# Adenopure®

ADENOVIRUS PURIFICATION KIT

INSTRUCTION MANUAL

# Dear Adenopure® Kit User,

*Puresyn, Inc is a separations and purification company that develops, manufactures and markets state-of-the-art products and services. We exceed our competition in quality, service and continued support which is why we are well known.*

*Puresyn is a leading supplier of custom contract manufacturing services for production and purification of plasmids for research, development and pre-clinical requirements. Puresyn markets and sells large-scale bioseperations systems, columns and chromatography media to leading drug and biotechnology companies to purify pharmaceuticals.*

*Thank you for purchasing one of Puresyn's premier products. As you will see, Adenopure® replaces time and labor intensive double cesium chloride gradient and generate adenovirus preparations with high purity and potency in a convenient kit format.*

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## INTRODUCTION AND BACKGROUND

Puresyn's Adenopure® Kit couples membrane adsorber technology, a simple sample loading mechanism and proprietary buffer formulations to provide rapid and simple isolation of highly purified adenovirus preparations suitable for in vitro biological assays.

Membrane adsorber modules are based on a technology that attaches ion exchange functional groups to the inner surface of synthetic microporous membranes in a syringe-filter format.

Membrane adsorber ion exchange modules provide many benefits including high binding capacity at high flow rates, and scaleability.

Each Adenopump™ Adenovirus Purification Unit is capable of purifying up to 5 x $10^{12}$ viral particles in less than two hours.

## ADENOPURE KIT COMPONENTS

There are six (6) components included in this Adenopure 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 888-245-9182 for replacement.

- 10X Dilution Buffer (50ml)
- Equilibration-Wash Buffer (270ml)
- Elution Buffer (30ml)
- Benzonase® Stock (4000 U)
- 10cc Syringes (3)
- Adenopump Adenovirus Purification Units (3)

## ADDITIONAL MATERIALS REQUIRED

- Low protein binding 0.2µm filter units with glass fiber pre-filters (Example: Millipore 0.22µm Durapore PVDF membrane Stericup™ filter unit with a glass fiber pre-filter; catalog numbers SCGV U01 RE and AP20 075 00).
- 15ml and 50ml sterile conical tubes
- Ring-stand/clamp
- Centrifuge
- 37°C water bath or incubator
- Sterile 100-200ml bottles
- Ethanol/dry ice bath

## SHIPPING AND STORAGE

The Adenopure Kit is shipped at ambient temperature. Upon arrival, store the supplied stock of Benzonase® at –20°C. Store all other Adenopure Kit components at room temperature.

## ADENOVIRUS PROPAGATION GUIDELINES

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% $CO_2$ incubator in DME medium supplemented with 10% fetal bovine serum.

For viral propagation, HEK293 cells may be seeded into 150cm$^2$ plates or flasks. When the HEK293 cells reach a confluency of approximately 80% they are infected with the adenovirus stock of interest at multiplicity of infection of 100 (as determined by HPLC analysis or OD260nm readings) in DME medium supplemented with 2% fetal bovine serum (20ml/plate or flask). Infected cells are maintained in a 37°C, 5% $CO_2$ incubator until harvest. For an average yielding vector, five (5) 150cm$^2$ plates or flasks of infected cells will generally yield an adequate amount of adenovirus for one (1) purification procedure utilizing the Adenopure Kit (approximately 1 x $10^{12}$ to 5 x $10^{12}$ viral particles per 100ml infected cell suspension).

Infected cells are harvested 2 to 5 days post-infection (usually when full cytopathic effect is observed). Virus is released by subjecting the infected HEK293 cell suspensions to three (3) freeze-thaw cycles alternately using a 37°C water bath and an ethanol/dry ice bath. Additional freeze-thaw cycles are not recommended as this may lead to adenovirus de radation.

## CONTRAINDICATIONS OF ADENOPURE KIT USE

As with any chromatographic purification process, Adenopure kit purification yield will suffer in severe under-load conditions. For the Adenopure kit to function efficiently, a minimum of 1 x $10^{12}$ viral particles should be present per 100ml of infected cell suspension.

The Adenopure 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 kit with adenovirus type 5 vectors that have modifications in viral capsid proteins resulting in the charge density of the virions also cannot be predicted or guaranteed.

