

- Determine titer by counting plaques within wells and multiplying by dilution factor. Most accurate results are obtained from wells with 20 to 80 plaques. In determining titer, take into account the 1:1 dilution of virus stock with trypsin.

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## Generation of Recombinant Vaccinia Viruses

UNIT 16.17

HeLa S3 cells are used for large-scale growth of vaccinia virus. However, several other cell lines may be required for plaque purification and amplification. For TK selection, HuTK<sup>-</sup> 143B cells are used; for XGPRT selection, BS-C-1 cells are used. CV-1 cells are used for transfection. BS-C-1 or CV-1 cells can be used for determination of virus titer (UNIT 16.16).

Approximately  $1-5 \times 10^{10}$  pfu of purified virus should be obtained per liter ( $5 \times 10^8$ ) of HeLa cells. Depending on the efficiency of the transfection, single, well-isolated plaques should be visible in cells infected with one of the recommended virus dilutions. With TK selection, 10-90% of the plaques will contain recombinant virus. If  $\beta$ -galactosidase screening is also used, only blue recombinant virus plaques should be picked. With XGPRT selection, all plaques picked should contain recombinant virus. If the titer of recombinant virus is low, amplification can be achieved by a round of growth in the presence of MPA prior to plaquing (use the procedure described in amplification of a plaque). Cytopathic effects should be clearly visible at each step of amplification except with final infection (infected HeLa cells do not exhibit clear cytopathic effects). The titer of the final crude stock should be  $1-2 \times 10^9$  pfu/ml.

**CAUTION:** Proceed carefully and follow biosafety level 2 (BL-2) practices when working with vaccinia virus (see UNIT 16.15 for safety precautions).

**NOTE:** Carry out all procedures described below in a tissue culture hood.

### TRANSFECTION OF INFECTED CELLS WITH A VACCINIA VECTOR

BASIC  
PROTOCOL

The foreign gene of interest is subcloned into a plasmid transfer vector (Figs. 16.17.1-16.17.3) so that it is flanked by DNA from a nonessential region of the vaccinia genome. This recombinant plasmid is then transfected into cells that have been infected with wild-type vaccinia virus. Homologous recombination between the vaccinia and plasmid DNA generates a recombinant virus. The recombinant virus is obtained in a cell lysate which is then subjected to several rounds of plaque purification using appropriate selection and/or screening protocols (second basic protocol).

*Materials (see APPENDIX 1 for items with ✓)*

pSC11, pMJ601, pTKgptF1S, or other suitable vector (Table 16.17.1; Figs. 16.17.1-16.17.3)

CV-1 cells (UNIT 16.16)

