Stock Preparation of Recombinant Vaccinia Virus

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I. INTRODUCTION

Recombinant vaccinia virus is useful for the transient expression of exogenous proteins in a variety of cell types. This article describes a simple method for preparing large stocks [about 10^9–10^{10} plaque-forming units (PFU)] of the virus. The virus is very stable at −80°C, and once a virus stock has been made, it can be used for experiments for several years, if required.

II. MATERIALS AND INSTRUMENTATION

Glasgow minimal essential medium (GMEM, Cat. No. 2170-025), fetal calf serum (Cat. No. 013-6290), 200 m*M* L-glutamine (Cat. No. 043-5030), penicillin–streptomycin (10,000 U/ml and 10,000 μg/ml, Cat. No. 043-5140), and tryptose phosphate broth (Cat. No. 18050-039) are from GIBCO-BRL. Bisbenzimide (Cat. No. B-1782), HEPES (Cat. No. H-3375), soybean trypsin inhibitor (Cat. No. T-9003), trypsin (Cat. No. T-8642), and Tris (Cat. No. T-6791) are from Sigma. Sucrose (Cat. No. 1.7654), sodium dihydrogenphosphate monohydrate (Cat. No. 1.06346) and disodium hydrogenphosphate dodecahydrate (Cat. No. 1.06599) are from Merck. BHK21 cells are from ATCC (Cat. No. CRL-6282). Mowiol (Cat. No. 32,459-0) is from Aldrich.

Sterile, 50-ml polypropylene tubes are from Falcon. Tissue culture dishes (15 cm) were from GIBCO-BRL. Cryotubes (1.5 ml) are from Nunc. SW27 centrifuge tubes are from Beckmann. Other equipment needed includes a Dounce homogenizer, sonifier, CO_2 incubator, LAF hood (vertical flow), rubber policeman, GS3 bottles (sterile), centrifuges equipped with rotors for 50-ml Falcon tubes, SW27 tubes, and GS3 bottles.

III. PROCEDURES

A. Infection of BHK21 Cells

Solutions

1. *BHK medium:* To make 1 liter, mix 830 ml GMEM with 100 ml tryptose phosphate broth, 50 ml fetal calf serum, 10 ml penicillin–streptomycin, and 10 ml L-glutamine under sterile conditions. Store at 4°C.

2. *Phosphate-buffered saline (PBS):* To make 4 liters, dissolve 0.632 g $NaH_2PO_4 \cdot H_2O$, 7.93 g $Na_2HPO_4 \cdot 12H_2O$, and 32.4 g NaCl in 4 liters H_2O. Check that the pH is between 7.2 and 7.4 Autoclave and store at 4°C.

3. *1 M HEPES stock solution, pH 7.2:* To make 100 ml, add 23.8 g HEPES to distilled water, adjust pH to 7.2 with 6 *M* NaOH, and add water to 100 ml. Autoclave and store at 4°C.

4. *GMEM/HEPES:* To make 1 liter, mix 980 ml GMEM with 20 ml 1 *M* HEPES under sterile conditions. Store at 4°C.

Steps

Note: All manipulations, except for the centrifugations, should take place in a safety sterile bench (vertical flow) in a laboratory certified for work with vaccinia virus (safety level 2 or equivalent). Gloves and eye protection are required. Virus-contaminated items should be autoclaved or chemically disinfected.

1. Plate approximately 2×10^6 BHK21 cells in each of 40 tissue culture plates (15 cm) in BHK medium. Grow the cells at 37°C overnight in a CO_2 incubator (5% CO_2).

2. Dilute 4×10^7 PFU of the recombinant vaccinia virus into 200 ml of GMEM/HEPES in a beaker.

3. Wash the cells twice with 10 ml PBS at room temperature. Add 5 ml of diluted virus. Incubate for 1 hr at 37°C in a CO_2 incubator.

4. Add 20 ml of BHK medium. Incubate further for 3 days at 37°C in the CO_2 incubator.

B. Harvest of Virus

Solutions

1. *10 mM Tris, pH 9.0:* To make 1 liter, add 1.09 g Tris to 1 liter distilled water. Adjust the pH with 1 *M* HCl (approximately 150 μl).

2. *Sucrose 36% (w/v) in 10 mM Tris, pH 9.0:* To make 100 ml, add 36 g sucrose in a cylinder and add 10 m*M* Tris, pH 9.0, to 100 ml. Autoclave and store at 4°C.

Steps

1. Scrape the infected cells off the plates with a rubber policeman. At this point of infection, the cells should already have started to detach from the plate. With a 20-ml pipette, transfer the cell suspension to four GS3 bottles.

2. Centrifuge at 3000 rpm for 10 min in a JS-4.2 rotor (Beckman). Remove the supernatant.

3. Using a 10-ml pipette, resuspend each of the 4 pellets in 10 ml ice-cold Tris buffer. Pool the suspensions and transfer to a 100-ml Dounce homogenizer on ice.

