



Microbix Biosystems Inc.

***Adenovirus Vector
Construction Manual***

Document: (03/00A) 01-1

341 Bering Avenue, Toronto, Canada M8Z 3A8
Tel: 416-234-1624 Fax: 416-234-1626
www.microbix.com customer.service@microbix.com



TABLE OF CONTENTS

PRODUCT INFORMATION.....	3
GROWTH OF 293 CELLS.....	4
GENERAL COMMENTS.....	4
PROCEDURE FOR SPLITTING CELLS.....	4
SOLUTIONS.....	4
GROWTH MEDIUM.....	5
FREEZING CELLS FOR STORAGE IN LIQUID N ₂	5
THAWING FROZEN CELLS.....	5
CSCL-BANDED PLASMID DNA PREPARATION FOR COTRANSFECTIONS.....	6
PROTOCOL.....	6
BUFFERS.....	7
COTRANSFECTION OF 293 CELLS WITH PLASMID DNA FOR VECTOR RESCUE BY THE ADMAX™ METHOD.....	9
PROTOCOL.....	9
NOTES.....	9
SCREENING ADENO PLAQUE ISOLATES FROM VECTOR RESCUES.....	10
PROTOCOL.....	10
PREPARATION OF HIGH-TITER VIRAL STOCKS (CRUDE LYSATES) FROM CELLS IN MONOLAYER.....	12
PROTOCOL.....	12
SOLUTIONS.....	12
PREPARATION OF HIGH-TITER CRUDE VIRAL STOCKS FROM CELLS IN SUSPENSION.....	13
PROTOCOL.....	13
SOLUTIONS.....	13
PREPARATION OF PURIFIED HIGH-TITER VIRAL STOCKS BY CSCL BANDING.....	14
PROTOCOL.....	14
MATERIALS.....	15
SOLUTIONS.....	15
REFERENCES.....	16
1. DNA TRANSFECTION.....	16
2. 293 CELLS.....	16
3. INFECTIVITY OF CIRCULAR FORMS OF AD DNA.....	16
4. THE TWO PLASMID METHOD FOR VECTOR RESCUE.....	17
5. PACKAGING CAPACITY OF AD VECTORS.....	17
6. AD VECTORS AS VACCINES.....	17
7. METHODS ARTICLES.....	18
8. GENERAL.....	18
ADMAX™ PLASMID MAPS AND TECHNICAL ASSISTANCE.....	19



Product Information

Storage: Plasmids are shipped at ambient temperatures. Plasmids should be stored frozen at <-20°C.

Hazards: We are aware of no specific hazards associated with this product. These materials should be handled only by qualified personnel using generally accepted good laboratory practices appropriate for biological reagents.

Stored In: Ethanol

Preparation: Plasmids are produced in E. coli strain DH5. Nucleic acids are released from lysed bacterial cells and DNA is precipitated. Plasmids are then purified by cesium chloride density gradient centrifugation. Finally, CsCl is removed through dialysis and the plasmids are aliquoted and precipitated with ethanol.

Description: Dr. F.L. Graham and his co-workers have developed a series of bacterial plasmids comprising circular forms of the adenovirus type 5 (Ad 5) genome (1 – 5) which simplify some of the steps required for construction of recombinant Ad5 vectors. When combined with a set of shuttle plasmids containing polycloning sites, these circularized viral genomes constitute three systems that allow insertion of DNA into early region 1 (E1) (cf ref. 2), E3 (cf refs 3&4), or E1 and E3 (cf ref. 5) of the viral genome.

Plasmid DNA preparations are provided as ethanol precipitates, 10µg/vial, and are intended to be used for transformation of E.colit from which plasmid DNA can be purified for cotransfections of 293 cells. These reagents are available in the form of kits. Individual plasmids may be ordered to augment a kit.



GROWTH OF 293 CELLS

General Comments.

293 cells grow in monolayer, preferably in plastic petri dishes, unless adapted to growth in suspension. They are particularly sensitive to the way they are handled (or mishandled). They should never be allowed to become overconfluent, should not be seeded too thinly, should have regular medium changes between splits (twice weekly if they are not growing rapidly enough to permit splitting every 2-4 days). Probably any standard growth medium is acceptable. For many years they had been grown in Joklik's modified MEM plus 10% heat inactivated horse serum but now are maintained in MEM-F11 or α -MEM plus heat inactivated new born bovine serum. Fetal calf serum should also be suitable.

Microbix 293 cells are low passage 293 cells that have been maintained under optimal conditions for strong adherence to plastic. They are particularly well suited for adenovirus plaque assays and DNA transfections. To ensure that these properties are retained it is essential that they be cultured appropriately as described below.

We recommend that a sufficient number of ampoules of the cells be frozen and stored in liquid N₂ to permit initiation of new cultures when the passage number of the lab stocks reaches 40-45 passages (including the passage number at which the cells were obtained from Microbix) or when the cells are no longer behaving well under agar overlays. Higher passage or poorly adherent cells may be suitable for growth of virus but the properties of the cells are more critical for plaque assays and transfections.

