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Isolation, Growth, and Purification of Defective Adenovirus Deletion Mutants

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Summary

Defective adenovirus deletion mutants can be grown by complementation in the presence of helper viruses that supply essential functions missing in the deletion mutant. In general, the deletion mutant then must be separated physically from the helper for use in subsequent experiments. This chapter includes suggestions for selection of helper viruses, protocols for the production of stocks by complementation, and procedures for physical separation of deletion mutants from their helpers.

Key Words: Deletion mutant; complementation; complementation plaquing; density gradient centrifugation; radiolabeling; helper virus.

1. Introduction

Adenovirus mutants that lack essential genes must be grown by complementation, the products of the missing genes supplied by a source other than the viral genome. Two complementation methods are available for the growth of defective adenovirus mutants. For mutations in E1, E2, E4, or proteins IV (fiber), IVa2, IX, or the 23k late protease, complementing cell lines that contain segments of viral DNA and that can supply the missing viral products can be used to produce pure stocks of mutant particles ***(1–11)***. This approach will probably be extended to other regions of the viral genome but may prove difficult to adapt to genes encoding abundant capsid proteins whose products are required in large amounts by the virus.

Alternatively, defective mutants can be grown as mixed stocks with a second "helper" virus that can supply *in trans* functions required by the mutant ***(12)***. Providing that a mutant contains all of the *cis*-active elements required for viral growth and is large enough to be packaged into an adenoviral capsid,

From: *Methods in Molecular Medicine, Vol. 130:*
Adenovirus Methods and Protocols, Second Edition, vol. 1:
Adenoviruses Ad Vectors, Quantitation, and Animal Models
Edited by: W. S. M. Wold and A. E. Tollefson © Humana Press Inc., Totowa, NJ

there are in principle no restrictions on the DNA sequences that can be deleted from a mutant grown by complementation with helper virus. Further, because the helper virus replicates, even products needed in large amounts can be effectively supplied *in trans*. Extreme examples of defective adenoviruses propagated by complementation from a helper virus are provided by gene therapy vectors that lack nearly all viral sequences ***(13,14)***. These helper-dependent or "gutless" gene transfer vectors were developed both to minimize antivector immune responses that restrict the duration of transgene expression from conventional vectors and to maximize capacity for accommodating transgenes. The size constraints imposed by some large genes such as dystrophin require that most of the adenoviral genome be deleted simply to make space for the therapeutic gene, and increased vector capacity also allows use of the endogenous promoter and control elements to naturally and precisely regulate the expression level of the therapeutic gene ***(15)***.

Growth of mutants or gene therapy vectors by complementation with helper virus requires that, for most purposes, the mutant and helper be physically separated before use. This generally is done by CsCl equilibrium density gradient centrifugation. The following protocols were developed for propagation of defective mutants with modest deletions (10–20% of the viral genome), but are applicable to larger deletions and to substitution mutants with genome sizes that differ from that of wild-type virus.

1.1. Growth of Deletion Mutants as Mixed Stocks

Because no two viral mutants have identical growth characteristics, the composition of a mixed virus stock changes over time. In particular, a defective mutant grown in the presence of replication-competent helper virus tends to disappear from the stock because any cell infected by such a mutant alone yields no progeny, while cells singly infected by the helper produce a normal yield of virus particles. Several approaches can be used to minimize that tendency. First, if a mutant helper that requires complementation for its own growth can be used, and if the deletion mutant of interest complements the helper, only dually infected cells will produce particles. Alternatively, a helper virus with defective packaging signals can be used. Such viruses are packaged into virions with much lower efficiency than the virus with normal packaging signals or, as in the case of some gene therapy vectors, two intact packaging signals. The multiplicity of infection (MOI) used to produce stocks can be made high enough to ensure that virtually all infected cells contain both a helper virus and the defective mutant. Finally, seed stocks can be enriched for the deletion mutant by physical methods before use in preparing new stocks. The use of multiple approaches when possible maximizes the yield of mutant particles.

