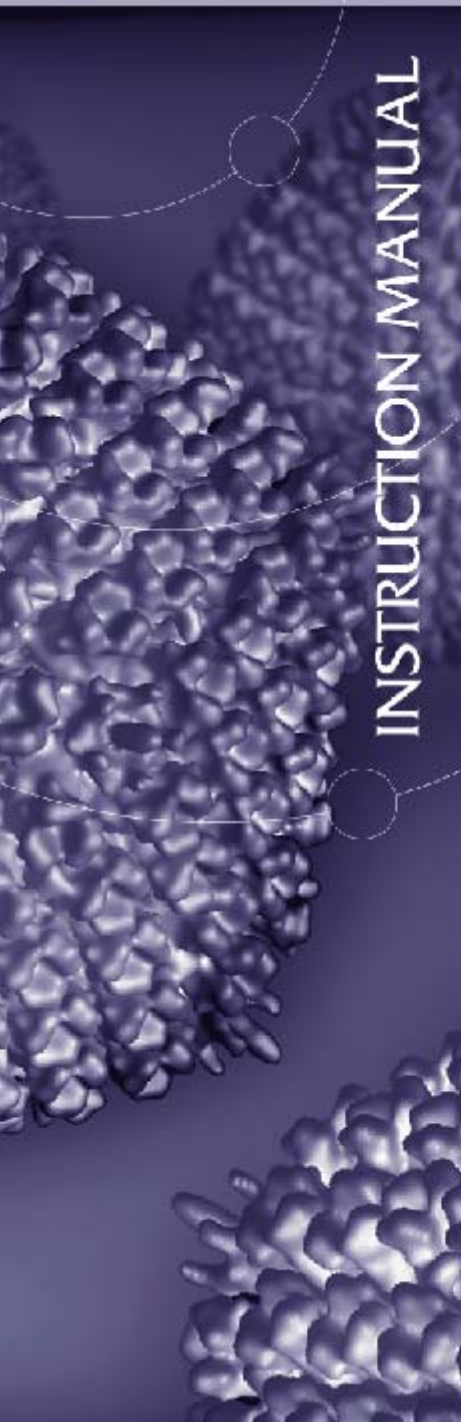


ADENOVIRUS PURIFICATION KIT

Adenopure LS[®]

Large Scale

INSTRUCTION MANUAL



Dear Adenopure LS® Kit User,

Thank you for choosing Puresyn, Inc. as your supplier for separation and purification products. Puresyn is dedicated to developing, manufacturing, and marketing state-of-the-art products for the purification of viruses, nucleic acids, and other biological molecules. Since our inception, Puresyn has performed over 400 purification processes for more than 175 companies in the major gene delivery vehicles: supercoiled plasmids, synthetic oligonucleotides, recombinant adenovirus, and adeno-associated virus.

You have chosen an elite product based on Puresyn's proprietary buffer formulations. The buffer formulations contained in this Adenopure LS® Kit produce superior results, demonstrated in the Scientific Support section of this manual. This advanced Adenovirus Purification Kit produces ultrapure product. We guarantee you will be satisfied with the Adenopure LS® Kit. If you do not agree that this Kit is efficient, convenient, and easy to use, call us at 1-888-245-9182.

We stand ready to help in all your scientific endeavors. Feel free to contact us with any questions or comments you have.

A handwritten signature in black ink, reading "Dennis M. Flynn". The signature is written in a cursive, flowing style.

Dennis M. Flynn
President



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INTRODUCTION AND BACKGROUND

Puresyn's Adenopure LS[®] Kit couples a *virus-binding module* and proprietary buffer formulations to provide rapid and simple isolation of highly purified adenovirus preparations suitable for *in vitro* biological assays in approximately two hours. *Virus-binding modules* are based on membrane adsorber technology that attaches ion exchange functional groups to the inner surface of synthetic microporous membranes. Membrane adsorber ion exchange modules provide many benefits including high binding capacity at high flow rates, and scalability.