Procedure for Splitting Cells

Remove the medium from the dishes. Rinse dish (containing cells at approximately 90% confluency), 2 times with 1X Citric Saline (approximately 5 ml for a 150 mm dish). Remove all but a trace of Citric Saline (enough to cover the cell monolayer, approximately 0.5 - 1 ml). Leave dish for approximately 15 min., examining frequently, until cells start to round up and lift off. (This can be done at room temperature. Cells will detach faster at 37 but conditions may be more difficult to control.) When essentially all the cells appear to be loosened, knock the cells off the plastic by tapping the dish sideways against a solid surface. Do not attempt to detach cells by scraping or by vigorous pipetting. We do not recommend the use of trypsin. Resuspend the cells in medium containing serum and distribute to new dishes. We recommend that you not dilute the cells more than 1:3, but the usual split ratio should be 1:2. Incubate the cells at 37°C and refresh the medium every 3 days if the cells are not ready to passage. (This is more likely to happen when the cells are initially started from a frozen ampoule.) Although it may seem that rapidly growing 293 cells are desirable we have found that they may be less suitable for transfections and plaque assays. **Never allow the cells to become completely confluent or overgrown, never allow them to pile up, and never leave them unattended for long periods. They will never forgive you!**

Solutions

10X Citric Saline:

50g KCl

22g Na Citrate

Dissolve in H₂O and bring to 500 ml.

Sterilize by autoclaving for 15 min. at 15 lb./sq. in. pressure



Growth Medium

F11 + 10% Heat Inactivated Newborn Bovine Serum or Fetal bovine serum.

MEM F11 = GIBCO Catalogue Number 61100-061

To 400ml of medium, add Penicillin/Streptomycin (as recommended by manufacturer), and 2 mM L-Glutamine. If fungal contamination is a problem Fungizone (Gibco) can be added to 0.25 µg/ml.

Freezing cells for storage in liquid N₂.

Detach as above, maintaining volume of suspension to a minimum. Resuspend cells in 100% fetal bovine serum. Add 1/10 volume sterile dimethylsulfoxide (DMSO). Freeze in ampoules using standard methods at a density of about 1/3 of a 150mm dish per ampoule. Optionally the cells can be resuspended in medium, centrifuged, and resuspended in an appropriate volume of 90% FBS - 10% DMSO.

Thawing frozen cells.

Thaw cells rapidly by dipping the lower half of the ampoule in a 37C bath. As soon as the sample is melted add the cells to 25 ml warm growth medium in a 75 cm sq plastic flask that has been equilibrated in a CO₂ incubator. Optionally, the cells can be diluted in 10 ml complete medium, centrifuged, and resuspended in fresh medium before seeding into flasks. Leave undisturbed in the incubator for 2-3 hrs then gently remove the flask and examine the cells in the microscope. If the majority of the cells have attached, gently remove the medium and add fresh growth medium. If a significant number of cells are still in suspension it is possible to seed a second flask by transferring the medium and incubating overnight.



CsCl-BANDED PLASMID DNA PREPARATION FOR COTRANSFECTIONS

Protocol

* recipes given at end of protocol

1. Set up 500 ml bacteria in the afternoon or evening the day before you want to purify plasmid. Higher yields are obtained with richer broths, such as SB or TB. Start this culture from a 5 ml culture that has been set up in the morning either from bacteria stored frozen at -70 in glycerol or from a colony on a plate. For plasmids >15 kb always use a colony from a freshly streaked plate (< 1 week old).
2. Next morning spin cells in a Cryofuge (or equivalent) in one 1000 ml bottle at 4200 rpm (6000 x g) for 10 min (or at 5000 rpm in a GSA rotor in a Sorvall centrifuge).
3. Resuspend each pellet in 40ml lysozyme buffer* (5mg/ml in 10mM EDTA, 50mM glucose, 25mM Tris, pH8.0, prepared fresh). Resuspend cells using a 10 ml pipette. Incubate 20 min at RT. (Lysozyme and the incubation are optional).
4. Add 80 ml alkaline SDS* (1% SDS, 0.2 N NaOH made up just before use). Mix by swirling, do not over-agitate. This should produce a relatively clear, viscous lysate. Incubate on ice 5-10 min.
5. Add 40 ml KOAc (Solution III)*. Mix thoroughly and incubate 20 min on ice. The viscosity should be greatly reduced. Add 10 ml H₂O and centrifuge 20 min at 4200 rpm in a Cryofuge.
6. Collect the supernatant, removing any pellicle and residual particulate matter by filtering through 2-3 layers of cheesecloth into a Nalgene 500 ml bottle.
7. Add 100 ml (0.6 volumes) isopropanol and incubate 30 min at RT. This step should precipitate DNA but not RNA. Centrifuge 10-15 min in a Cryofuge or equivalent at 4200 rpm at RT.
8. Pour off the supernatant and drain at RT for 15 min. Keeping the bottle upside down, wipe inside the rim with a clean KimWipe to remove all residual isopropanol.
9. Re-dissolve the pellet in 5 ml 0.1 x SSC and transfer to a 50 ml Corning polypropylene tube (this size tube facilitates removal of the pellicle in step 12).
10. Add 2 ml pronase-SDS (0.5 mg/ml pronase* in working buffer: 0.5% SDS, 10 mM Tris, pH 7.4, 10 mM EDTA). Mix well and incubate 30 min at 37°C.
11. Add 8.6 g CsCl. (This will result in the required density if steps 8 and 9 are done reproducibly.) Dissolve completely, then incubate 30 min on ice. This results in precipitation of RNA and proteins.
12. Spin in a Beckman tabletop centrifuge or equivalent at 3300 rpm (top speed) for 20-30 min at 5°C. Carefully collect supernatant using a 5 ml syringe and 16g needle, avoiding any pellicle if possible (the material in the pellicle can be troublesome if not removed, as it sometimes co-sediments with the plasmid band in CsCl gradients).