1.2. Selection of Helper Virus

Three criteria should be used in selecting helper virus:

1. If possible, the helper should be defective and require complementation by the mutant of interest for growth or should carry a defective packaging signal. For example, helpers carrying temperature-sensitive (*ts*) mutations in late genes have been used in the isolation of Ad2 E4 deletion mutants ***(12)***, and packaging-defective helpers have been used for complementing gutless vectors ***(16–18)***.
2. Because separation of the mutant and helper depends on differences in buoyant density that in turn reflect differences in DNA content, the difference in genome size between the helper and mutant should be as large as possible. A helper with wild-type genome length can be used for mutants with deletions greater than about 10% of the viral genome; helpers with genomes slightly larger than wild-type can be used for mutants with somewhat smaller deletions ***(19)***. It is important to ensure that no recombinants with a genome size nearer that of the mutant can arise during the growth of the mixed stock, because such recombinants make purification of the mutant more difficult.
3. The helper should have no properties that make low levels of contamination of the eventual purified mutant stock unacceptable, because no physical purification scheme is completely effective.

2. Materials

1. CsCl solutions: all CsCl solutions should be 20 m*M* Tris-HCl (final concentration), adjusted to pH 8.1. Adjustment of the pH should be made after dissolving the CsCl because some lots produce very acidic solutions:
   a. CsCl density 1.25: refractive index 1.3572; 33.8 g CsCl per 100 mL of solution.
   b. CsCl density 1.34: refractive index 1.3663; 46.0 g CsCl per 100 mL of solution.
   c. CsCl density 1.7: refractive index 1.3992; 95.1 g CsCl per 100 mL of solution.
2. 1,1,2-Trichlorotrifluoroethane (Sigma, cat. no. T5271).
3. 200X IGEPAL (Sigma, cat. no. I-3021): 10% IGEPAL CA-630 solution in water.
4. TE: 10 m*M* Tris-HCl, pH 8.1, 1 m*M* ethylene diamine tetraacetic acid (EDTA).
5. Phosphate-buffered saline (PBS) (per liter): 160 g NaCl, 4 g KCl, 18.2 g Na_2HPO_4, 4 g KH_2PO_4 37.2 g EDTA. pH should be 7.2.

3. Methods

3.1. Isolation of Defective Mutants by Complementation Plaquing

3.1.1. With Defective Helpers (see ***Notes 1*** and ***2***)

1. Prepare host cell monolayers in 6-cm tissue culture dishes. If applicable, the host cells should be nonpermissive for the helper.
2. Determine the number of cells in one dish.

3. Transfect monolayers with mutant DNA by the calcium phosphate procedure (*see* Chapter 1). DNA fragments can be used if mutants are being constructed by a recombinational strategy.
4. After transfection, infect the monolayer at an MOI of 2–5 PFU/cell with the helper virus. Remove the medium from the transfected dishes, add the virus in 1 mL of medium, adsorb for 2 h, remove the inoculum, and fill the dishes with agar medium.
5. Continue by a standard plaquing protocol. If the helper is a *ts* mutant, incubate the dishes at the restrictive temperature.
6. When plaques are visible, pick and screen for the presence of the mutant (*see* Chapter 1).

3.1.2. With Nondefective Helpers

1. Prepare helper virus DNA–protein complex (DNAPC; *see* Chapter 1).
2. Mix mutant DNA and helper DNAPC in a molar ratio of 10:1 to 50:1 and transfect appropriate monolayers. The optimal amount of DNA for transfection varies depending on the plaque-forming efficiency of the helper DNA; adjust the DNA level to produce about 100 plaques per dish.
3. Treat as a normal plaque assay.
4. Pick and screen plaques for the presence of the mutant.