✓ Complete MEM-10 and -2.5

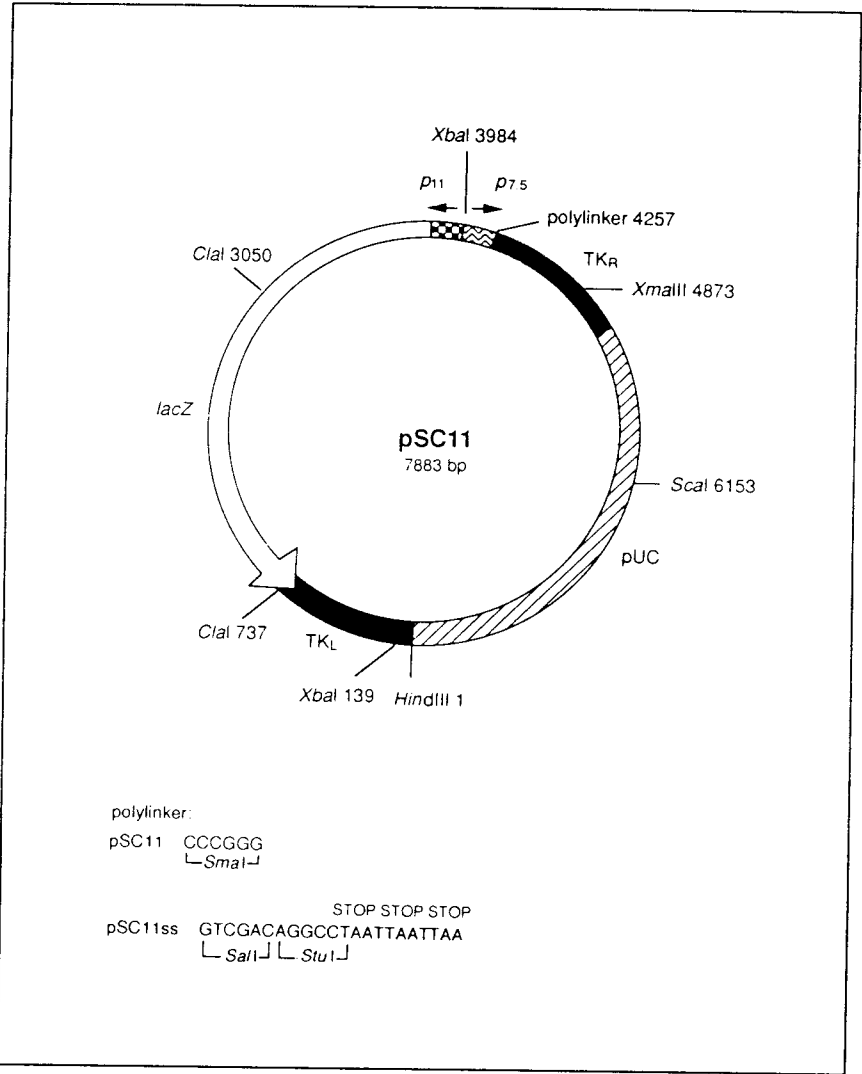
16.17

Protein Expression

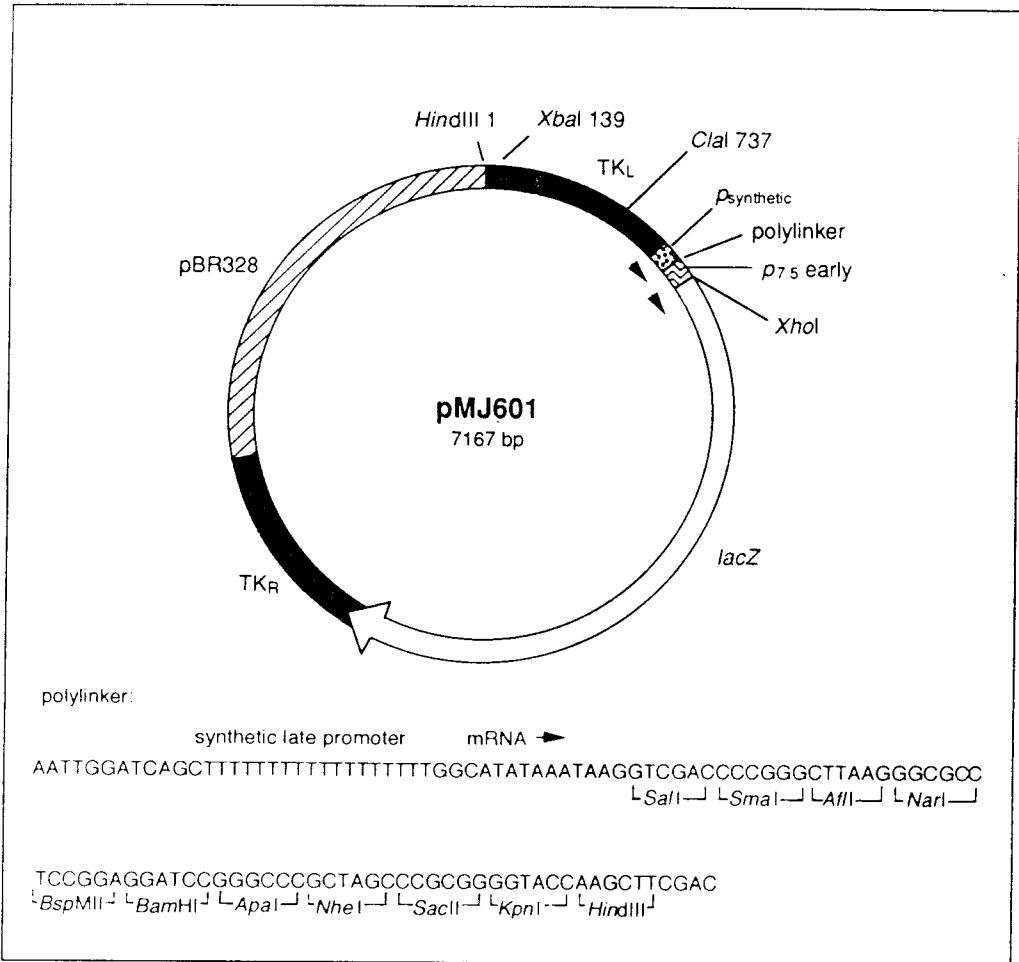
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- Wild-type vaccinia virus stock (UNIT 16.16)
- 0.25 mg/ml trypsin (2× crystallized and salt-free, Worthington; filter sterilize and store at -20°C)
- ✓ Transfection buffer
- ✓ 2.5 M CaCl<sub>2</sub>
- 25-cm<sup>2</sup> tissue culture flask
- Humidified 37°C, 5% CO<sub>2</sub> incubator
- 12 × 75-mm polystyrene tube
- Disposable scraper or rubber policeman, sterile
- Sorvall H-6000A rotor or equivalent



**Figure 16.17.1** pSC11 contains a moderate-strength compound early/late promoter, *p7.5*, with one or more unique restriction endonuclease sites for insertion of genes (Chakrabarti et al., 1985). A late vaccinia virus promoter, *p11*, is used to express the *E. coli lacZ* gene. The expression cassette is flanked by segments of the vaccinia TK gene. TK selection and β-galactosidase screening can be used for isolation of recombinant virus plaques. Nucleotide numbers for these and other plasmid vectors have been estimated and may not be exact.