The Adenopure LS[®] Kit differs from the original Adenopure[®] Kit in several ways including scale, cell lysis method, sample delivery mechanism and inclusion of a clarification filter. The original Adenopure[®] Kit is capable of purifying up to 5×10^{12} viral particles whereas the Adenopure LS[®] Kit is capable of purifying up to 1×10^{14} viral particles. In the original Adenopure[®] Kit procedure, cell lysis is accomplished by subjecting the sample to three (3) freeze-thaw cycles, and a syringe-based Adenopump[®] is used to deliver the sample to the *virus-binding module*. In order to accommodate the larger volumes involved at the scale of the Adenopure LS[®] Kit, cell lysis is accomplished by subjecting the sample to a rapid, volume-independent chemical lysis step and a peristaltic pump is used to deliver the sample to the *virus-binding module*. In addition, the Adenopure LS[®] Kit includes an optimized clarification filter for convenience.

The seven basic steps in the Adenopure LS[®] Kit procedure include:

- Propagation and harvest of adenovirus infected cells.
- Benzonase[®] treatment and chemical cell lysis of the infected cells.
- Clarification of the infected cell lysate to remove cell debris.
- Addition of 10X Dilution Buffer to adjust the conditions of the infected cell lysate for binding of adenovirus to the *virus-binding module*.
- Loading the infected cell lysate onto the *virus-binding module*.
- Washing the adenovirus bound to the *virus-binding module*.
- Elution of purified adenovirus.

CONTRAINDICATIONS OF ADENOPURE LS[®] KIT USE

As with any chromatographic purification process, Adenopure LS[®] Kit purification yield will suffer in severe under-load conditions. For the Adenopure LS[®] Kit to function efficiently, a minimum of 1×10^{13} viral particles should be present per 1000ml of infected cell suspension. The Adenopure LS[®] Kit has been optimized for adenovirus type 5 vector purification, and the performance of the kit with non-adenovirus type 5 constructs cannot be predicted or guaranteed. The performance of the Adenopure LS[®] Kit with adenovirus type 5 vectors that have modifications in viral capsid proteins resulting in a change in the charge density of the virions also cannot be predicted or guaranteed.

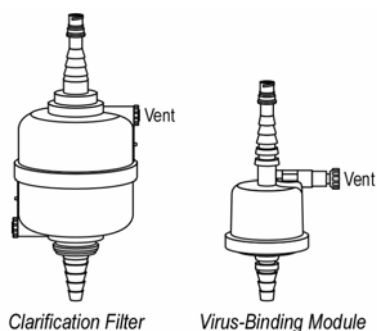
SHIPPING AND STORAGE

The Adenopure LS[®] Kit is shipped at ambient temperature. Upon arrival, store the supplied stock of Benzonase[®] at -20°C. Store all other Adenopure LS[®] Kit components at room temperature. The shelf life of the Adenopure LS[®] Kit when stored properly is at least 12 months.

ADENOPURE LS[®] KIT COMPONENTS

There are ten (10) components included in this Adenopure LS[®] Kit. Prior to use, please familiarize yourself with each component and its use. If any component is missing or damaged, contact Puresyn's Technical Support at 1-888-245-9182 for replacement.

- 600cm² 0.45µm Clarification Filter (1)
- Virus-Binding Module (1)
- Feed Tubing and Adaptor (1)
- 10X Dilution Buffer (270ml)
- Equilibration Buffer (270ml)
- Wash Buffer (270ml)
- Elution Buffer (50ml)
- Chemical Lysis Reagent (3.5ml)
- Benzonase[®] Stock (60,000 U)
- 60cc Syringe (1)



ADDITIONAL MATERIALS REQUIRED

- Peristaltic pump capable of a 50ml/minute flow rate with a pump head able to accommodate either 0.25" or 0.50" O.D. tubing
- 50ml sterile conical tubes
- Ring-stand/clamp
- 37°C water bath or incubator
- Sterile 1000-2000ml bottles
- 100ml graduated cylinder
- 1000ml graduated cylinder
- Timer

ADENOVIRUS PROPAGATION AND HARVEST

The following discussion of adenovirus propagation is for guideline purposes only. Specific adenovirus constructs may need to be propagated differently.