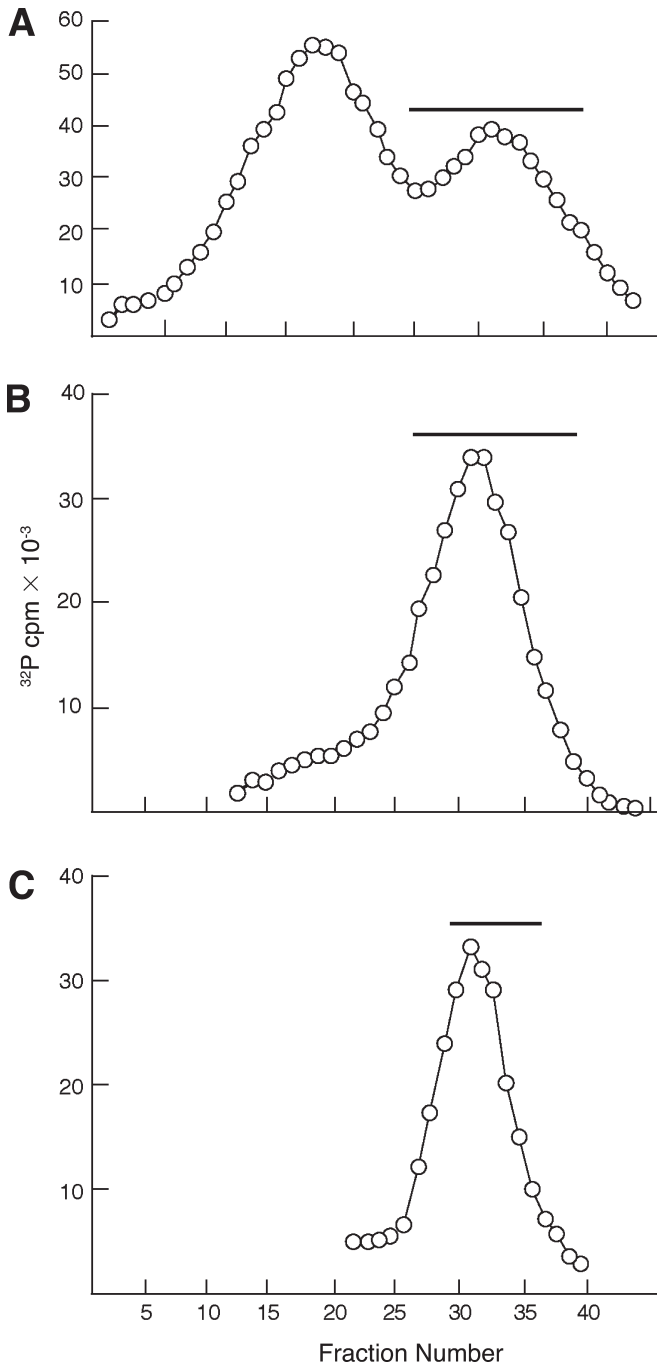
3.2. Growth of Mixed Stocks

1. Prepare host cell monolayers in 6-cm tissue culture dishes. If applicable, the host cells should be nonpermissive for the helper.
2. Remove the medium from each dish. Place the dishes with one edge slightly raised (for example, resting on a pencil) on a tray. This makes it possible to restrict the inoculum to a small area of the dish and raises the MOI in that region.
3. Pipet 0.1–0.25 mL of a mixed inoculum onto the lower edge of the monolayer. The inoculum can be a ministock (*see* Chapter 1), a portion of a previously made mixed stock, or a stock enriched for the deletion mutant by one round of CsCl density gradient centrifugation, diluted with medium to approx 10^9 infectious units per mL.
4. With the dish still tilted, incubate at 37°C in a humidified incubator for 2 h. (Proper humidification is important to prevent the raised side of the monolayer from drying out.)
5. Remove the inoculum, refill the dish with medium, place the dish flat, and incubate until all of the cells have detached from the plate. If a *ts* helper is being used, incubate at the restrictive temperature. Feed twice weekly by replacement of the medium until evidence of viral infection is seen over a substantial portion of the monolayer.
6. Harvest the infected cells and medium when all of the cells have detached from the dish. This mixed stock can be stored at –80°C until use.