13. Transfer DNA solution to a Beckman 13 ml quick seal tube. Add light parafin oil to fill the tube up to the shoulder. Add 100-200 ul 10 mg/ml ethidium bromide solution, but do not mix (to avoid exposure of DNA to light and ethidium at the same time). Top up with parafin oil to the neck of the tube then seal the tube.
14. Mix the contents of the tube well by inversion, and spin in a Beckman VTi 65.1 rotor at 55,000 rpm for 10-14hrs at 14°C (or 55K in NVT65.1 rotor).
15. Remove rotor and transport to the lab bench without agitation to preserve the gradient. Keep tubes covered with foil (or otherwise in the dark, such as in the rotor) except when recovering plasmid DNA bands. Do not expose to fluorescent or UV light more than necessary. The supercoiled plasmid DNA band should be the thicker, lower red band in the gradient, but it should not be at the very bottom of the tube. The upper red band (if present) is nicked plasmid or chromosomal DNA.
16. Support the tube in a rack or on a stand. Puncture the top of the tube to allow entry of air. Collect plasmid DNA through the side of the tube with 18g needle attached to a 3ml or 5ml syringe by puncturing the tube with the needle bevel side down just below the band, then rotate the syringe such that you pull the band with the needle bevel side up (alternatively, if you are worried about losing the sample, puncture above the band with the needle bevel side down). Consider using a small dollop of silicone grease at the point of needle entry if leakage seems to be a problem. (It normally is not.)
17. Transfer plasmid DNA to a 15-ml polypropylene tube containing 5 ml isopropanol which has been saturated with CsCl in 25mM Tris-10mM EDTA, and mix immediately to extract the ethidium into the solvent layer.
18. Repeat steps 16 and 17 for each plasmid sample. Let tubes stand for a few minutes or spin briefly to separate the layers. Remove the ethidium-solvent (pink) layer, then continue to re-extract with 5 ml CsCl-saturated isopropanol until the solvent layer is completely colourless.
19. Add 3 volumes of 10 mM Tris-1 mM EDTA, and 8 volumes ethanol (e.g., to 1 ml DNA add 3 ml TE, then 8 ml cold ethanol). Mix well. Centrifuge in Beckman tabletop centrifuge at top speed for 15 min, then aspirate off supernatant. (It is also possible to dialyze rather than precipitate to get rid of the CsCl.)
20. Wash pellet with 70% ethanol, then 95% ethanol and air dry at 37 (approximately 1 hr).
21. Dissolve DNA in about 1ml 0.1 x SSC or 10 mM Tris-1 mM EDTA, depending on size of pellet.
22. Digest about 1ul with appropriate enzyme and run on gel with a marker. Determine the plasmid DNA concentration by OD or by fluorimetry. Concentration of DNA will depend on the plasmid size and whether the plasmid is high copy number or low.

Buffers

Lysozyme Buffer: 500 ml Autoclave and store at RT until opened. (Add lysozyme just before use.)

.25M EDTA.....	20 ml
0.5M Glucose.....	50 ml
1M Tris pH 8.0.....	12.5 ml
ddH ₂ O.....	417.5 ml



Alkaline SDS	120ml	140ml	160ml	200ml	240ml	280ml
Make fresh daily.						
20% SDS.....	6ml	7ml	8ml	10ml	12ml	14ml
4N NaOH.....	6ml	7ml	8ml	10ml	12ml	14ml
ddH ₂ O.....	108ml	126ml	144ml	180ml	216ml	252ml

KOAc (Solution III): 500 ml Store at 4°C

5M potassium acetate.....300 ml

glacial acetic acid.....57.5 ml

H₂O.....142.5 ml

Pronase stock solution: 5mg/ml in 0.01 M Tris, pH7.5: preincubate at 56°C for 15 min followed by 37°C for 1hr. Aliquot and store at -20°C. Add to pronase-SDS working buffer before use (this dilute pronase solution can be used for about a month if stored at 4°C).

