Adenovirus Standard Purification Virakit™

Adenovirus 5 and Recombinant Derivatives

Protocol

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR HUMAN CLINICAL PROCEDURES.
Please read entire kit instructions before performing virus infection and purification.

Contains four single Adenovirus purification applications with the following components:		
	Catalog No. 003051	**Catalog No. 003054**
Components	**Quantity**	**Quantity**
30 ml Syringe	1	4
5 ml Syringe	1	4
Clarification Filter	2	8
Purification Filter Assembly	1	4
Dilution Buffer A	12.5 ml	50 ml
Wash Buffer B	25 ml	50 ml x 2
Elution Buffer C	5 ml	20 ml
Freeze Buffer D	5 ml	20 ml
Optional: 50 ul Benzonase®* *Virapur Cat. No. 003005		

1. Introduction – We make it easy

Virapur provides an easy and quick membrane based method for laboratory scale purification of adenovirus:

- **Adenovirus Standard Purification ViraKit™** (cat# 003051 & 003054), up to 10^{12} adenovirus particles which is the about the amount of adenovirus you should get from five 15cm cell culture dishes.
- **Adenovirus Mini Purification ViraKit™** (cat# 003058 & 003059), up to 10^{10} adenovirus particles which is about the amount of adenovirus you should get from one well of a 6 well plate. This kit is designed for small volume samples, quick analysis of new viral constructs, continuous monitoring of large scale bioprocesses, and small pilot experiments.

2. Overview and Precautions

a. Virapur's Adenovirus purification kit is designed to purify and concentrate your recombinant virus derived from Serotype 5. In order to perform animal studies and some *in vitro* studies with adenovirus, whether recombinant or wild type, it is usually necessary to purify the virus away from cellular contaminants and the expressed recombinant transgene. Purification results in concentrated virus in simple storage buffer that can be used for experimental studies.

b. Adenovirus purification using filter technology is effective and highly comparable to purification by cesium chloride gradient centrifugation. Cesium chloride density gradients have been the time-tested way to purify adenovirus, but this cumbersome and tricky procedure involving large expensive equipment can take days. Our Adenovirus Purification ViraKit™ utilizes ion exchange chromatography technology, identical to procedures used to purify clinical grade adenovirus for human trials. Virus obtained through our method is appropriate for *in vitro* and *in vivo* studies and is of high titer and high purity. The ViraKit™ surpasses previous methods of adenovirus purification, saving the user time, money and generating an end product of equal quality.

c. Our ViraKit™ now purifies virus from the infected cell pellet, increasing the efficiency and speed of the virus extraction and purification procedure. The kit will purify up to 1 x 10^{12} viral particles from the number of infected cells indicated.

d. The amount of adenovirus purified with this kit depends on how well your virus grows. Viruses may have reduced yield if the foreign transgene effects cell machinery and the efficiency of virus packaging. Virus isolates that produce less than 1 x 10^{10} particles per ml in the unpurified total cell and supernatant extracts may give lesser yields in the Adenovirus Purification ViraKit™.

e. PRECAUTIONS: This kit permits the quick purification of Adenovirus, an infectious agent which according to the National Institutes of Health (NIH) guidelines must be handled under Biosafety Level 2 safety precautions (*see:* http://www4.od.nih.gov/oba/rac/guidelines_02/NIH_Guidelines_Apr_02.htm). Although adenovirus recombinants are 99%+ replication incompetent, research viruses may over express their transgene, the transgene may be toxic or be involved in cellular regulation. All these unknowns indicate that a conservative safety approach should be followed and Biosafey Level 2 practices should be used when appropriate. Wear hand, eye, face and body personal protection devices when manipulating adenovirus within a Class II Biosafety cabinet. Dispose of infected liquid and solid wastes according to NIH guidelines. Provider of kit takes no responsibility for improper use of kit.

f. STORAGE: The **Adenovirus Purification ViraKit™** should be stored at room temperature between 10^{O}C-25^{O}C