3.3. Purification of Deletion Mutants From Mixed Stocks

Because adenovirus particles with differing DNA contents have differing buoyant densities in CsCl, adenovirus deletion mutants grown in the presence of helper virus can be separated from the helper by equilibrium sedimentation in CsCl density gradients. Only mutants with fairly large deletion mutations (>10%) can be efficiently purified from helpers with a wild-type genome size by this method, although helper virus with longer than wild-type genomes have been used to make the purification of mutants with smaller deletions possible ***(19)***.

1. Prepare a mixed lysate. One dish should be labeled with ^{32}P, as described below.
2. To the mixed stock, add IGEPAL to a final concentration of 0.05%.
3. Extract the stock vigorously with 1/5 vol of 1,1,2-trichlorotrifluoroethane. Recover the aqueous phase after centrifugation at 4000*g* for 5 min in a Sorvall GSA rotor. Re-extract the cell debris and organic phase with a small volume of PBS; recover the aqueous phase and pool with the supernatant from the previous centrifugation.
4. Centrifuge the pooled supernatants at 10,000 rpm (*g*) for 5 min (Sorvall SS34 rotor) to remove small particulate cell debris.
5. Prepare a discontinuous CsCl gradient in a 35-mL polypropylene centrifuge tube by adding (in order) approx 20 mL extracted virus suspension, 4 mL of CsCl density 1.25, and 5 mL of CsCl density 1.7. Add each solution slowly through a pipet placed all of the way to the bottom of the tube. After the CsCl solutions have been added, fill the tube to the desired level with extracted virus suspension.
6. Centrifuge for 90 min at 29,000*g* (Sorvall SV288 rotor or equivalent) or 3 h at 82,000*g* (Beckman SW27 rotor or equivalent).
7. In a darkened tissue culture hood, illuminate the gradient with narrow beam of light from one side. A microscope lamp is a suitable light source. The virus will form a sharp, blue-white, translucent band at the interface of the two CsCl solutions. A broader, yellowish or tan, frequently granular layer of cell debris will appear at the top of the lighter CsCl cushion.
8. Collect the virus, avoiding the cell debris (*see* **Note 3**).
9. Adjust the concentrated virus suspension to a density of 1.34 (refractive index 1.3663) with 20 m*M* Tris-HCl, pH 7.5, or with the density 1.7 CsCl solution. Place in a centrifuge tube and fill to the required volume with CsCl density 1.34.
10. Centrifuge the suspension at 35,000 rpm (*g*) for 16 h in a Sorvall TV865 rotor (or equivalent). Two closely spaced virus bands should be visible in the center of the tube.
11. Fractionate the gradient into single-drop fractions through a hole made in the bottom of the tube.
12. Measure the radioactivity in each fraction by Cherenkov counting. Two more or less well-separated peaks should appear (**Fig. 1**).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Fraction Number

Fig 1.

13. Pool the fractions that comprise the lighter peak (*see* **Fig. 1**) and repeat **steps 7–10** (*see* **Note 4**).
14. Estimate the infectious titer of the virus suspension from its A_{260}. An A_{260} of 1 corresponds to a plaque-forming titer of 3.5×10^9 PFU/mL for Ad5 purified over three CsCl gradients.
15. The purity of the mutant stock can be assessed by plaquing under conditions permissive for the helper, or by restriction enzyme digestion of purified DNA.
16. Purified virus is stable for months in buoyant CsCl at 4°C. However, high-titer suspensions dialyzed against solutions of low ionic strength (e.g., TE) frequently precipitate. If it is necessary to remove the CsCl from a purified stock, first adjust the A_{260} of the suspension to 0.5 or lower and minimize storage time at low ionic strength.