# ADENOPUMP ADENOVIRUS PURIFICATION UNIT OPERATION

1. Mount the Adenopump in a clamp attached to a ring-stand.

2. Make sure all the connections of the Adenopump are tight.

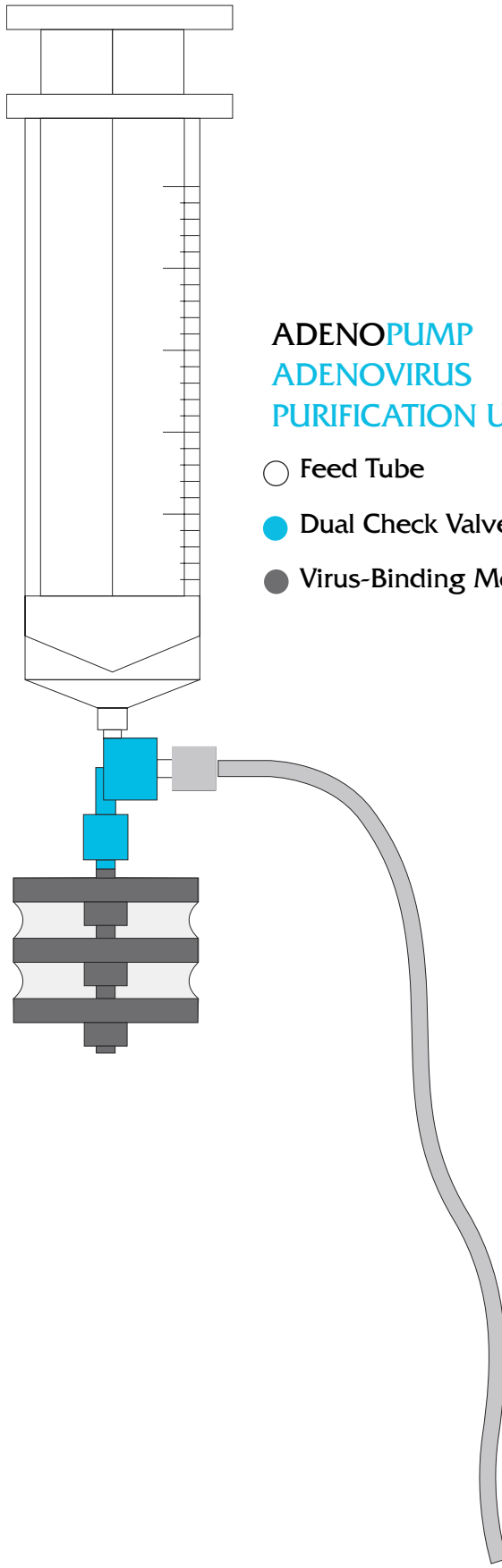
3. Fully depress the syringe plunger.

4. Place the feed tube into a reservoir containing the solution to be passed over the virus-binding module. Note: Do not place the feed tube directly into any of the supplied buffer containers because cross contamination may occur between adenovirus purification procedures. When purification steps require it, pour the appropriate amount of supplied buffer into a clean feed reservoir.

5. Pull up on the syringe plunger drawing the solution through the oneway dual check valve T-fitting into the syringe. Push down on the syringe plunger to pass the solution back through the one-way dual check valve T-fitting and over the virus-binding module. Repeat the process as many times as necessary to pass all of the solution over the virus-binding module. Try not to push air through the virus-binding module as this can prevent even distribution of solution across the membranes in the module.

6. For the virus elution step, detach the virus-binding module from the one-way dual check valve T-fitting of the Adenopump, and reattach the module to a 10cc syringe containing Elution Buffer.

7. Optimum Flow Rate: In general, slower is better with a flow rate of 10ml/minute being suitable. A flow rate of 10ml/minute may be approximated if you can “count the drops” as they come out of the virusbinding module. A flow rate that results in a stream of material coming out of the virus-binding module is too fast. If you are unsure how fast you are pushing material through the purification unit, a direct measure Virus-Binding ModuleDual Check Valve T-FittingFeed Tube may be made with a timer, and a disposable 15ml conical tube.



ADENOPUMP
ADENOVIRUS
PURIFICATION UNIT

- Feed Tube
- Dual Check Valve T-Fitting
- Virus-Binding Module

## 2-5 DAY INFECTED CELL SUSPENSION SAMPLE PREPARATION

1. Collect the infected cells and media from five (5) 150cm$^2$ tissue culture plates or flasks. If necessary, detach infected cells from the tissue culture plates or flasks by pipetting or by using a cell scraper.

   **Note:** For an average yielding vector, five (5) 150cm$^2$ plates or flasks of infected cells will generally yield an adequate amount of adenovirus for one (1) purification procedure utilizing the Adenopure Kit (approximately 1 x $10^{12}$ to 5 x $10^{12}$ viral particles per 100ml infected cell suspension).

2. Process the cell suspension through three (3) freeze-thaw cycles alternately using a 37°C water bath and an ethanol/dry ice bath. Note: Additional freeze-thaw cycles are not recommended as this may lead to adenovirus degradation.

3. Pellet cell debris by centrifugation at 2000xg for 5 minutes at room temperature.

4. Filter the adenovirus containing supernatant through a 0.2µm filter unit with glass fiber pre-filter. Pre-wetting the filter is usually unnecessary.