COTRANSFECTION OF 293 CELLS WITH PLASMID DNA FOR VECTOR RESCUE BY THE ADMAXTM METHOD

Protocol

1. Set up 60mm dishes of 293 cells to be about 70-80% confluent at time of use (1 to 2 days) using 5 ml/dish F11 + 10% bovine serum. The denser and older the cell monolayer the longer it takes for virus cpe to reach completion.
2. Approximately 1-2 hrs prior to cotransfection change the medium. (This step is optional but may increase the efficiency of transfection moderately.)
3. Prepare 1X HeBs + 10µg/ml salmon sperm DNA, and mix by vortexing for 1 min.
4. Aliquot into 15 ml conical polystyrene tubes (0.5 ml/60 mm dish). We recommend a volume of 1-2 ml, enough to transfect 2-4 cultures.
5. Add plasmid DNA (2-5 µg/dish of each plasmid) and mix well. There is no need to adjust the salmon sperm DNA concentration to compensate for the plasmid DNA.
6. As a positive control, prepare a sample of pFG140 (provided free with all Microbix kits) at 0.5 - 1 µg/dish. This should produce at least 50-100 viral plaques.
7. Add 2.5M CaCl₂ (50 µl/ml).
8. Mix well and incubate at room temperature for 15 to 30 min. (A fine precipitate should form.)
9. Add to adherent 293 cell cultures dropwise around the dish without removing the medium. (0.5ml/5ml medium)
10. Incubate at 37°C in a CO₂ incubator overnight (12-14 hr).
11. Aspirate medium and overlay with 10 ml F11 + 5% Horse Serum + 0.5% agarose* or refeed with 5 ml F11 + 5% Horse Serum**.
12. Incubate at 37°C. Plaques or CPE should appear after about 5 days. Plaques can be isolated for expansion by 10 days. Note that some vectors may replicate slowly and in such cases an additional 5 ml overlay can be added to the dishes at 10 days to allow for further plaque development.

Notes

* Prepare by mixing equal volumes of 2X F11 + 10% Horse Serum + Pen-Strep + L-Glutamine + 0.2% Yeast Extract and 1% Agarose. (If contamination with molds is an occasional problem fungizone can be added.)

** It is good laboratory practice to use plaque purified virus prior to extensive experimentation (hence we recommend using an agar overlay at step 11) but recombinant virus can also be recovered by incubation of transfected cells under liquid medium. Cpe can usually be detected by 5 days and should be complete by 7-8 days. The AdMaxTM virtually guarantees that all recovered viruses will be the desired expression vector.

SCREENING ADENO PLAQUE ISOLATES FROM VECTOR RESCUES

General comments. Although 100% of viruses recovered by the AdMax™ method should contain and express the desired transgene it is good laboratory practise to confirm the structure of the virus isolates to be used in large scale vector production and to prepare titered stocks of virus before embarking on extensive experimentation. Of course those who are impatient (who isn't?) may wish to carry out preliminary experiments (expression assays using Westerns, ELISAs or IPs for example) as soon as expanded virus preparations are available, such as those obtained at step 5 below.

The protocol below is designed for 293 cells.

Protocol

1. Set up 60mm dishes 293s as for plaque assays or cotransfections, i.e. to be about 80% confluent at time of use. The denser and older the cell monolayer the longer it takes for virus cpe to reach completion. Use dishes the next day or after 2 days.
2. Pick well isolated plaques by punching out agar plugs using a sterile Pasteur and transfer mashed agar to 1ml of PBS⁺⁺ plus 10% glycerol. This can be stored at -70°C until results of analysis are available.
3. Remove medium from 293 dishes and add 0.2 ml virus suspension from step 2. Distribute over monolayer and adsorb at room temp for 30-60 min occasionally rocking the dishes to spread the inoculum over the cells. Add 5 ml F11 + 5%HS and incubate at 37°C.
4. Depending on size of the plaque and growth properties of the recombinant virus you should start to see cpe within 2-3 days. Do not attempt to harvest before cpe is absolutely complete, ie essentially all cells rounded and many floating (usually 3-4 days).
5. Process dishes with complete cpe as follows: leave dishes undisturbed in a laminar flow hood for 20-30 min to allow any cells in suspension to settle. Gently remove medium with a pipette and save about 4ml or so in a sterile glass vial containing 0.5 ml sterile glycerol, for storage at -70°C. Remove residual medium by suction. If all this is done carefully the majority of loose cells will be left behind in the dish. The recovered medium should contain significant amounts of virus at titres at least 10⁷ and 10⁸ PFU/ml and can be used in preliminary experiments or for further virus expansion.
6. Add 0.5 ml pronase-SDS (see plasmid DNA purification protocol for recipe) and digest at 37°C for 3-4 hrs or overnight.
7. Transfer viscous lysate to a 1.5 ml Eppendorf tube and extract once with 0.5 ml phenol (saturated with buffer). Collect the aqueous phase with an Eppendorf pipetter and transfer to a fresh tube.
8. Add 1 ml 96% ethanol, vortex lightly or mix by tipping tube. You should get an easily visible fibrous precipitate. Spin and wash 2x with 96% ethanol to remove phenol.
9. Dry completely in a 37°C warm room or heating block and redissolve DNA in 50ul 0.1 x SSC or 10mM Tris 1mM EDTA. It is advisable to tap the tube sharply to suspend the DNA then leave the sample overnight at 4C to allow the DNA to dissolve.