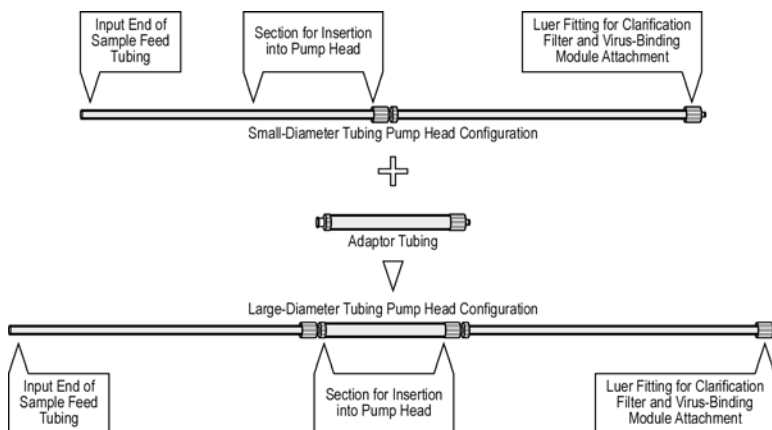
Wild type adenovirus or adenovirus recombinants are usually propagated in the HEK293 cell line (Graham, et al.). HEK293 cells are maintained in a 37°C, 5% CO₂ incubator in DME medium supplemented with 10% fetal bovine serum.

For viral propagation, HEK293 cells may be seeded into 150cm² plates or flasks. When the HEK293 cells reach a confluency of approximately 80% they are infected with the adenovirus stock of interest at a multiplicity of infection of 100 (as determined by HPLC analysis or OD_{260nm} readings) in DME medium supplemented with 2% fetal bovine serum (20ml/plate or flask). Infected cells are maintained in a 37°C, 5% CO₂ incubator until harvest. Infected cells are harvested at 2 to 5 days post-infection (usually when full cytopathic effect is observed). If necessary, infected cells may be detached from the tissue culture plates or flasks by pipetting or by using a cell scraper.

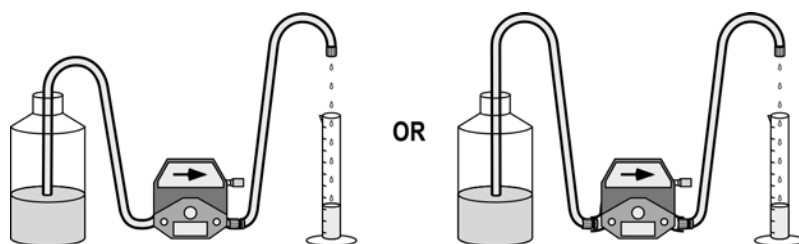
For an average adenovirus construct, fifty (50) 150cm² plates or flasks of infected cells will yield 1 x 10¹³ to 1 x 10¹⁴ viral particles per 1000ml of media; the appropriate amount of starting material for one (1) Adenopure LS[®] Kit purification procedure. After harvest, infected cells and media may be stored short-term at 4°C or long-term at -70°C.

PERISTALTIC PUMP CALIBRATION

Notes: (a) Familiarize yourself with the operation of your peristaltic pump. (b) The sample feed tubing provided with the Adenopure LS[®] Kit is configured for use with peristaltic pump heads requiring 0.25" O.D. tubing. Add the adaptor section of tubing if your peristaltic pump head requires 0.50" O.D. tubing (see figure below).



1. Pass the appropriate section of feed tubing through the peristaltic pump head and place the feed end of the tubing into a container filled with 200ml of H₂O.
2. Place the end of the feed tubing with the luer fitting into a 100ml graduated cylinder.



3. Turn on the peristaltic pump and adjust it to a flow rate of 50ml/minute using a timer.
4. Drain the feed tubing by pumping air through it, then turn off the peristaltic pump. Leave the feed tubing in place as it will be used in the Clarification and Purification steps described below.

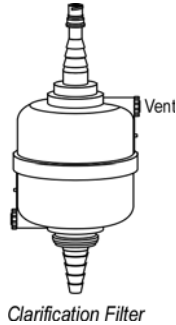
BENZONASE® TREATMENT & CHEMICAL LYSIS OF SAMPLE

Note: The amount of Benzonase® utilized in this step is based on fifty (50) 150cm² plates or flasks of infected cell suspension (1000ml of suspension).

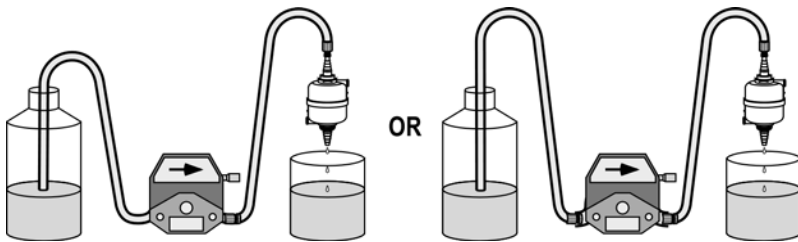
1. If the infected cell suspension has been frozen or if it has been stored at 4°C, it must be uniformly warmed to 37°C prior to the commencement of this procedure.
2. Pipette 2.4ml of Benzonase® into the 1000ml infected cell suspension and gently swirl to mix.
3. Incubate the infected cell suspension for 10 minutes at 37°C in a water bath.
4. Pipette 3ml of chemical lysis reagent (Fitchmun, et al.) into the infected cell suspension and gently swirl to mix.
5. Incubate the infected cell suspension at 37°C for 25 minutes, gently swirl to mix, and incubate at 37°C for an additional 25 minutes.