3.4. Preparation of ^{32}P-Labeled Tracer Virus Particles (see Note 5)

1. Inoculate a 10-cm dish of cells with a mixed stock as described above.
2. Examine the dish daily, replacing the medium every 3 d until one-fourth to one-half of the cells show evidence of viral infection. Gently remove the medium from the dish and replace it with 10 mL of phosphate-free medium supplemented with 2% serum and 40 µCi/mL ^{32}P orthophosphate.
3. When all of the cells have become detached from the dish, harvest the cells and medium.
4. Collect the cells by low-speed centrifugation. Rinse the labeled cells twice by low-speed centrifugation and resuspension in PBS.
5. After the second rinse, resuspend the cells in 5 mL of PBS, add IGEPAL to 0.05%, and extract vigorously with 5 mL of 1,1,2-trichlorotrifluoroethane. Centrifuge and recover the aqueous phase. If intended for use as tracer, add this material to one tube of unlabeled virus and concentrate (**step 3**).

4. Notes

1. Some defective viruses kill cells that they infect even though they do not form plaques. If monolayers infected with helper at the MOI recommended here do not survive, the following modification should be used.
   a. Prepare monolayers in 24-well tissue culture dishes.
   b. Determine the number of cells in one well.

Fig 1. (*opposite page*) Purification of H2*dl*807 by CsCl density gradient centrifugation. A mixed lysate, lightly labeled with ^{32}P, containing H2*dl*807 and an Ad5 *ts* helper virus, was subjected to three successive bandings in CsCl density gradients. The radioactivity of single-drop fractions covering the middle portion of the gradients is shown. The top, center, and bottom panels represent the first, second, and third bandings, respectively. The mutant (upper) peaks have been aligned for clarity; the fraction numbers are arbitrary. The fractions pooled after each gradient are indicated by a black bar. H2*dl*807 lacks 12.5% of the viral genome. The contamination of the final H2*dl*807 pool with helper was approx 0.03%.

c. Transfect the wells with mutant DNA by the calcium phosphate procedure.
d. After transfection, infect the monolayer at an MOI of 2–5 with the helper virus. Remove the medium from the transfected dishes, add the virus in 0.5 mL of medium, adsorb for 2 h, remove the inoculum, and refill with medium. If the helper is a *ts* mutant, incubate at the restrictive temperature (*see* **Note 2**).
e. 12–16 h after infection, trypsinize each well and reseed the transfected/infected cells in a 6-cm dish along with enough uninfected cells to form a confluent or nearly confluent monolayer.
f. After the cells have attached (8–24 h), overlay with agar medium and proceed as described in **Subheading 3.1.1.**, **step 5**.

2. If the mutant is available as virus particles (as in the isolation of naturally occurring mutants), use one of the protocols in **Subheading 3.1.1.**, or in **Note 1**, replacing the transfection step with infection by the mutant stock at approx 50 infectious particles per dish (or well).
3. It is convenient to collect the virus through a hole made in the bottom of the tube with a pushpin. Plug the tube with a rubber stopper pierced by a large-gage syringe needle, close the needle with a finger over its hub, and make the hole in the bottom of the tube. The rate at which liquid flows out of the hole can be controlled by finger pressure on the hub of the syringe needle. Alternatively, a needle can be inserted through the side of the tube and the virus band drawn out with a syringe. For lysates from two or more 10-cm dishes, the virus band should be visible in the drops as they leave the tube; for small preps, ^{32}P-labeled virus tracer (*see* **Fig. 1**) can be added before centrifugation and fractions can be collected and the virus located by Cherenkov counting.
4. Depending on the purity required, two or more gradient steps may be necessary. In the experiment shown in **Fig. 1**, contamination of the deletion mutant stock by helper was about 0.03% after three gradient steps.
5. If labeled virus to be used as tracer is being prepared in parallel with a large unlabeled stock, the labeled cells should be harvested at the same time as the large stock, even if not all cells appear to be infected. If the labeled dishes are ready for harvesting before the remaining dishes, collect and rinse the cells and store at –80°C until needed.

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http://www.springer.com/978-1-58829-598-9

Adenovirus Methods and Protocols
Volume 1: Adenoviruses, Ad Vectors, Quantitation, and Animal Models
Wold, W.S.M.; Tollefson, A.E. (Eds.)
2007, XIV, 242 p. 30 illus., Hardcover
ISBN: 978-1-58829-598-9
A product of Humana Press