10. Digest 5-10 μ l with any suitable restriction enzyme (Hind III is often the best all purpose diagnostic enzyme) for 3-4 hrs or preferably overnight. The careful worker will have already constructed the expected vector on the computer and will have created predicted maps and diagnostic restriction patterns, using, for example Vector NTi. Complete sequences for all AdMax plasmids are available from Microbix as ASCII files or as Vector NTi files.

11. Run on 1% agarose gel **with an appropriate marker.**

Comments: If cpe was complete you should get a relatively “pure” preparation of viral DNA (roughly 50% viral DNA - 50% cellular) with a background of cellular DNA running as a smear (some enzymes, eg Pvu I, cut human DNA infrequently in which case the cellular DNA will remain near the top of the gel). There should be very little RNA. If Hind III was used there will be a band of cellular repetitive DNA at around 1.8kb (derived from a LINE-1 element), not to be confused with viral DNA.



PREPARATION OF HIGH-TITER VIRAL STOCKS (CRUDE LYSATES) FROM CELLS IN MONOLAYER

General comments. Because most of the virus remains associated with the infected cells until very late in infection ie until the cells lyse, high-titer stocks can be prepared easily by concentrating infected 293 cells as described here.

Protocol

1. Set up 150-mm dishes of 293 cells to be 80-90% confluent at time of infection. We generally use eight or more dishes for each virus.
2. To prepare high-titer stocks, remove medium from the 293 cells and infect at a multiplicity of infection (MOI) of 1-10 PFU per cell (1 ml virus suspension per 150-mm dish). For the initial stock preparation, we dilute virus (from the untitered 4 ml sample stored at -70°C after the last round of viral screening) 1:8 with PBS^{++} . To minimize the probability of E1^{+} replication competent virus (RCA), prepare subsequent high-titer stocks from this same original viral screening sample.
3. Adsorb for 30-60 min, then refeed with complete MEMF11 + 5% HS. Incubate at 37°C , and examine daily for signs of cytopathic effect.
4. When cytopathic effect is nearly complete, i.e., most cells rounded but not yet detached, harvest by scraping the cells off the dish, combining the cells plus spent medium (if it contains significant numbers of floating cells), and centrifuging at 2000 g for 15 min. Aspirate the medium, and resuspend the cell pellet in 2 ml PBS^{++} + 10% glycerol per 150-mm dish. Freeze (-70°C) and thaw (37°C) the crude virus stock three times prior to titration. Store aliquots at -70°C .

Solutions

PBS^{++} , PBS^{++} + 10% glycerol, and complete MEMF11 + 5% HS

PREPARATION OF HIGH-TITER CRUDE VIRAL STOCKS FROM CELLS IN SUSPENSION

General comments. Many experimental studies can be carried out using virus in the form of crude infected cell lysates. The following protocol results in high titre virus preparations. Recombinant Ads can be produced using either monolayer (see above) or suspension cultures. However, due to the greater ease of handling suspension cultures, these are preferable for the production of large amounts of high-titer viral stocks. Suspension adapted 293 cells (293N3S) are available from Microbix.

Protocol

1. For infection with replication-defective viruses (E1 insertion recombinants) grow 293N3S cells in spinner culture to a density of $2-4 \times 10^5$ cells/ml in 4 liters complete Joklik's modified MEM + 10% HS. For growth of wt adenovirus or replication-proficient E1⁺ viruses (eg E3 insertion recombinants), grow 4 liters KB cells to a density of $5-6 \times 10^5$ cells/ml in complete Joklik's modified MEM + 10% NBS. Centrifuge cell suspension at 750 g for 20 min, saving half of the conditioned medium. Resuspend the cell pellet in 0.1 vol fresh medium, and transfer to a sterile 500-ml bottle containing a sterile stir bar.
2. Add virus at MOI of 10-20 PFU/cell and stir gently at 37°C. After 1 hr, return the cells to the 4-liter spinner flask and bring to the original volume using 50% conditioned medium and 50% fresh medium. Continue stirring at 37°C.
3. Monitor infection twice daily by inclusion body staining as follows:
 - a. Remove a 5-ml aliquot from the infected spinner culture. Spin for 10 min at 750 g and resuspend the cell pellet in 0.5 ml of 1% sodium citrate.
 - b. Incubate at room temperature for 10 min; then add 0.5 ml Carnoy's fixative and fix for 10 min at room temperature.
 - c. Add 2 ml Carnoy's fixative, spin 10 min at 750 g, aspirate, and resuspend the pellet in a few drops of Carnoy's fixative. Add one drop of fixed cells to a slide and air-dry for about 10 min; then add one drop orcein solution and a coverslip and examine in the microscope. Inclusion bodies appear as densely staining nuclear structures resulting from accumulation of large amounts of virus and viral products at late times in infection. A negative control should be included in initial tests.
4. When inclusion bodies are visible in 80-90% of the cells (2 to 3 days depending on the input MOI), harvest by centrifugation at 2000 g for 20 min in sterile 1-liter bottles. Combine pellets in a small volume of medium, and spin again. Resuspend pellet in 20 ml PBS⁺⁺ and 10% glycerol. Freeze and thaw 2-3 times then aliquot and store at -70°C until use. Titrates by plaque assays on 293 monolayer cells.