CLARIFICATION OF SAMPLE

1. Connect the clarification filter to the feed tubing using the attached luer fitting.



2. Clamp the clarification filter in a ring stand and position it above a collection vessel large enough to accommodate the crude cell lysate. Place the feed end of the tubing into the container filled with the crude cell lysate.



3. Open the top vent screw on the clarification filter and turn on the peristaltic pump calibrated to 50ml/minute. When all of the air is displaced from the clarification filter by the crude lysate, close the vent screw. Pass all of the crude cell lysate through the clarification filter collecting the clarified cell lysate.
4. Tip the feed container when it is nearly empty to load as much of the crude cell lysate as possible.
5. When the feed container is empty, keep the peristaltic pump on to push air through the clarification filter to drain it of all crude cell lysate.
6. After the clarification is complete, turn off the peristaltic pump. To relieve any built up pressure, slowly disconnect the clarification filter from the feed tubing luer fitting. Dispose of the clarification filter in an appropriate biohazard waste receptacle.
7. Leave the feed tubing in place as it will be used in the Adenovirus Purification step described below.

DILUTION OF SAMPLE

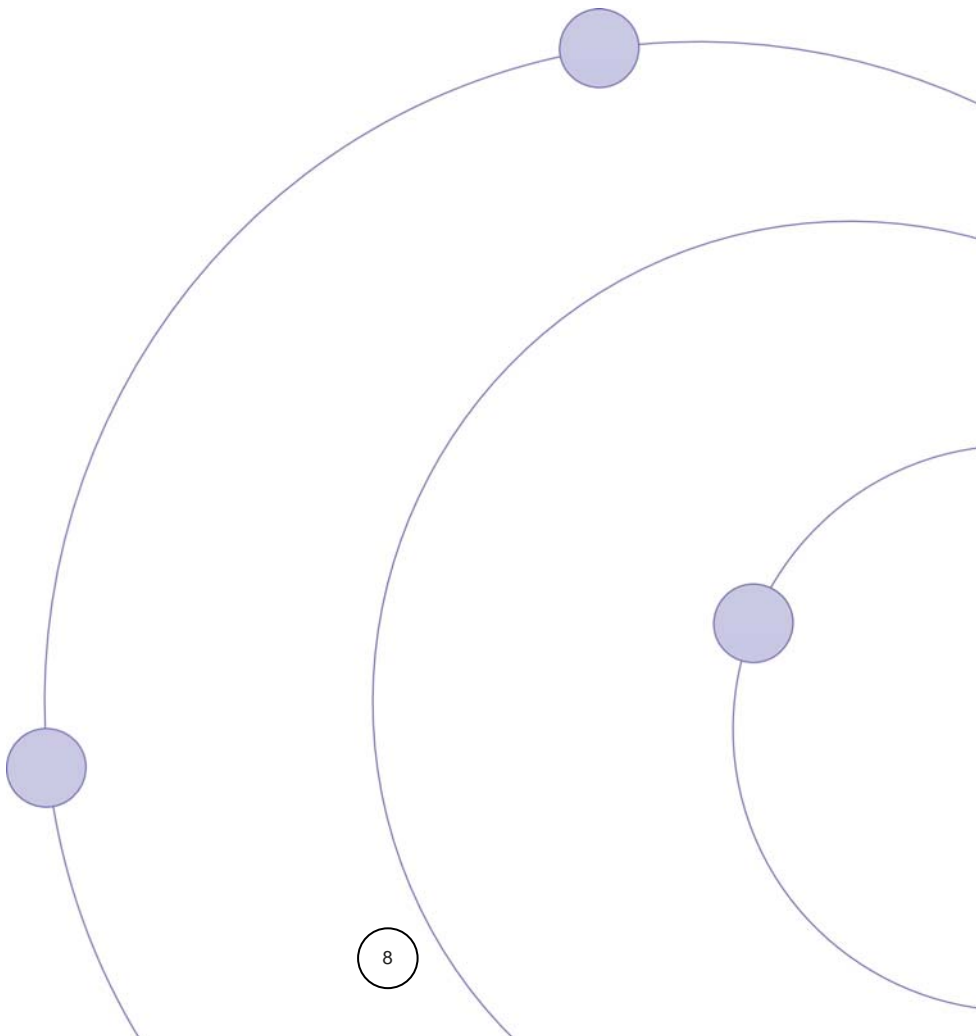
Note: Accurately measure the volume of the clarified lysate with a graduated cylinder. Accurate volume determination of the clarified lysate is critical, as the addition of the incorrect amount 10X Dilution Buffer will lead to poor binding of adenovirus to the *virus-binding module* of the Adenopure LS[®] Kit.