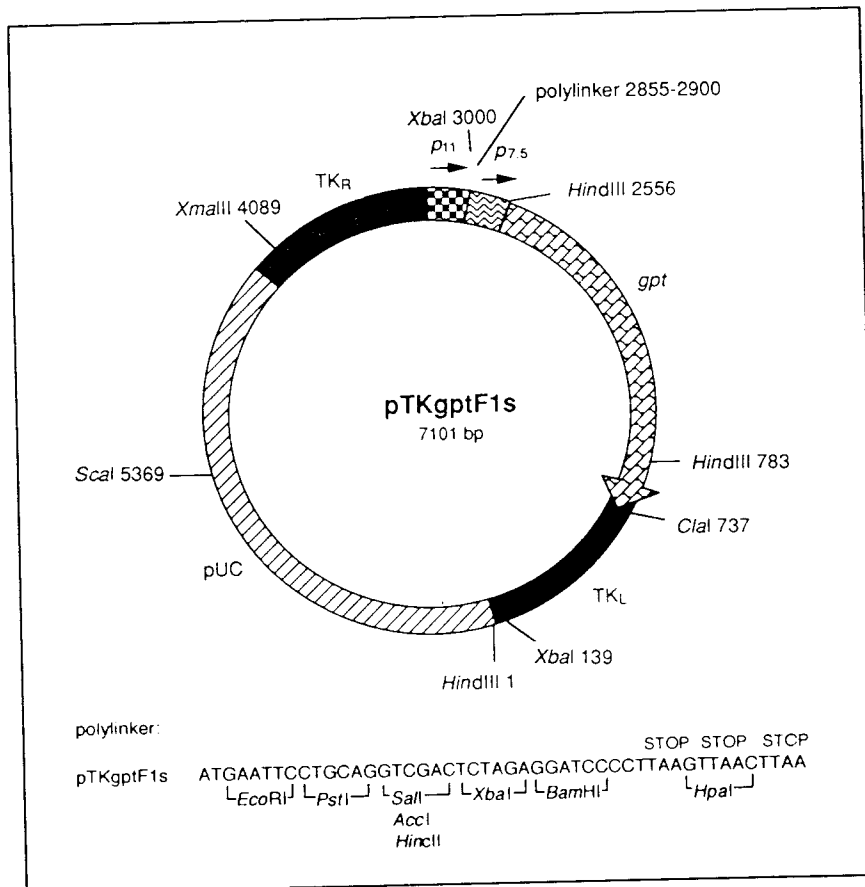
**Figure 16.17.2** pMJ601 contains a very strong synthetic late promoter and a polylinker to be used as a multiple cloning site (Davison and Moss, 1990). The early portion of the promoter, *p7.5*, is used to regulate expression of the *E. coli lacZ* gene. The entire expression cassette is flanked by segments of the TK gene. TK selection and β-galactosidase screening can be used for isolation of recombinant virus plaques.

1. Subclone gene of interest into polylinker in pSC11, pMJ601, pTKgptF1S, or other suitable vector (UNIT 3.16).
2. Seed 25-cm<sup>2</sup> flask with 10<sup>6</sup> CV-1 cells in complete MEM-10. Place in CO<sub>2</sub> incubator at 37°C until nearly confluent (usually overnight).
3. Prepare trypsinized virus as follows: just prior to use, mix an equal volume of wild-type vaccinia virus stock and 0.25 mg/ml trypsin and vortex vigorously. Incubate 30 min in 37°C water bath, vortexing at 5- to 10-min intervals (for detailed information, see step 3 of basic protocol on preparation of vaccinia virus stock in UNIT 16.16).
4. Dilute trypsinized virus in complete MEM-2.5 to 1.5 × 10<sup>5</sup> pfu/ml. Aspirate medium from confluent monolayer of CV-1 cells and infect with 1 ml diluted vaccinia virus (0.05 pfu/cell). Place 2 hr in CO<sub>2</sub> incubator at 37°C, rocking at ~15-min intervals.

- Approximately 30 min before end of infection, prepare calcium phosphate-precipitated DNA solution as follows: place 1 ml transfection buffer into 12 x 75-mm polystyrene tube and add 5 to 10 µg recombinant plasmid DNA (in <50 µl; containing gene of interest from step 1); slowly add 50 µl of 2.5 M CaCl<sub>2</sub> and mix *gently*. Leave 20 to 30 min at room temperature—a fine precipitate should appear.

*Inclusion of wild-type vaccinia DNA (second support protocol) in transfection results in higher efficiency of recombination. If added, combine 1 µg wild-type vaccinia DNA with 5 to 10 µg recombinant plasmid DNA and vortex vigorously.*

- Aspirate virus inoculum from CV-1 cells (step 4). Add precipitated DNA solution (step 5) and leave 30 min at room temperature. Add 9 ml complete MEM-10 and place 3 to 4 hr in CO<sub>2</sub> incubator at 37°C.
- Aspirate medium, replace with 5 ml complete MEM-10, and incubate 2 days in CO<sub>2</sub> incubator at 37°C.



**Figure 16.17.3** pTKgptF1s contains the strong late vaccinia virus promoter, *p<sub>11</sub>*, followed by a polylinker for insertion of protein-coding segments that are in-frame with the ATG (Falkner et al., 1988). Additional variants, pTKgptF2s and pTKgptF3s, contain one or two additional G residues, respectively, following the ATG to allow all three phasing possibilities. The *E. coli gpt* gene is regulated by the compound early/late promoter, *p<sub>7.5</sub>*. The expression cassette is flanked by segments of the vaccinia virus TK gene. Either TK or XGPRT selection can be used for isolation of recombinant virus plaques.

8. Dislodge cells with disposable scraper or sterile rubber policeman and transfer to cone-bottom centrifuge tube. Centrifuge 5 min at  $1800 \times g$  (2500 rpm in an H-6000A rotor), 5 to  $10^{\circ}\text{C}$ , and aspirate medium. Resuspend cells in 0.5 ml complete MEM-2.5.
9. Lyse cell suspension by three freeze-thaw cycles as follows: freeze in dry ice/ethanol, thaw in  $37^{\circ}\text{C}$  water bath, and vortex.
10. Store cell lysate at  $-70^{\circ}\text{C}$  until needed in selection and screening procedure (second basic protocol).