Solutions

1. *Complete Joklik's modified MEM + 10% HS (or 10% NBS):* Add 50 ml HS (or NBS) to 450 ml complete Joklik's modified MEM (described in Section B). Store at 4°C.
2. *1% sodium citrate:* Dissolve 1 g trisodium citrate dihydrate in H₂O to a final volume of 100 ml.
3. *Carnoy's fixative:* Add 25 ml glacial acetic acid to 75 ml methanol.
4. *Orcein solution:* Add 1 g orcein dye to 25 ml glacial acetic acid plus 25 ml H₂O. Filter through Whatman No. 1 paper.

PREPARATION OF PURIFIED HIGH-TITER VIRAL STOCKS BY CSCL BANDING

General comments. Many experimental studies can be carried out using virus in the form of crude infected cell lysates prepared as described above. However, for some experiments, particularly for animal work it is usually desirable to use purified virus. The following protocol is adapted from Hitt et al. (1998).

Recombinant Ads can be purified from crude lysates of either monolayer or suspension cultures. Suspension cultures are easier to harvest, but it is easier to determine the correct time to harvest (i.e., complete or near complete cpe) when using monolayer cultures. A subline of 293 cells, 293N3S cells, adapted to growth in suspension is available from Microbix. The following protocol describes a method for banding virus obtained from 3-liters of infected suspension culture, or about 30 x 150-mm dishes.

Protocol

1. Prepare crude cell lysate from infected cells:

a) For 3-liter spinner cultures: when inclusion bodies are visible in 80-90% of the cells, harvest by centrifugation at 750 x g for 20 min in sterile 1-liter bottles. Combine pellets in a small volume of medium, and spin again. Resuspend pellet in 15-30 ml 0.1M Tris-HCl, pH 8.0: if wild-type levels of virus are expected, resuspend in 30 ml, if lower yields are expected (some vectors may replicate to lower yields than others), use 15 ml. Store sample at -70°C.

b) For 30 x 150-mm dishes: when all infected cells are rounded and most are floating, scrape dishes, then transfer cells and supernatant to 50-ml centrifuge tubes and spin 10 min at 750 x g. Resuspend cell pellet in 15 ml 0.1M Tris-Cl, pH 8.0. Store sample at -70°C.

2. Thaw sample and add 1.5 ml 5% Na deoxycholate for each 15 ml of cell lysate. Mix well and incubate at room temperature for 30 min. This results in a relatively clear, highly viscous suspension.

3. Add 150 µl 2 M MgCl₂ and 75 µl DNase I solution for each 15 ml of cell lysate, then mix well. Incubate at 37°C for 30-60 min, mixing every 10 min. The viscosity should be reduced to only slightly more than that of water.

4. Spin 15 min, top speed, at 5°C in the Beckman table top centrifuge.

5. Meanwhile, prepare CsCl step gradients (one SW41 ultraclear tube for each 5 ml of sample) in the virus hood: Add 0.5 ml of 1.5g/cc solution to each tube. Gently overlay with 3.0 ml of 1.35g/cc solution. Gently overlay this with 3.0 ml of 1.25g/cc solution. Do not disturb gradients once they are formed.

6. Apply 5 ml of supernatant from step 4 to the top of each gradient. If necessary, top up tubes with 0.1M Tris-Cl, pH 8.

7. Spin at 35K rpm in SW41 rotor, 10 °C, for 1 hour (accelerate/decelerate set to 1).

8. Collect the viral bands (should be at the 1.25g/cc-1.35g/cc interface) and pool. (One can collect by sealing the top of the tube with a piece of parafilm, puncturing the parafilm with a needle, then puncturing the bottom of the tube with a red hot 16 gauge needle, controlling the flow of solution out the bottom with a gloved finger over the top hole.)