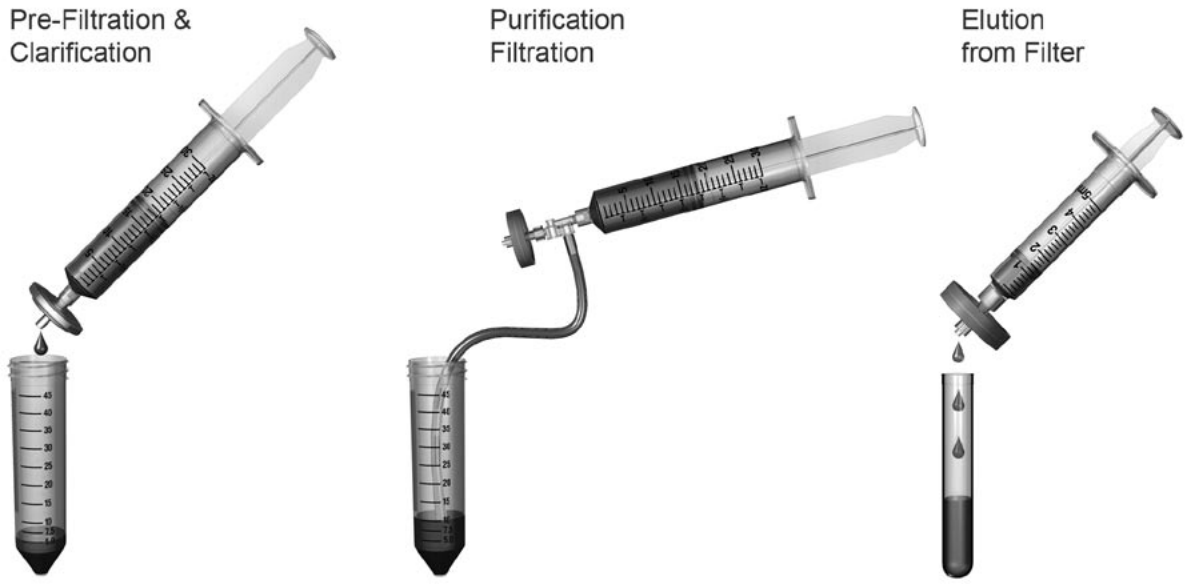
3. User Provided Equipment and Supplies

- Table top centrifuge capable of spinning 100 to 200 ml at 2000 RPM
- Class II Biosafety Cabinet for Adenovirus manipulation
- Sterile Centrifuge tubes or bottles 15 ml, 50 ml or if needed 200-250 ml
- Sterile PBS (50 ml)
- Two 250 ml sterile clean bottles
- Sterile Microfuge tubes if desired
- Microfuge capable of 14,500 RCF if desired.
- Benzonase® Nuclease, Virapur Catalog number 003005, or Novagen Catalog numbers 70664-3, 70746-4, 70746-3.[1]

4. Summary of the Purification Procedure

a. Virus can be purified easily and quickly. Two to four days after infection of 293 cells with your virus, the cells display cytopathic effect. Infected cells are harvested from the container and centrifuged away from the tissue culture media. After several rounds of freeze/thaw, lysed infected cells are pelleted in the centrifuge and the supernatant extracted from the cells is briefly incubated with Benzonase® Nuclease to digest cellular DNA and RNA. The supernatant is filtered through a syringe-tip preliminary filter to remove debris. Then the supernatant is passed over the virus purification filter where adenovirus particles are preferentially adsorbed to the filter. The filter is washed with buffer and virus is eluted off the filter with a small syringe containing elution buffer. You can accomplish this procedure within 90 minutes and be ready to take your virus into experiments!

b. Virus can be further purified and exchanged into the formulation buffer for storage to –80^{O}C.



5. Determine amount of adenovirus stock to infect your cells

a. Each virus stock varies slightly in infectious titer.
   i. Preparations recently derived from a plaque may be at a titer of 10^6 to 10^7 infectious units (IU) per ml.
   ii. Routinely passaged adenovirus stocks may be at titer of 10^8 to 10^9 IU per ml
   iii. Purified stocks could be as high as 10^{10} IU per ml.

b. Aim to infect 293 cultures with a multiplicity of 3 IU per cell. Do not over-infect your cultures with too much virus because the cells will not produce high virus titers. One approximation is to use about 100 µl of crude infected virus stock (a.ii., above) to infect a 10 cm dish.

