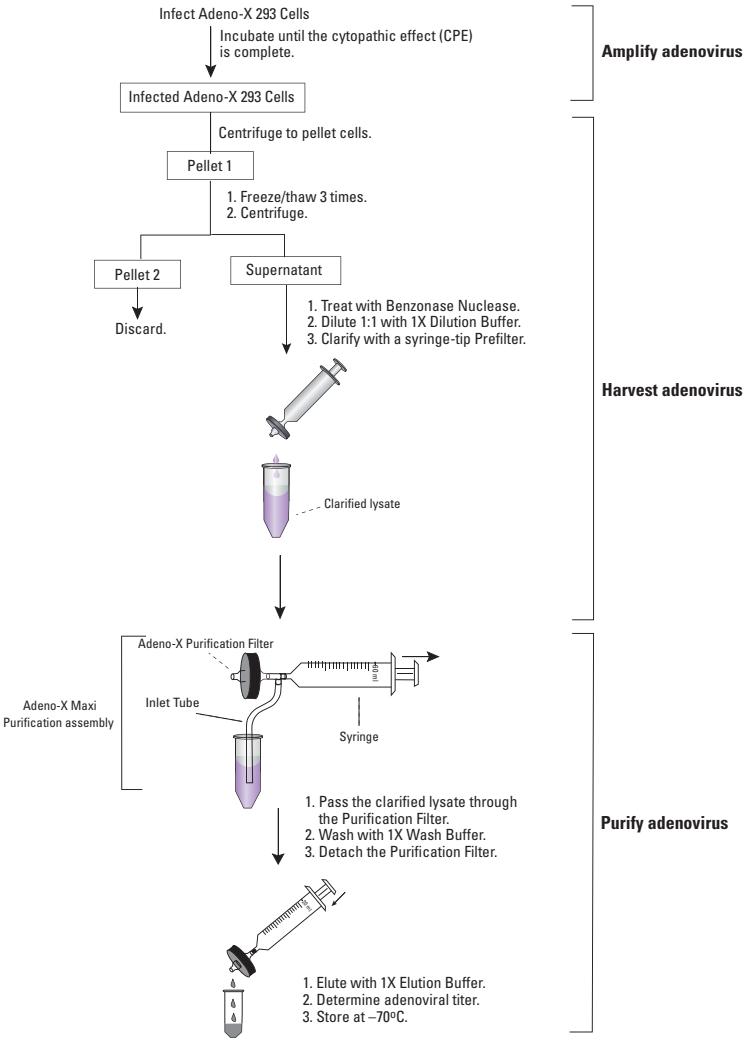

Figure 3. Overview of the Adeno-X Maxi Purification Protocol.

V. Adenovirus Purification Protocol *continued*

B. Amplify Adenovirus in Adeno-X 293 Cells

1. Detach low passage Adeno-X 293 cells with trypsin, wash, and count.
2. In a 50 ml conical tube, dilute the cells to a final density of 1.83×10^6 cells/ml in a total of 40 ml.
3. Add virus to the cells and mix.

   Note: Perform the test titer in Part A to determine the optimal amount of virus to add in this step.
4. Aliquot 33 ml of fresh medium onto each of five 150 mm cell culture dishes.
5. Aliquot 8 ml of virus-infected cells (from step 3) onto each of the five 150 mm cell culture dishes; make sure the cells are evenly distributed.

   Note: This is equivalent to 1.46×10^7 cells/plate.
6. Incubate the cells at 37°C and 5% CO_2 until the CPE is complete; this should take approximately 3-5 days.

   Note: Optimally, the purification assay should be performed when 50% of the cells are detached, and the remainder are attached but rounded.

C. Harvest Adenovirus (See Figure 3 for a procedural diagram)

1. Pellet the cells by centrifugation in a swinging-bucket centrifuge at 1,500 rpm (500 x g for Table-Top Beckman Centrifuge GH3.8 rotor) for 10 min.
2. Discard the supernatant.
3. Resuspend the pellet in 5 ml of fresh medium.