9. Transfer the pooled virus to a SW50.1 ultraclear tube (using the 1.35g/cc CsCl solution to top up the tube), mix well, and centrifuge in the SW50.1 rotor at 35K rpm, 4°C, for 16-20 hr. (Alternatively, the pooled virus can be centrifuged in the SW41 rotor at 35K rpm, 10°C, 16-24 hr.)
10. Collect the virus band in the smallest volume possible (usually 0.5 to 1 ml), transfer to a Slide-A-Lyzer dialysis cassette, and dialyze at 4°C against 3 changes of 500 vol (or more) 10 mM Tris-HCl, pH 8.0, for at least 24 hr total.
11. After dialysis, add sterile glycerol to a final concentration of 10%. Store the purified virus at -70°C in small aliquots.

Materials

The following special equipment is required: Beckman SW 41 rotor, SW50.1 rotor, ultraclear tubes for each rotor, Pierce Slide-A-Lyzer dialysis cassettes, sterile 1-liter beaker with stir bar, syringes and needles.

Solutions

1. *0.1 M Tris-HCl, pH 8.0*: Add 1.2 g Tris base to 80 ml H₂O. Adjust pH to 8.0 with HCl. Adjust volume to 100 ml and autoclave.
2. *5% Na deoxycholate*: Add 5 g Na deoxycholate to 100 ml H₂O.
3. *2M MgCl₂*: Add 40.6 g MgCl₂·6H₂O to 100 ml H₂O and sterile filter.
4. *DNase I solution*: Dissolve 100 mg bovine pancreatic deoxyribonuclease I (DNase I) in 10 ml of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 50% glycerol. Store in small aliquots at -20°C.
5. *CsCl solutions for banding*:

<u>density</u>	<u>CsCl</u>	<u>10 mM Tris, pH8</u>
1.50 g/cc	90.8 g	109.2 g
1.35 g/cc	70.4 g	129.6 g
1.25 g/cc	54.0 g	146.0 g

Add 10 mM Tris to indicated amount of CsCl and stir to dissolve. Verify density by weighing 1 ml of each solution. Sterile filter and store at room temperature.

6. *Dialysis buffer*: 10 mM Tris, pH 8.0. Use at 4°C.
7. *Sterile glycerol*: Prepare by autoclaving.

REFERENCES

1. DNA Transfection

Graham, F.L. and van der Eb., A.J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456-467, 1973.

Graham, F.L., Veldhuisen, G., and Wilkie, N.M. Infectious Herpes Virus DNA. *Nature New Biology* 245, 265-266, 1973.

Graham, F.L., and van der Eb, A.J. Transformation of rat cells by DNA of human adenovirus 5. *Virology* 54, 536-539, 1973.

Graham, F.L., van der Eb, A.J., and Heijneker, J.L. Size and location of the transforming region in human adenovirus type 5 DNA. *Nature* 251, 687-691, 1974.

Bacchetti, S., and Graham, F.L. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. *Proc. Natl. Acad. Sci. U.S.A.* 74, 1509-1594, 1977.

Graham, F.L., and Bacchetti, S. DNA mediated gene transfer using the calcium technique. In: *Techniques in the Life Sciences: Nucleic acid biochemistry*, B5. Ed. R.A. Flavell, Elsevier Scientific Publishers, Ireland, Ltd., 1983.

2. 293 cells

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

Graham, F.L. Growth of 293 cells in suspension culture. *J. Gen. Virol.* 68: 937-940, 1987.

Krougliak, V. and Graham, F. L. Development of cell lines capable of complementing E1, E4 and protein IX defective adenovirus type 5 mutants. *Human Gene Therapy* 6: 1575-1586, 1995.

Louis, N., Eveleigh, C. and Graham, F. L. Cloning and sequencing of the cellular/viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology*, 233:423-429, 1997.

Chen, L., Anton, M. and Graham, F. L. Production and characterization of human 293 cell lines expressing the site-specific recombinase Cre. *Somat. Cell and Molec. Genet.* 22: 477-488, 1996.

Matthews, D. A., Cummings, D., Eveleigh, C., Graham, F. L. and Prevec, L. Development of a modified 293 cell line expressing the lac repressor and its use in the rescue of recombinant adenoviruses expressing high levels of rabies glycoprotein (G). *J. Gen. Virol.* 80: 345-53, 1999.

3. Infectivity of circular forms of Ad DNA

Ruben, M., Bacchetti, S., and Graham, F.L. Covalently closed circles of human adenovirus type 5 DNA. *Nature* 301,172-174, 1983.

Graham, F.L., Covalently closed circles of human adenovirus DNA are infectious. *The EMBO J.* 3, 2917- 2922, 1984.

Ghosh Choudhury, G., Haj-Ahmad, Y., Brinkley, P., Rudy, J. and Graham, F.L. Human adenovirus cloning vectors based on infectious bacterial plasmids. *Gene*. 50: 161-171, 1986.

Graham, F.L., Rudy, J. and Brinkley, P. Infectious circular DNA of human adenovirus Type 5: Regeneration of viral DNA termini from molecules lacking terminal sequences. *The EMBO Journal* 8: 2077-2085, 1989.

4. The two plasmid method for vector rescue

McGrory, J., Bautista, D., and Graham, F. L. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology*, 163: 614-617, 1988.