1. **Dilute:** Determine the amount of 10X Dilution Buffer required using the following formula:

Volume of clarified cell lysate \div 9 = Volume of 10X Dilution Buffer required.

Add the required amount 10X Dilution Buffer to the clarified cell lysate and mix by gently swirling.

2. Proceed to Adenovirus Purification.



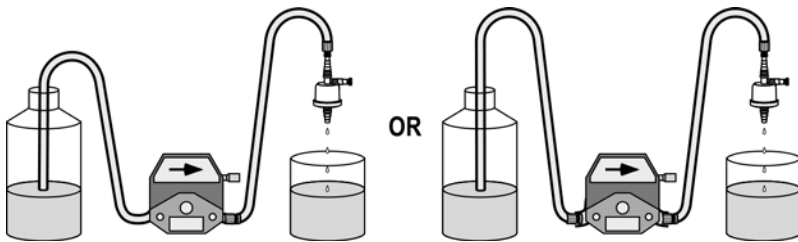
ADENOVIRUS PURIFICATION

1. Fill the supplied 60cc syringe with 30ml of Elution Buffer and 30ml of air. Set aside for the elution step.
2. Connect the *virus-binding module* to the feed tubing using the attached luer fitting. Clamp the *virus-binding module* in a ring stand and position it above a collection vessel.



Virus-Binding Module

- Notes:** (a) Once the *virus-binding module* is wet in the equilibration step, do not allow it to dry out between steps. (b) During the Adenopure LS[®] purification procedure, try to minimize the amount of air passing over the *virus-binding module* by stopping the peristaltic pump prior to air entering the feed tubing end.



3. **Equilibrate:** Place the free end of the feed tubing in the Equilibration Buffer container. Open the vent screw on the *virus-binding module* and turn on the peristaltic pump calibrated to 50ml/minute. When all of the air is displaced from the *virus-binding module* by the Equilibration Buffer, close the vent screw. Equilibrate the *virus-binding module* by passing the remainder of the Equilibration Buffer through it. Turn off the peristaltic pump prior to air entering the feed tubing end.
4. **Load:** Place the free end of the feed tubing in the container containing the diluted sample. Turn on the peristaltic pump and pump the diluted sample over the *virus-binding module* at 50ml/minute. Turn off the peristaltic pump prior to air entering the feed tubing end.
5. **Wash:** Place the free end of the feed tubing in the Wash Buffer container. Turn on the peristaltic pump and wash the *virus-binding module* by passing 250ml of Wash Buffer through it at 50ml/minute. Turn off the peristaltic pump prior to air entering the feed tubing end.

- Elute:** Disconnect the *virus-binding module* from the feed tubing luer fitting and reattach the *virus-binding module* to the 60cc syringe containing Elution Buffer prepared in step 1. Collect all material at this step as one fraction as it contains the purified adenovirus. Slowly pass 15ml of Elution Buffer through the *virus-binding module*. Incubate the *virus-binding module* for 5 minutes at room temperature before continuing. Slowly pass the remainder of the Elution Buffer through the *virus-binding module* and push the air in the syringe through as well to expel all remaining Elution Buffer from the module.
- Dispose of the feed tubing and *virus-binding module* in an appropriate biohazardous waste receptacle.



POST-PURIFICATION GUIDELINES

Adenopure LS[®] purified adenovirus preparations should be 0.2 μ m syringe-filtered prior to use in any tissue culture or animal experiments. Adenopure LS[®] purified adenovirus preparations may be stored at 4°C for short-term use (1 to 2 days), or at -70°C for long-term storage. Repeated freeze-thaw cycles are not recommended.