## PURIFICATION OF VACCINIA VIRUS

Purified virus is useful for preparation of vaccinia DNA (second support protocol), studies in which contaminating infected cell proteins are undesirable, and as a very high-titer stock. For large-scale purification, it is preferable to use HeLa cell suspensions for infection.

*Additional Materials (see APPENDIX 1 for items with ✓)*

Vaccinia virus stock (UNIT 16.16)

0.25 mg/ml trypsin (2 $\times$  crystallized and salt-free, Worthington; filter sterilize and store at  $-20^{\circ}\text{C}$ )

HeLa S3 cells (UNIT 16.16)

✓ Complete spinner medium containing 5% horse serum (complete spinner medium-5)

✓ 10 mM and 1 mM Tris-Cl, pH 9.0

95% ethanol

36% (w/v) sucrose solution in 10 mM Tris-Cl, pH 9.0

40%, 36%, 32%, 28%, and 24% (w/v) sucrose solutions in 1 mM Tris-Cl, pH 9.0, sterile

Hemocytometer (UNIT 1.2)

Vented spinner flasks (Bellco #1965 series and #A523-A59)

Dounce homogenizer, glass and tight-fitting

Probe sonicator (Ultrasonic processor VC-600, Sonics and Materials)

Beckman SW-27 or SW-28 rotor and sterile centrifuge tubes

### *Infect cells*

1. Just prior to use, mix an equal volume of vaccinia virus stock and 0.25 mg/ml trypsin by vortexing vigorously. Incubate 30 min at  $37^{\circ}\text{C}$ , vortexing at 5- to 10-min intervals (for detailed information, see step 3 of basic protocol on preparation of vaccinia virus stock in UNIT 16.16).
2. Count HeLa S3 cells. Centrifuge  $5 \times 10^8$  cells 10 min at  $1800 \times g$  (2500 rpm in H-6000A rotor), room temperature. Resuspend cells in complete spinner medium-5 at  $2 \times 10^7$  cells/ml.
3. Add trypsinized virus (step 1) at an MOI of 5 to 8 pfu/cell and stir 30 min at  $37^{\circ}\text{C}$ . Transfer cells to vented spinner flask containing 1 liter complete spinner medium-5 and stir 2 to 3 days at  $37^{\circ}\text{C}$ .
4. Centrifuge cells 5 min at  $1800 \times g$ ,  $5^{\circ}$  to  $10^{\circ}\text{C}$ . Resuspend in 14 ml of 10 mM Tris-Cl, pH 9.0. Keep on ice for remainder of protocol.

**SUPPORT  
PROTOCOL**

**16.17**

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**Table 16.17.1** Vaccinia Virus Transfer Vectors

Vector	Promoter <sup>a</sup>	Unique restriction sites	Flanking DNA	Selection/screening	Reference
pGS20	<i>p</i> <sub>7.5</sub> (E/L)	<i>Bam</i> HI, <i>Sma</i> I <sup>b</sup>	TK	TK <sup>-</sup>	Mackett et al., 1984
pGS61	<i>p</i> <sub>7.5</sub>	<i>Bam</i> HI, <i>Hind</i> III	TK	TK <sup>-</sup>	Smith et al., 1987
pGS62	<i>p</i> <sub>7.5</sub>	<i>Bam</i> HI, <i>Sma</i> I, <i>Eco</i> RI	TK	TK <sup>-</sup>	Smith et al., 1987
pVV3	<i>p</i> <sub>7.5</sub>	Polylinker	TK	TK <sup>-</sup>	Rice et al., 1985
pBCB01, 2, 3 <sup>c</sup>	<i>p</i> F	Polylinker	TK	TK <sup>-</sup>	Boyle et al., 1989
pBCB06	<i>p</i> <sub>7.5</sub>	Polylinker	TK	TK <sup>-</sup>	Boyle et al., 1989
pSC11 <sup>d</sup>	<i>p</i> <sub>7.5</sub>	<i>Sma</i> I <sup>b</sup>	TK	TK <sup>-</sup> /β-gal	Chakrabarti et al., 1985
pSC11ss <sup>d</sup>	<i>p</i> <sub>7.5</sub>	<i>Stu</i> I, <i>Sal</i> I	TK	TK <sup>-</sup> /β-gal	Earl et al., 1990
pCF11	<i>p</i> <sub>7.5</sub>	<i>Sma</i> I <sup>b</sup>	<i>Hind</i> III C	β-gal	Flexner et al., 1987
pYF6	<i>p</i> <sub>7.5</sub>	<i>Sma</i> I <sup>b</sup>	HA	HA <sup>-</sup> , β-gal	Flexner et al., 1987
pPro18	<i>p</i> <sub>7.5</sub>	<i>Sma</i> I <sup>b</sup>	HA	HA <sup>-</sup>	Shida et al., 1987
pTK-7.5A	<i>p</i> <sub>7.5</sub>	Polylinker	<i>Hind</i> III F	TK <sup>+</sup>	Coupar et al., 1988
pTK-7.5B	<i>p</i> <sub>7.5</sub>	Polylinker	<i>Hind</i> III F	TK <sup>+</sup>	Coupar et al., 1988
pUVI	<i>p</i> <sub>1</sub> (L)	Polylinker <sup>e</sup>	TK	TK <sup>-</sup> /β-gal	Falkner et al., 1987
pTK-gpt-F1s, 2s, 3s <sup>d</sup>	<i>p</i> <sub>11</sub>	Polylinker	TK	TK <sup>-</sup> or gpt	Falkner & Moss, 1988
pJ16 <sup>f</sup>	<i>p</i> <sub>11</sub> , <i>p</i> <sub>25</sub> (E)	Multiple	TK	TK <sup>-</sup>	Tsao et al., 1988
p1200	CAE I (L)	<i>Cl</i> aI	TK	TK <sup>-</sup>	Patel et al., 1988
pMP528HRH	<i>p</i> H6 (E/L)	<i>Xho</i> I, <i>Kpn</i> I, <i>Sma</i> I <sup>b</sup>	<i>Hind</i> III K	Host range	Perkus et al., 1989
pHES1, 2, 3	<i>p</i> H6	Polylinker <sup>g</sup>	<i>Hind</i> III K	Host range	Perkus et al., 1989
pHES4	<i>p</i> H6	Polylinker	<i>Hind</i> III K	Host range	Perkus et al., 1989
pMJ601 <sup>d</sup>	Synthetic (L)	Polylinker	TK	TK <sup>-</sup> /β-gal	Davison & Moss, 1990
pMJ602	Synthetic (L)	Polylinker	TK	TK <sup>-</sup> /β-gal	Davison & Moss, 1990
pSC59	Synthetic (E/L)	Polylinker	TK	TK <sup>-</sup>	Chakrabarti & Moss, unpub. obs.
pSC65	Synthetic (E/L)	Polylinker	TK	TK <sup>-</sup> /β-gal	Chakrabarti & Moss, unpub. obs.