5. Add 50µl of 25U/µl Benzonase to the filtered lysate and mix by gently swirling. Incubate the mixture at 37°C for 30 minutes. Note: The amount of Benzonase added is dependant on the overall amount of substrate, not substrate concentration.

6. **Dilute:** Add 10X Dilution Buffer to the Benzonase-treated filtered lysate and mix by gentle swirling. Determine the amount of 10X Dilution Buffer required using the following formula: Volume of filtered cell lysate ÷ 9 = Volume of 10X Dilution Buffer required. **Note:** Accurate sample volume determination is critical, as incorrect volume addition of 10X Dilution Buffer will lead to poor binding of adenovirus to the virus-binding module of the syringe-check valve purification unit.

7. Proceed to adenovirus purification.

## ADENOVIRUS PURIFICATION

1. Fill one of the supplied 10cc syringes with 3ml of Elution Buffer and 7ml of air. Set aside for the elution step.

2. **Equilibrate:** Equilibrate the virus-binding module of an Adenopump by passing 30ml of Equilibration-Wash Buffer through the unit. Notes: a) Avoid pushing air through the virus-binding module as this can prevent even distribution of solution across the membranes in the module. Leaving a small amount of Equilibration-Wash Buffer in the syringe when performing step 3 will not interfere with efficient adenovirus purification; b) Do not place the feed tube directly into any of the supplied buffer containers because cross contamination may occur between adenovirus purification procedures. When purification steps require it, pour the appropriate amount of supplied buffer into a clean feed reservoir.

3. **Load:** Load the diluted-filtered lysate onto the virus-binding module using the Adenopump. Repeat the loading step as many times as required to pass the entire sample over the virus-binding module.
   **Note:** In general, a slower flow rate will increase the efficiency of binding of adenovirus to the virus-binding module with a flow rate of 10ml/minute being suitable.

4. Wash: Wash the adenovirus bound to the virus-binding module by passing 50ml of Equilibration-Wash buffer over the module using the Adenopump.

   **Notes:** a) Avoid pushing air through the virus-binding module as this can prevent even distribution of solution across the membranes in the module; b) Do not place the feed tube directly into any of the supplied buffer containers because cross contamination may occur between adenovirus purification procedures. When purification steps require it, pour the appropriate amount of supplied buffer into a clean feed reservoir.

5. **Elute:** Detach the virus-binding module from the one-way dual check valve T-fitting of the Adenopump, and reattach the module to the 10cc syringe containing Elution Buffer prepared in step 1. Collect all material at this step as one fraction as it contains the purified

adenovirus. Pass 15 drops of Elution Buffer through the virus-binding module. Incubate the virus-binding module for 5 minutes at room temperature before continuing. 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.

Notes: a) During the 5 minute incubation, the syringe virus-binding module may be laid on its side or held vertically; b) The final elution volume will be approximately 4.5ml if 3ml of Elution Buffer was utilized in the elution step. The volume increase is due to approximately 1.5ml of residual Wash Buffer remaining in the virus-binding module from the Wash step.

## POST-PURIFICATION GUIDELINES

Adenopure purified adenovirus preparations should be 0.2µm syringefiltered prior to use in any tissue culture or animal experiments. Adenopure purified adenovirus preparations may be stored at 4°C for short-term use (up to one week), or at -20°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.

Successful purification of adenovirus from crude cell lysate is challenging. 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 he Adenopure Kit.

**Figure 1A** shows an HPLC chromatogram of adenovirusinfected crude cell lysate. 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 upon Benzonase treatment of the crude cell lysate, the adenovirus-DNA complex resolves into a peak enriched for free adenovirus. The free DNA peak is also significantly reduced.

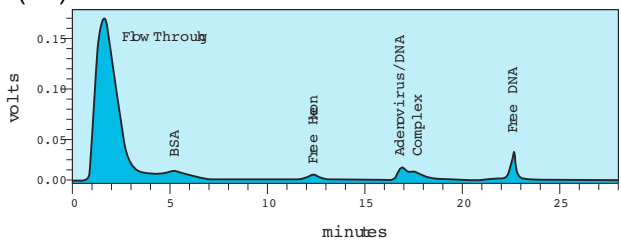
**Figure 1C** shows that the majority of free cellular proteins, BSA, free adenovirus hexon and free DNA do not bind to the virus-binding module of the Adenopump under the buffer conditions utilized, and are present in the sample flow through fraction.

**Figure 1D** shows that the emainder of free cellular proteins, BSA, free adenovirus hexon and free DNA are removed from the virus-binding module of the Adenopump during the wash step under the buffer conditions utilized.