Bett, A. J., Haddara, W., Prevec, L. and Graham, F.L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. US* 91: 8802-8806, 1994.

Ng, P., Parks, R. J., Cummings, D. T., Eveleigh, C. M., Sankar, U. and Graham, F. L. A high efficiency Cre/loxP based system for construction of adenoviral vectors. *Human Gene Therapy* 10: 2667-2672, 1999.

Ng, P., Parks, R. J., Cummings, D. T., Eveleigh, C. M., Sankar, U. and Graham, F. L. An enhanced system for construction of adenoviral vectors by the two plasmid rescue method. In Press. *Human Gene Therapy* 11:xxx, 2000.

Ng, P., Cummings, D. T., Eveleigh, C. M. and Graham, F. L. The yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors. Submitted.

5. Packaging capacity of Ad vectors

Ghosh Choudhury, G., Haj-Ahmad, Y., and Graham, F. L. Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *The EMBO J.* 6: 1733-1739, 1987.

Bett, A. J., Prevec, L., and Graham, F. L. Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* 67: 5911- 5921, 1993.

Parks, R. J. and Graham, F. L. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J. Virol.* 71: 3293-3298, 1997.

6. Ad vectors as vaccines

McDermott, M.R., Graham, F.L., Hanke, T. and Johnson, D.C. Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. *Virology* 169: 244-247, 1989.

Prevec, L., Schneider, M., Rosenthal, K.L., Belbeck, L.W., Derbyshire, J.B. and Graham, F.L. Use of human adenovirus based vectors for antigen expression in animals. *J. Gen. Virol.* 70: 429-434, 1989.

Prevec, L., Campbell, J.B., Christie, B.S., Belbeck, L. and Graham, F.L. A recombinant human adenovirus vaccine against rabies. *J. Inf. Dis.* 161: 27-30, 1990.

Prevec, L. Christie, B.S., Laurie, K.E., Smith, M., Graham, F.L. and Rosenthal, K.L. Immune response to HIV-1 gag antigens induced by recombinant adenovirus vectors in mice and Rhesus macaque monkeys. *J. Acquired Imm. Def. Syndr.* 4: 568-576, 1991.



Ebata, S. N., Prevec, L., Graham, F.L., and Dimock, K. Function and Immunogenicity of Human Parainfluenza Virus-3 Glycoproteins Expressed by Recombinant Adenoviruses. *Virus Research* 24: 21-33, 1992.

Zheng, B., Graham, F. L., Johnson, D. C., Hanke, T., McDermott, M. R. and Prevec, L. Immunogenicity of tandem repeats of a linear neutralizing epitope of the herpes simplex gD protein when expressed in recombinant adenovirus vectors. *Vaccine* 11: 1191-1198, 1993.

Both, G. W., Lockett, L. J., Janardhana, V., Edwards, S. J., Bellamy, A. R., Graham, F. L., Prevec, L., and Andrew, M. E. Protective immunity to rotavirus-induced diarrhoea is passively transferred to new-born mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. *Virology* 193, 940-950, 1993.

Wesseling, J. G., Godeke, G.-J., Schijns, V. E. C. J., Prevec, L., Graham, F. L., Horzinek, M. C., and Rottier, P. J. M. Mouse Hepatitis Virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection. *J. Gen. Virol.* 74: 2061-2069, 1993.

Yarosh, O. K., Wanderer, A. I., Graham, F. L., Campbell, J. B. and Prevec, L. Human adenovirus type 5 vectors expressing rabies glycoprotein. *Vaccine*. 14: 1257-1264, 1996.

7. Methods articles.

Hitt, M., Bett, A.J., Prevec, L. and Graham, F.L Construction and propagation of human adenovirus vectors. In: "Cell Biology: A Laboratory Handbook" Ed. J. E. Celis. Academic Press. 2nd Edition, Vol. 1, pp 500-512, 1998.

8. General

Hitt, M.M., R.J. Parks, and F.L. Graham. Structure and genetic organization of adenovirus vectors. *In The Development of Human Gene Therapy*, T. Friedman (ed.), Cold Spring Harbor Press, Cold Spring Harbor pp 61-86, 1998.

Hitt, M., Addison, C. and Graham, F.L Human adenovirus vectors for gene transfer into mammalian cells. In: "Advances in Pharmacology - Gene Therapy" Ed. J. Thomas August, Academic Press. San Diego, CA. 40: 137-206, 1997.

ADMAXTM PLASMID MAPS AND TECHNICAL ASSISTANCE

Plasmid maps and sequences can be downloaded from: www.microbix.com/products/av293c/d_p_maps_s.html.

If you have any questions regarding the production, testing or use of this vector system, please consult www.microbix.com/products/av293c/technical_support.html for technical bulletins and FAQs. If you require additional assistance, please send your questions and any relevant data to customer.service@microbix.com, or by fax 416-234-1626, to Microbix Technical Services. Your complete satisfaction with the performance of this product is important to us.