If a particular storage or formulation buffer is desired for the final adenovirus preparation, the purified adenovirus may be dialyzed into the buffer of interest. A small-scale tangential flow filtration (TFF) device may also be used for buffer exchange and/or additional sample concentration.

QUANTITATION OF ADENOVIRUS

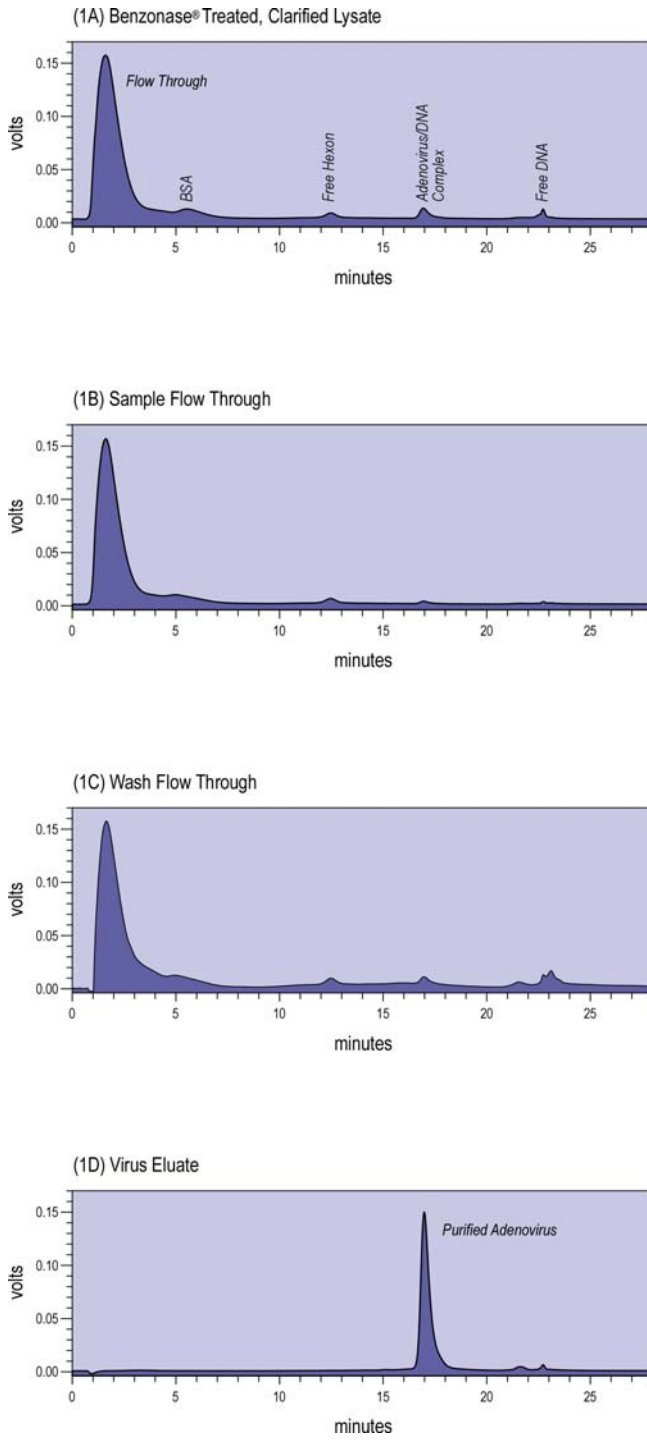
Purified adenovirus may be quantitated using several methods including HPLC (Shabram, et al.) and UV spectrophotometry (Maizel, et al.). Biological activity may be quantitated using several methods including plaque and CPE assays and gene expression specific bioassays.

SCIENTIFIC SUPPORT

Successful purification of adenovirus from crude cell lysate is a challenging problem. Crude cell lysate typically contains contaminating cellular proteins and nucleic acids as well as excess viral components not incorporated into intact progeny virions. Additionally, purification processes must deal with typical tissue culture media additives such as fetal bovine serum components.