   Attention: Do not use PBS, as it inhibits some endonucleases. Endonuclease is used below to remove contaminating cellular nucleic acids from filter-clarified cell lysate.
4. Lyse the cells with three consecutive freeze-thaw cycles; briefly freeze the cells in a dry ice/ethanol bath, then thaw them in a 37°C water bath.
5. After thawing for the final time, centrifuge the lysate at 3,000 rpm (~1,500 x g for Table-Top Beckman Centrifuge GH3.8 rotor) for 5 min to pellet the debris.
6. Collect and save the supernatant in a sterile centrifuge tube. Discard the pellet.
7. Add 5 µl Benzonase Nuclease and incubate for 30 min at 37° C.

   Note: Nuclease treatment decreases the viscosity of the solution so that it can be drawn more easily through the Adeno-X Purification Filter.
8. Measure the volume of the sample with a pipet, then add an equal volume of 1X Dilution Buffer (approximately 5 ml).

   Note: The solution may turn slightly purple if you resuspended the original cell pellet in fresh medium (step 3).
9. Clarify the lysate by filtering it through the 0.45 µm Syringe-tip Pre-filter, using a 20 ml syringe (provided).

V. Adenovirus Purification Protocol *continued*

D. Purify Adenovirus

1. Equilibrate the Filter Assembly with 5 ml 1X Equilibration Buffer. To do this, place the inlet tube into the buffer and draw the buffer into the syringe by pulling back on the plunger. Press lightly on the plunger to push the buffer through the Filter at a flow rate of approximately 3 ml/min (this is approximately 1 drop/sec).

   Note: Do not force residual air through the Filter. To remove air from the syringe, disconnect the Filter, force the air out of the syringe with the plunger, then reposition the Filter on the syringe.
2. Place the inlet tube into the clarified lysate, and draw the lysate into the syringe.
3. Load the adenovirus onto the Purification Filter. This is done by pushing the lysate through the Filter at a rate of ~3 ml/min to allow the virus to bind.
4. Transfer the inlet tube to a sterile tube containing 20 ml 1X Wash Buffer. Push the Wash Buffer through the filter at a rate of ~3 ml/min.
5. Remove the Filter from the Assembly.
6. To elute the adenovirus, attach the Filter to a new 5 ml syringe containing 3 ml 1X Elution Buffer. Push 1 ml of Elution Buffer through the filter into a sterile 15 ml conical tube. Incubate the Filter at room temperature for 5 min, then push the remaining elution buffer through. Use residual air in the syringe to push any remaining virus through the Filter. Combine the eluate to obtain approximately 3 ml total.
7. Determine the adenoviral titer.

   Note: We recommend using the **Adeno-X Rapid Titer Kit** (Cat. No. 632250) or the **Adeno-X qPCR Titration Kit** (Cat. No. 632252).
8. The adenovirus can be used immediately, or aliquoted and stored at –70°C.

Note:

For improved long-term stability, and proper tonicity for *in vivo* applications, we recommend a buffer exchange of the eluted adenovirus into 1X Formulation Buffer.

1X Formulation Buffer:
2.5% glycerol (w/v), 25 mM NaCl, and 20 mM Tris-HCl, pH 8.0 (GTS buffer; Hoganson, *et al.,* 2002)

VI. References

Graham, F. L. & Prevec, L. (1991) Manipulation of adenovirus vectors. In *Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols.* Ed. Murray, E. J. (Human Press Inc., Clifton, NJ), pp. 109–128.

Hoganson, D. K., Ma, J. C., Asato, L., Ong, M., Printz, M. A., Huyghe, B. G., Sosnowski, B . A. & D'Andrea, M. J. (2002) Development of a stable adenoviral vector formulation. *Bioprocessing J.* **1**(1):43–48.

Hutchins, B. (2002) Development of a reference material for characterizing adenovirus vectors. *Bioprocessing J.* **1**(1):25–28.

This document has been reviewed and approved by the Clontech Quality Assurance Department.