6. Initial Growth of (Human Embryonic Kidney) 293 Cells in Tissue Culture Vessels

a. 293 cells or their variants can be grown in tissue culture treated flasks. For the production of adenovirus, cells should be at a relatively early passage level and be 95-100% confluent. They should be kept on a regular passage program. Cells should not remain confluent for more than a few days. Cells that have remained confluent and unpassaged for more than several days can be passed at least one time at a low seeding density to reset the cells into an active growing state. Cells that have been confluent for more than 24 hours will give reduced virus yields if infected.

Cell Culture vessels to set up for one Adenovirus Standard Purification ViraKit™:

Goal is 1- 2 x 10^8 total infected cells	Square surface area per vessel (cm^2)	Maximum number of infected vessels per single ViraKit™	Media Volume per Vessel During Growth and Infection (ml)
T flask T75	75	11	15
T flask T225	225	4	45
10 cm dish	78	11	10
15 cm dish	176	5	30
Roller bottle 850	850	1	150

b. Cells should be seeded into the tissue culture flask at approximately 4 to 6 x 10^4 cells per cm^2. Recommended media: DMEM, high glucose with 4 mM glutamine and 10% Fetal Calf Serum plus antibiotics if desired. The cell monolayer will become nearly confluent within approximately 2 - 4 days. You will need to decide when the monolayer is 95-100% confluent. It is now time to infect with your adenovirus.

c. Your sterile adenovirus can now be added to the culture to initiate the infection.

d. Replace the vessels back in the incubator for 2-4 days until cytopathic effect is complete. Cells will progress from Day 2 through Day 4 of infection from isolated rounding up of cells to clumps of floating cells. Cultures should be harvested when all of cells are rounded and few are floating. This is usually at Day 3

7. Harvest and Centrifugation of Infected Cell Lysate

a. At harvest, ensure that all the cells are detached from the culture vessel by shaking the vessel or pipetting the cells. Pool the cells and media into one or more sterile capped tubes (50 ml). Spin at 1250 rpm for 10 to 15 minutes in a swinging bucket or stationary rotor equipped centrifuge. Reserve 10 ml of the supernatant, and discard the rest of the supernatant. Resuspend the pellet in the remaining 10 ml of supernatant and freeze/thaw the pellet for three consecutive cycles. A cycle consists of placing the pellet in a dry ice/ethanol bath or in the -80^{O}C freezer for a complete freeze and then into the 37^{O}C water bath until thawed.

b. After the final thaw, centrifuge the lysate at 2000 rpm for 10 minutes to initially clarify the solution. Retain the supernatant in a new 50 ml tube. This lysate supernatant will be further clarified in Step 9.

8. Recommended Benzonase® Nuclease

a. Before running the virus over the purification filter, you can remove much of the contaminating cellular DNA by adding Benzonase® Nuclease to the unpurified virus solution. Many laboratory grades of Bovine Pancreatic DNase will work, but one that is proven low in endotoxin may be preferred. As an optional item, you may obtain Benzonase® Nuclease directly from Virapur in the correct aliquot for this kit, Cat # 003005.

b. Add 10 µl Benzonase® to the 10 ml of clarified virus fluid. Incubate at 37^{O}C for 30 minutes.

9. Pre-filtration and Further Clarification of Virus Lysate

a. Attach the **Clarification Filter** to the **30 ml syringe** provided and set aside. Two **Clarification Filters** are provided for each application in case your first **Clarification Filter** becomes clogged and resists flow.

b. Add an exact and equal volume of **Dilution Buffer A** to your lysate supernatant in a 50 ml conical – you should have approximately 10 ml of initially clarified lysate supernatant from Step 7 above. Measure the volume of the lysate supernatant with a 10 ml pipette to determine the exact amount of **Dilution Buffer A** to add. Gently mix. The solution should become more basic if your media contains phenol red.

c. Load the mixture into the **30 ml syringe** with the **Clarification Filter** attached and gently push the mixture through the **Clarification Filter** into a new 50 ml conical at a rate of approximately 5 ml per minute. If your first **Clarification Filter** becomes clogged, detach and discard it. Reattach a new **Clarification Filter** and continue.