A series of HPLC chromatograms of fractions collected during the purification process are presented in **Figure 1** to illustrate the functionality of the Adenopure LS[®] Kit. **Figure 1A** shows an HPLC chromatogram of adenovirus-infected crude cell suspension that has been Benzonase[®] treated, chemically lysed and clarified. Under the chromatographic conditions employed in this analysis, peaks resolve for cellular proteins and other debris (flow through), BSA, free adenovirus hexon, adenovirus, adenovirus-DNA complex and free DNA. **Figure 1B** shows that the majority of free cellular proteins, BSA, free adenovirus hexon and free DNA do not bind to the *virus-binding module* of the Adenopure LS[®] Kit under the buffer conditions utilized, and are present in the sample flow through fraction. **Figure 1C** shows that the remainder of free cellular proteins, BSA, free adenovirus hexon and free DNA are removed from the *virus-binding module* of the Adenopure LS[®] Kit in the wash step under the buffer conditions utilized. **Figure 1D** shows the eluted adenovirus as a uniform peak free from other contaminants.

Figure 1: Adenopure LS[®] Kit HPLC Chromatograms



TROUBLESHOOTING

Problem	Cause	Solution
No virus yield	Adenopure LS [®] procedure was not followed	Read and follow Adenopure LS [®] instructions
	Virus stock used for infection is not viable	Obtain a viable virus stock
Low virus yield	Virus-binding module under-loaded due to low virus yield	Re-optimize virus production
	Flow rate through virus-binding module too fast	Slow flow rate down to 50ml/minute
	Air pushed through virus-binding module during purification	Avoid pushing air through the virus-binding module
	Elution step performed too fast	Slow flow rate down during elution step
	Wrong buffers used during procedure	Read and follow Adenopure LS [®] instructions
	Benzonase [®] under- or over-digestion	Read and follow Adenopure LS [®] instructions
	Virus-binding module allowed to dry out during procedure	Minimize pauses during the procedure
	Buffer left in virus-binding module during elution	Collect all buffer from virus-binding module during elution
Virus-binding module clogs	Too much residual cellular DNA	Increase Benzonase [®] digestion time
Purified adenovirus contains a high level of cellular DNA	Benzonase [®] treatment incomplete, due to incorrect incubation time or temperature	Follow instructions for Benzonase [®] digestion step or increase Benzonase [®] digestion time
	Extract from too many plates of infected cells being purified	Lower the amount of extract being purified to (50) 150cm ² plate equivalents
	Benzonase [®] stock inactive	Store Benzonase [®] at recommended temperature

CURRENT PURIFICATION PRODUCTS FROM PURESYN

- Adenopure[®] Adenovirus Purification Kit
- Adenopure LS[®] Large Scale Adenovirus Purification Kit
- PolyFlo[®] Resin
- Pre-packed columns with PolyFlo[®] resin designed for small to gram-scale plasmid purification
- Contract services for fermentation, supercoiled plasmid purification, RNA purification and oligonucleotide purification
- Adenoserve[®] contract services for adenovirus propagation and purification
- Vectopur[®] purified recombinant adenovirus type 5 constructs

Visit our website for product updates at www.puresyn.com



Technical Support

Representatives from Puresyn are available via phone or e-mail for technical support Monday through Friday from 8:30 am to 4:30 pm Eastern Standard Time. Please call 888-245-9182 and ask for technical support, or write to:

Technical Support
Puresyn, Inc.
87 Great Valley Parkway
Malvern, PA 19355

You may also contact us via fax at 610-640-0808, or e-mail Adenopure@puresyn.com

Important Notice

The components of this adenovirus purification kit are intended for **research laboratory use only** and should not be used for any human or animal diagnostic or therapeutic application.

Warning: Utilization of this Kit exposes the user to potentially hazardous biological agents, i.e. recombinant adenovirus, and may generate biohazardous waste material. Wear safety glasses, gloves, and a lab coat when using this Kit. Follow established safety protocols for handling of biological agents and disposal of biohazardous waste as determined by federal, state and local regulations. It is the user's responsibility to consult the applicable MSDS(s) before using this Kit.

References

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Trademarks and Patents

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Acknowledgements

The cover figure shows the three-dimensional structure of the human adenovirus type 5 virion at 15Å resolution, as obtained by three-dimensional reconstruction from cryo-electron microscopy images. Only short portions of the fibers are properly reconstructed because the fibers do not follow icosahedral symmetry. For a review of the methodologies utilized in three-dimensional reconstruction of icosahedral viruses, see Baker, et al. Figure courtesy of Carmen San Martin, Ph.D., and Roger M. Burnett, Ph.D., The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104.

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