<sup>a</sup>E, early; L, late; E/L, early and late.

<sup>b</sup>*Sma*I digestion gives a blunt-end for cloning any fragment that has been blunt-ended.

<sup>c</sup>Initiation codons upstream of polylinker.

<sup>d</sup>Represented in Figures 16.17.1-16.17.3.

<sup>e</sup>Initiation codon immediately precedes *Eco*RI site.

<sup>f</sup>Bidirectional promoters. *p*<sub>11</sub> has consecutive initiation and termination codons.

<sup>g</sup>Three-vector set with translation initiation codon followed by polylinker in all three open-reading-frames.

### *Lyse cells*

5. Homogenize cell suspension with 30 to 40 strokes in a tight-fitting, glass Dounce homogenizer. Check for cell breakage by light microscopy.
6. Centrifuge 5 min at  $300 \times g$  (900 rpm in H-6000A rotor),  $5^{\circ}$  to  $10^{\circ}\text{C}$ , to remove nuclei. Save the supernatant. Resuspend cell pellet in 3 ml of 10 mM Tris-Cl, pH 9.0. Centrifuge 5 min at  $300 \times g$ ,  $5^{\circ}$  to  $10^{\circ}\text{C}$ . Save supernatant and pool with previous supernatant.
7. Sonicate lysate—keeping lysate on ice the entire time—using probe sonicator as follows: (a) sterilize probe by dipping it in 95% ethanol and passing it through a flame; (b) let probe cool; (c) remove cap from tube containing lysate, place probe into lysate, and sonicate at full power for 15 sec; (d) wait 15 sec and repeat sonication three to four times.

*If a probe sonicator is unavailable, sonication can be done in a cup. It is best to split the sample into 3-ml aliquots and sonicate each separately. To use cup sonicator, fill cup with ice-water (~50% ice). Place tube containing lysate in ice-water, and sonicate at full power for 1 min. Repeat 3 to 4 times, placing lysate on ice  $\geq 30$  sec between sonications. Sonication melts ice, so it is necessary to replenish ice in cup.*

### *Obtain purified virus*

8. Layer sonicated lysate onto cushion of 17 ml of 36% sucrose (in 10 mM Tris-Cl, pH 9.0) in sterile SW-27 centrifuge tube. Centrifuge 80 min at  $32,900 \times g$  (13,500 rpm in SW-27 rotor),  $4^{\circ}\text{C}$ . Aspirate supernatant.
9. Resuspend viral pellet in 1 ml of 1 mM Tris-Cl, pH 9.0. Sonicate once for 15 sec with probe sonicator as in step 7 (if cup sonicator is used, sonicate 1 min).
10. Prepare sterile 24% to 40% continuous sucrose gradient in sterile SW-27 centrifuge tube the day before needed by carefully layering 6.8 ml of each of the following sucrose solutions (in 1 mM Tris-Cl, pH 9.0) in tube: 40%, 36%, 32%, 28%, and 24%. Let sit overnight in refrigerator.
11. Overlay sucrose gradient with 1 ml sonicated viral pellet from step 9. Centrifuge 50 min at  $26,000 \times g$  (12,000 rpm in SW-27 rotor),  $4^{\circ}\text{C}$ .
12. Observe virus as a milky band in about the middle of tube. Aspirate sucrose above band and discard. Carefully collect virus band (~10 ml) with sterile pipet and place in sterile tube and save.
13. Collect aggregated virus from pellet at bottom of gradient by aspirating remaining sucrose from tube. Resuspend viral pellet by pipetting up and down in 1 ml of 1 mM Tris-Cl, pH 9.0. Sonicate as in step 7.
14. Reband virus as in steps 10 to 12 and pool band with band from step 12. Add 2 vol of 1 mM Tris-Cl, pH 9.0, and mix. Transfer to sterile SW-27 centrifuge tubes (total volume should be ~60 ml; if less is obtained, fill tubes with 1 mM Tris-Cl, pH 9.0). Centrifuge 60 min in SW-27 rotor at  $32,900 \times g$ ,  $4^{\circ}\text{C}$ , and aspirate supernatant.
15. Resuspend virus pellets in 1 ml of 1 mM Tris-Cl, pH 9.0. Sonicate and divide into 200- to 250- $\mu\text{l}$  aliquots. Save one aliquot for step 16 and freeze remainder at  $-70^{\circ}\text{C}$ .
16. Estimate amount of virus in aliquot spectrophotometrically: one  $A_{260}$  unit is  $\sim 1.2 \times 10^{10}$  virus particles, which is  $\sim 2.5 \times 10^8$  pfu (value is