Figure 1

d. Set your clarified virus lysate aside. You are now ready to use the virus **Purification Filter Assembly**.

10. Purification Procedure using the virus Purification Filter Assembly

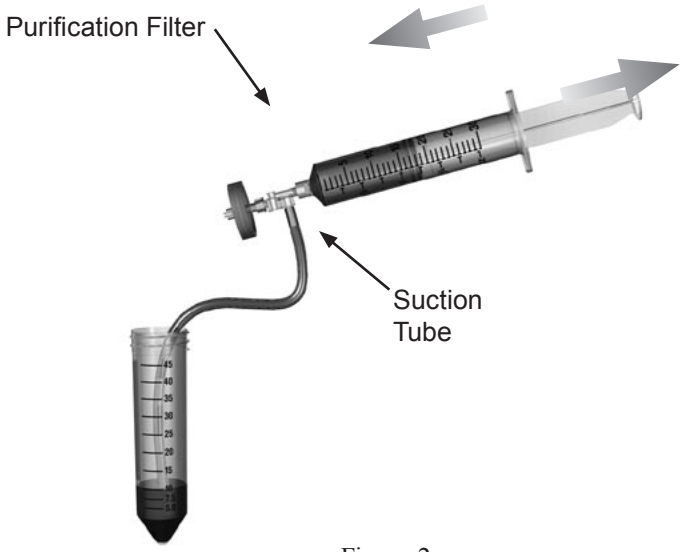


Figure 2

a. Pre-wet the **Purification Filter** with PBS by first placing approximately 10 ml of PBS into a 50 ml conical and inserting the thin plastic suction tube *(Figure 2)*. Pull back on the syringe plunger to draw PBS into the syringe, and push forward on the plunger to push the PBS through the **Purification Filter** at a rate of 5 ml per minute into a waste container. Aim for about one drop exiting the filter per second. Do not pass air over the filter. If air remains in the syringe, remove the syringe from the fitting by twisting it off and fully empty the syringe. Reattach the syringe on the luer lock connection.

b. Now place the suction tube into the 50 ml conical containing your clarified virus lysate. Draw all the lysate into the syringe then slowly push the plunger so your clarified virus lysate passes over the **Purification Filter** at the rate of 5 ml per minute or one drop per second. Your virus is sticking to the filter as the lysate passes over it. Collect the flow through in a new 50 ml conical.

c. Place the suction tube into a sterile container containing 20 ml of **Wash Buffer B**. Draw 20 ml of **Wash Buffer B** into the syringe and slowly push **Wash Buffer B** over the **Purification Filter** at a rate of 5 ml per minute.

d. Detach the **Purification Filter** from the two-way valve. Draw 3 ml of **Elution Buffer C** into the new **5 ml syringe** provided in the kit. Attach the 5 ml syringe to the **Purification Filter** and position the assembly over a convenient sterile collection tube *(Figure 3)*. Slowly add about 1 ml of **Elution Buffer C** to the **Purification Filter,** incubate at room temperature for one minute and then slowly push the remaining 2 ml of **Elution Buffer C** into the collection tube. You can use air in the syringe to push all remaining fluid in the **Purification Filter** into the collection tube.

Figure 3

e. Your virus is now in a moderate salt solution and can be stored. Add glycerol to 10% final concentration or add a volume of **Freeze Buffer D** equal to 25% of the volume of your eluted virus to achieve a sugar concentration which will be cryoprotective. Virus should be aliquoted, snap frozen in dry ice and stored at -70^{O}C or lower.

DISCLAIMER

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1. Benzonase® Nuclease is manufactured by Merck KGaA and its affiliates. The technology is covered by US Patent 5,173,418 and EP Patent 0,229,866. Nycomd Pharma A/S (Denmark) claims worldwide patent rights to Benzonase® Nuclease, which are licensed exclusively to Merck KGaA, Darmstadt, Germany. Benzonase® is a registered trademark of Merck KGaA, Darmstadt, Germany.