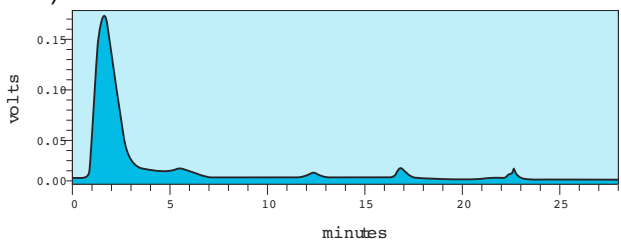
**Figure 1E** shows the eluted adenovirus as a uniform peak free from other contaminants.

Figure 1: Adenopure Kit HPLC Chromatograms

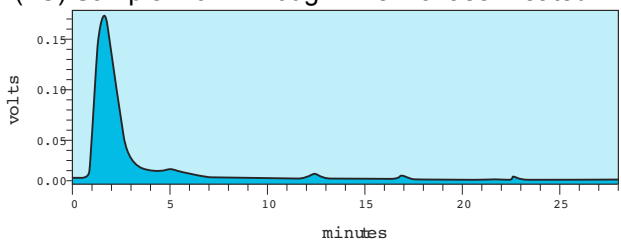
(1A) Preload - Untreated



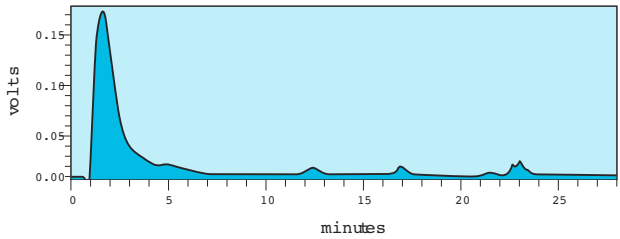

1B) Preload - Benzonase Treated



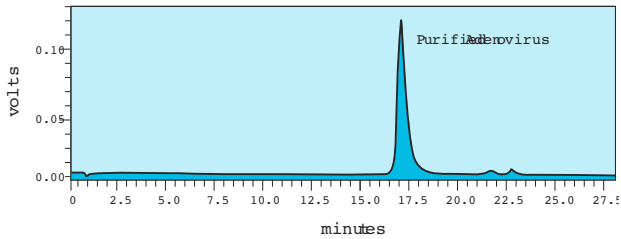

(1C) Sample Flow Through - Benzonase Treated




(1D) Wash Flow Through - Benzonase Treated




(1E) Virus Eluate - Benzonase Treated



## TRUBLESHOOTING

| Problem | Cause | Solution |
|---|---|---|
| No Virus Yield | Adenopure procedure was not followed | Read and follow Adenopure instructions |
| | Virus stock used for infection is not viable | Obtain a viable virus stock |
| Low Virus Yield | Adenopump under-loaded due to low virus yield | Re-optimize virus production |
| | Flow rate through Adenopump too fast | Slow flow rate down to 10ml/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 instructions |
| | Benzonase under- or over-digestion | Read and follow Adenopure instructions |
| | Buffer left in virus-binding module during elution | Collect all buffer from virusbinding module during elution |
| Virus-binding Module Clogs | Too much residual cellular debris | Pass cell lysate through a 0.2µm filter prior to purification |
| | Too much residual cellular DNA | Follow instructions for Benzonase digestion step |
| Purified adenovirus contains a high level of cellular DNA | Benzonase treatment incomplete, due to incorrect incubation time or temp. | Follow instructions for Benzonase digestion step |
| | Extract from too many plates of infected cells being purified | Lower the amount of extract being purified to (5) 150cm$^2$ plate equivalents |
| | Benzonase stock inactive | Store Benzonase at recommended temperature |
| Leaks in Adenopump™ | Junctions loose in Adenopump | Tighten all junctions of the Adenopump prior to use |

## CURRENT PURIFICATION PRODUCTS FROM PURESYN

- Adenopure® - Adenovirus Purification Kit
- PolyFlo® Resin - DNA

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:

Fax at 610-640-0808
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

Graham, F. L., Smiley, J., Russel, W. C., and Narin, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-72

Shabram, P.W., Giroux, D.D., Goudreau, A.M., Gregory, R.J., Horn, M.T., Huyghe, B.G., Liu, X., Nunnally, M.H., Sugarman, B.J., and Sutjipto, S. (1997). Analytical anion-exchange HPLC of recombinant type-5 adenovirus particles. Hum. Gene Ther. 8, 453-465.

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## Trademarks and Patents

Adenopure®, Adenopump™, Adenopure LS®, and PolyFlo® are trademarks of Puresyn, Inc., Malvern, PA 19355. Stericup™ is a trademark of Millipore Corporation, Bedford, MA 01730. Benzonase® is a trademark of and manufactured by Merck KGaA, Darmstadt, Germany. Benzonase is covered by US patent 5173418 and EP patent 0229866. Nycomed Pharma A/S (Denmark) claims worldwide patent rights to Benzonase, which are licensed exclusively to Merck KGaA, Darmstadt, Germany.

## 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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