**SUPPORT  
PROTOCOL**

approximate due to light scattering). Use to determine amount of virus needed in support protocol for isolation of vaccinia virus DNA.

17. Titer virus by sonicating 20 to 30 sec on ice, preparing 10-fold serial dilutions to  $10^{-10}$ , and infecting in duplicate confluent BS-C-1 cell monolayers in 6-well tissue culture dishes using the  $10^{-8}$ ,  $10^{-9}$ , and  $10^{-10}$  dilutions (UNIT 16.16).

**ISOLATION OF VACCINIA VIRUS DNA***Additional Materials*

Purified vaccinia virus (first support protocol)  
50 mM and 1 M Tris-Cl, pH 7.8  
10% sodium dodecyl sulfate (SDS)  
60% sucrose  
10 mg/ml proteinase K  
Phenol equilibrated with 50 mM Tris-Cl, pH 7.8 (UNIT 2.1)  
1:1 (v/v) phenol/chloroform  
1 M sodium acetate, pH 7.0  
100% and 95% ethanol

1. Measure purified vaccinia virus at  $A_{260}$  and bring 20  $A_{260}$  units of virus to 1.2 ml final volume with 50 mM Tris-Cl, pH 7.8. Do not vortex DNA throughout this protocol to avoid shearing of large viral genome.
2. Add the following to vaccinia suspension (2 ml final): 0.1 ml of 1 M Tris-Cl, pH 7.8; 0.1 ml of 10% SDS; 0.2 ml of 60% sucrose; and 0.4 ml of 10 mg/ml proteinase K. Incubate 4 hr at 37°C.
3. Extract twice with phenol as follows: add an equal volume equilibrated phenol, mix *gently* by rocking tube, centrifuge 10 min at  $300 \times g$  (900 rpm in H-6000A rotor), room temperature, and save aqueous phase using a wide-bore pipet.
4. Extract once with 1:1 phenol/chloroform (step 3).
5. Add  $\frac{1}{10}$  vol of 1 M sodium acetate, pH 7.0, and 2.5 vol of 100% ethanol. Mix gently and cool several hours at  $-20^{\circ}\text{C}$ .
6. Microcentrifuge 10 min at top speed, 4°C. Aspirate supernatant. Wash pellet twice with 95% ethanol, air dry, and dissolve in 100  $\mu\text{l}$  water.
7. Prepare dilutions (using only a small amount of the total material) and measure  $A_{260}$  to determine DNA concentration (APPENDIX 3).

**BASIC  
PROTOCOL****SELECTION AND SCREENING OF RECOMBINANT  
VIRUS PLAQUES**

Two procedures involving guanine phosphoribosyltransferase (XGPRT) or thymidine kinase (TK) for selecting plaques that contain recombinant viruses are described. In addition,  $\beta$ -galactosidase screening can be used alone or in conjunction with TK selection to discriminate  $\text{TK}^-$  recombinants from spontaneous  $\text{TK}^-$  mutants. For each method, recombinant virus (obtained in the transfection basic protocol) is used to infect a monolayer culture of cells—for XGPRT selection, BS-C-1 cells are used because large plaques



are obtained; for TK selection, it is necessary to use a cell line such as HuTK<sup>-</sup> 143B that is deficient in thymidine kinase.

*Materials (see APPENDIX 1 for items with ✓)*

BS-C-1 confluent monolayer culture (UNIT 16.16)

HuTK<sup>-</sup> 143B confluent monolayer culture (UNIT 16.16)

✓ Complete MEM-2.5

10 mg/ml mycophenolic acid (MPA; Calbiochem #475913) in 0.1 N NaOH (400×; store at -20°C)

10 mg/ml xanthine in 0.1 N NaOH (40×; store at -20°C)

10 mg/ml hypoxanthine in water (670×; store at -20°C)

Transfected cell lysate (first basic protocol)

2% LMP agarose in water (GIBCO/BRL #5517UA), sterilized by autoclaving

✓ Complete 2× plaque medium containing 5% FCS (complete 2× plaque medium-5)

5 mg/ml BrdU in water (filter sterilize and store at -20°C)

10 mg/ml neutral red in water

4% Xgal in dimethylformamide (optional; Table 1.4.2)

Hemocytometer (UNIT 1.2)