4. Homogenize the cells on ice by 20 strokes (up and down) with a Teflon pestle. Place a small drop on a microscopy slide, cover with a coverslip, and examine by phase-contrast microscopy. Nuclei of broken cells will appear dark, whereas intact cells appear bright. Repeat the homogenization with a few strokes more if the suspension still contains more than 10% of intact cells.

5. Transfer to a 50-ml centrifuge tube (Falcon). Centrifuge at 2000 rpm at 4°C for 5 min in a Heraeus tissue culture centrifuge. Recover the supernatant and repeat the centrifugation procedure until no visible pellet appears.

6. Sonify the postnuclear supernatant on ice, using a sonifier equipped with a standard tip. We use the following conditions: A Branson sonifier adjusted to output level 6 for 2 min, at 20% intervals. *Warning:* Make sure that the cover of the sterile bench is completely closed before switching on the sonicator!

7. Using a 20-ml pipette, carefully layer the postnuclear supernatant on top of an equal

volume of a 36% sucrose cushion in SW27 centrifuge tubes, on ice. Centrifuge in a SW27 rotor at 25,000 rpm for 40 min at 4°C.

8. Remove the supernatant carefully with a Pasteur pipette connected to a suction pump. Using a 5-ml pipette, resuspend the pellet in 4 ml of the Tris buffer. Make aliquots of 50 or 100 μl in 1.5-ml cryotubes. Snap freeze in liquid nitrogen, and store the tubes at −80°C. The virus will be stable for years, and the same aliquot can be thawn and frozen several times without a significant reduction in the virus titer.

C. Determination of the Working Dilution of the Virus

Solutions

1. *PBS:* See Section A.

2. *Carnoy solution:* To make 8 ml, mix 2 ml of glacial acetic acid with 6 ml of methanol.

3. *Trypsin at 2.5 mg/ml:* To make 5 ml, dissolve 12.5 mg trypsin in 5 ml distilled water. Sterile filter and store in aliquots at −80°C.

4. *Soybean trypsin inhibitor at 20 mg/ml:* To make 5 ml, dissolve 100 mg soybean trypsin inhibitor in 5 ml of distilled water. Sterile filter and store in aliquots at −80°C.

5. *1 mM bisbenzimide stock solution:* To make 10 ml, dissolve 5.3 mg bisbenzimide in 10 ml of water. Store at 4°C protected from light.

6. *Virus incubation medium:* To make 10 ml, mix 9.8 ml of GMEM with 0.2 ml of 1 *M* HEPES stock solution (Section A) and 10 μl of soybean trypsin inhibitor (20 mg/ml).

Steps

1. Plate BHK cells on eight round glass coverslips (11 mm) in a 24-well plate (5×10^4 cells/well). Grow the cells overnight at 37°C in a CO_2 incubator.

2. Trypsinize an aliquot of the virus preparation by adding 1/10 vol of trypsin solution. Incubate at 37°C for 30 min.

3. Wash the cells once with 1 ml of GMEM, then add 200 μl of virus incubation medium. To different wells, add increasing amounts of the virus, e.g., 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μl. Incubate at 37°C in a CO_2 incubator for 8 hr.

4. Wash the cells twice with PBS, then air dry them for >20 min at room temperature.

5. Add 500 μl of Carnoy solution to each well. Leave at room temperature for 30 min.

6. Remove the solution, wash twice with PBS, and air dry >20 min again.

7. Incubate with 5 μM bisbenzimide in PBS for 30 min.

8. Wash the coverslips three times with water. Blot off excess water on a paper tissue and place the coverslip (cells facing down) on 4 μl of Mowiol on a microscopy slide. Let dry for >20 min at 37°C.

9. Examine the cells in a fluorescence microscope, using a UV filter. Viral factories should be evident as bright spots in the cytoplasm of infected cells. Choose the lowest dilution of virus giving infection of >95% of the cells as the working dilution for later experiments.

IV. COMMENTS

This method yields mainly the intracellular mature form of the virus. The virus will not be entirely pure, and some cellular debris is present in the pellet after the sucrose cushion. However, for most practical applications, the virus should be of sufficient purity. A typical preparation requires a working dilution of 0.1–0.5 μl of virus for infection of 5×10^4 cells on a coverslip. If an accurate determination of the virus titer is desirable, a plaque assay (Fuerst *et al.*, 1986) is required. We have estimated that a typical working concentration (infecting >95% of the cells) corresponds to approximately 5 PFU/cell (Bucci *et al.*, 1992).

V. PITFALLS

1. Vaccinia virus may cause severe infections if in contact with the eyes or with open wounds. Wear eye protection and gloves. Vaccination against smallpox is recommended. The virus is stable and resists drying. Any spills, therefore, have to be chemically disinfected.

2. The cells should be less than confluent (approximately 80%) at the time of infection. Infection is less efficient if the cells are too confluent, resulting in a lower virus yield and low purity of the virus.

3. Although a good yield of virus depends on an efficient breakage of the cells, it is essential not to overhomogenize the cells. Broken nuclei, resulting from overhomogenization, are not efficiently pelleted at 2000 rpm, and DNA from these nuclei will be released during the sonication step. Such DNA may cause the final pellet to become yellowish–brown and sticky, making it difficult to pipette precise amounts.

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References

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