6-well, 35-mm tissue culture dishes

Humidified 37°C, 5% CO<sub>2</sub> incubator

45°C water bath

Cotton-plugged Pasteur pipets, sterile

*Prepare cells*

1. Trypsinize confluent monolayer culture and resuspend in appropriate complete medium as in UNIT 16.16, steps 3 to 4 of the first basic protocol.
  - a. For XGPRT selection, use BS-C-1 cells.
  - b. For TK selection, use HuTK<sup>-</sup> 143B cells.
2. Count cells. Plate  $5 \times 10^5$  cells/well in 6-well tissue culture dish (2 ml/well final). Place in CO<sub>2</sub> incubator at 37°C and allow to reach confluency (<24 hr).
3. Prepare cells as indicated below.
  - a. For XGPRT selection, preincubate monolayer for 12 to 24 hr in filter-sterilized complete MEM-2.5 containing:  $\frac{1}{400}$  vol 10 mg/ml MPA;  $\frac{1}{40}$  vol 10 mg/ml xanthine; and  $\frac{1}{670}$  vol 10 mg/ml hypoxanthine.
  - b. For TK selection do not preincubate.

*Prepare lysate and infect cells*

4. Trypsinize 100  $\mu$ l of transfected cell lysate as for wild-type vaccinia virus in step 3 of first basic protocol. Sonicate 20 to 30 sec on ice.
5. Make four 10-fold serial dilutions ( $10^{-1}$  to  $10^{-4}$ ; UNIT 1.11) of trypsinized cell lysate in complete MEM-2.5 as indicated below.
  - a. For XGPRT selection, add MPA, xanthine, and hypoxanthine at concentrations indicated in step 3a above.
  - b. For TK selection, make no additions.

6. Aspirate medium from cell monolayers (step 2) and infect with 1.0 ml diluted lysate/well (use dilutions between  $10^{-2}$  and  $10^{-4}$ ). Place 2 hr in  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ , rocking at 30-min intervals.
7. Before 2-hr infection is finished, melt 2% LMP agarose (1.5 ml  $\times$  number of wells) and place in  $45^\circ\text{C}$  water bath to cool—be sure it cools to  $45^\circ\text{C}$  before using to overlay cells. Prepare and warm to  $45^\circ\text{C}$  the necessary amount of selective plaque medium (1.5 ml  $\times$  number of wells) by making the following additions to complete 2 $\times$  plaque medium-5:
  - a. For XGPRT selection, include MPA, xanthine, and hypoxanthine at twice the concentrations indicated in step 3a; filter sterilize.
  - b. For TK selection, include  $\frac{1}{100}$  vol of 5 mg/ml BrdU.
8. Prepare appropriate selective agarose by mixing equal volumes of 2% LMP agarose and selective plaque medium from step 7a or 7b.
9. Aspirate viral inoculum from cells (step 6). Overlay each well with 3 ml appropriate selective agarose and allow to solidify at room temperature or  $4^\circ\text{C}$ . Place 2 days in  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ .
10. Prepare second agarose overlay by mixing an equal volume of 2% LMP agarose (1 ml  $\times$  number of wells, melted and cooled to  $45^\circ\text{C}$ ) and 2 $\times$  plaque medium-5 (1 ml  $\times$  number of wells, warmed to  $45^\circ\text{C}$ ) with  $\frac{1}{100}$  vol of 10 mg/ml neutral red. If  $\beta$ -galactosidase screening is used, add  $\frac{1}{20}$  vol of 4% Xgal to agarose/ plaque medium. Overlay each well with 2 ml second agarose overlay, allow to solidify, and place overnight in  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ .

#### *Obtain the plaques*

11. Add 0.5 ml complete MEM-2.5 to sterile microcentrifuge tubes. When incubation period is complete (step 10), pick well-separated plaques by squeezing the rubber bulb on a sterile, cotton-plugged Pasteur pipet and inserting tip through agarose to plaque. Scrape cell monolayer and aspirate the agarose plug into pipet. Transfer to tube containing 0.5 ml complete MEM-2.5. Repeat for 6-12 plaques, placing each in separate tube.
12. Vortex, then carry out freeze-thaw cycling of the plaque isolates three times as described in step 9 of first basic protocol.
13. Place tube containing virus into cup sonicator containing ice-water and turn on full power for 20 to 30 sec (a probe sonicator dipped into a 50 ml plastic beaker also may be used; see step 7 of first support protocol). Cool on ice after sonication.

*If TK selection only has been used, plaque isolates should be tested by DNA dot-blot hybridization (UNIT 16.18) or polymerase chain reaction (UNIT 16.18) because some plaques will contain spontaneous TK<sup>-</sup> mutations and not recombinant virus.*

#### *Carry out several rounds of plaque purification*

14. Prepare monolayers of appropriate cell line as in steps 1 to 3; one 6-well dish is needed for each plaque isolate.
15. Make three 10-fold serial dilutions at  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  of each of several plaque isolates as in step 5. If XGPRT selection is used,

preincubate cells with selective drugs and add selective drugs to serial dilutions of viral isolates (step 3a).

16. Aspirate medium from cell monolayers and infect two wells with 1.0 ml of each dilution of virus. Place 2 hr in CO<sub>2</sub> incubator at 37°C, rocking by hand at 30-min intervals.
17. Repeat steps 7 to 13 for two or three rounds of plaque purification to ensure a clonally pure recombinant virus.

## AMPLIFICATION OF A PLAQUE

*Materials (see APPENDIX 1 for items with ✓)*

Resuspended recombinant plaque (second basic protocol)  
Confluent monolayer cultures of cells in both a 12-well, 22-mm tissue culture dish and a 25-cm<sup>2</sup> tissue culture flask (UNIT 16.16)

- ✓ Complete MEM-2.5 and -10
- Spinner culture of HeLa S3 cells (UNIT 16.16)
- Humidified 37°C, 5% CO<sub>2</sub> incubator
- Hemocytometer (UNIT 12)
- Sorvall H-6000A rotor or equivalent
- 150-cm<sup>2</sup> tissue culture flask

### *Infect monolayer culture of cells with a plaque*

1. Sonicate resuspended recombinant plaque 20 to 30 sec on ice as in step 13 of second basic protocol.
2. Infect appropriate confluent monolayer culture in 12-well dish with 250 µl (½) of each plaque isolate. If XGPRT selection is used, preincubate monolayer culture 12 to 24 hr in complete MEM-2.5 containing MPA, xanthine, and hypoxanthine (step 3a of second basic protocol); carry out infection in presence of these drugs. Place 1 to 2 hr in CO<sub>2</sub> incubator at 37°C, rocking at ~15-min intervals.
3. Overlay with 1 ml complete MEM-2.5 containing appropriate drugs as indicated in previous basic protocol—for XGPRT selection, follow step 3a and for TK selection, use ½<sub>00</sub> vol of 5 mg/ml BrdU. Place 2 days in CO<sub>2</sub> incubator at 37°C or until cytopathic effect (cell rounding) is obvious.
4. Scrape cells, transfer to microcentrifuge tube, and microcentrifuge 30 sec at top speed. Aspirate medium. Resuspend cells in 0.5 ml complete MEM-2.5 and carry out freeze-thaw cycle three times as in step 9 of first basic protocol.
5. Sonicate 20 to 30 sec on ice as in step 13 of second basic protocol.

### *Scale-up the culture*

6. Dilute 0.25 ml lysate with 0.75 ml complete MEM-2.5. Infect appropriate confluent monolayer culture in a 25-cm<sup>2</sup> flask and place 30 min to 1 hr in CO<sub>2</sub> incubator at 37°C.
7. Prepare selective MEM-2.5: (a) For XGPRT selection, see step 3a of the second basic protocol; (b) For TK selection, include ½<sub>00</sub> volume of 5 mg/ml BrdU. Remove medium and overlay with 4 ml selective

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MEM-2.5. Incubate 2 days in CO<sub>2</sub> incubator at 37°C or until cytopathic effect is obvious.

8. Scrape cells, transfer to 15-ml conical centrifuge tube, centrifuge 5 min at 1800 × g (2500 rpm in H-6000A rotor), 5-10°C. Resuspend cells and repeat freeze-thaw cycling and sonication as in steps 4 and 5.
9. Count HeLa S3 cells from spinner culture. Centrifuge 5 × 10<sup>7</sup> cells 5 min at 1800 × g (2500 rpm in H-6000A rotor), room temperature. Resuspend cells in 25 ml complete MEM-10, dispense in one 150-cm<sup>2</sup> flask, and place overnight in CO<sub>2</sub> incubator at 37°C.
10. Remove medium from cells and replace with mixture of 0.25 ml lysate and 1.75 ml complete MEM-2.5. Place 1 hr in CO<sub>2</sub> incubator at 37°C, rocking flask at 15- to 30-min intervals.
11. Overlay with 25 ml complete MEM-2.5 (selection is not required at this step) and incubate 3 days in CO<sub>2</sub> incubator at 37°C.
12. Detach cells from flask by shaking. Transfer to centrifuge tube by pipetting, centrifuge 5 min at 1800 × g, 5° to 10°C. Aspirate supernatant. Resuspend cells in 2 ml complete MEM-2.5 and carry out freeze-thaw cycling three times as in step 9 of first basic protocol.
13. Determine titer of viral stock as in UNIT 16.16. Freeze viral stock at -70°C.

*Reference:* Piccini et al., 1987.

*Contributors:* Patricia L. Earl and Bernard Moss

## Characterization of Recombinant Vaccinia Viruses and Their Products

**CAUTION:** Proceed carefully and follow biosafety level 2 (BL-2) practices when working with vaccinia virus (see UNIT 16.15 for safety guidelines).

**NOTE:** Carry out all procedures for growth of vaccinia virus using sterile technique in a tissue culture hood.

### DETECTION OF VACCINIA DNA USING DOT-BLOT HYBRIDIZATION

*Materials (see APPENDIX 1 for items with ✓)*

- Confluent BS-C-1 or HuTK<sup>-</sup> 143B cell monolayer (UNIT 16.16)
- ✓ Phosphate-buffered saline (PBS)
- Trypsin/EDTA (0.25%:0.02%; Quality Biological #18-112-1), 37°C
- ✓ Complete MEM-10, -2.5, and -5, without and with selective drugs
- ✓ Complete MEM-10 containing 25 µg/ml 5-bromodeoxyuridine (complete MEM-10/BrdU)
- Recombinant virus plaques (UNIT 16.17)
- 10 mg/ml mycophenolic acid (MPA; Calbiochem #475913) in 1 N NaOH (400×; store at -20°C)
- 10 mg/ml xanthine in 0.1 N NaOH (40×; store at -20°C)
- 10 mg/ml hypoxanthine in water (670×; store at -20°C)