MEM-2.5. Incubate 2 days in CO_2 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 5×10^7 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^2 flask, and place overnight in CO_2 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_2 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_2 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

16.18

UNIT 16.18

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.

BASIC PROTOCOL

DETECTION OF VACCINIA DNA USING DOT-BLOT HYBRIDIZATION

Materials (see APPENDIX 1 for items with ✓)

Confluent BS-C-1 or HuTK$^-$ 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)

5 mg/ml BrdU in water (filter sterilize and store at −20°C)
0.5 N NaOH
✓ 1 M Tris·Cl, pH 7.5
✓ 2× SSC

Hemacytometer (*UNIT 1.2*)
24-well, 16-mm tissue culture dishes
Humidified 37°C, 5% CO_2 incubator
Nitrocellulose membrane
Dot-blot apparatus

1. Aspirate medium from a confluent cell monolayer.
   a. For XGPRT selection, use BS-C-1 cells.
   b. For TK selection, use HuTK$^-$ 143B cells.
2. Wash cells once with PBS or trypsin/EDTA to remove remaining serum. Overlay cells with 37°C trypsin/EDTA using a volume that is just enough to cover monolayer (1.5 ml for 150-cm^2 flask). Allow to sit ~30 sec (the cells should detach). Shake to detach cells completely.
3. Add 8.5 ml medium (see below). Pipet cell suspension up and down several times to disrupt clumps.
   a. For BS-C-1 cells, use complete MEM-10.
   b. For HuTK$^-$ 143B cells, use complete MEM-10/BrdU.
4. Count cells and plate 1.25×10^5 cells/well in 24-well tissue culture dishes (0.5 ml/well final). Place in CO_2 incubator at 37°C and allow to reach confluency (<24 hr).
5. Lyse by three freeze-thaw cycles as follows: freeze in dry ice/ethanol, thaw in 37°C water bath, and vortex. Sonicate virus before infection as in step 13 of second basic protocol, *UNIT 16.17*.
6. Aspirate medium and infect each well with one recombinant virus plaque, using half the volume in which each plaque is suspended. Place 1 to 2 hr in CO_2 incubator at 37°C, rocking at 15- to 30-min intervals.
7. Overlay with 1 ml complete MEM-2.5 to which appropriate selection drugs have been added. Place in CO_2 incubator at 37°C until cytopathic effect (cell rounding) is evident (24 to 48 hr).
   a. For XGPRT selection, add 1/400 vol of 10 mg/ml MPA, 1/40 vol of 10 mg/ml xanthine, and 1/670 vol of 10 mg/ml hypoxanthine.
   b. For TK selection, add 1/200 vol of 5 mg/ml 5-BrdU.
8. Scrape cells and transfer to a microcentrifuge tube. (Cells in a 24-well dish can be scraped using the plunger of a 1-ml syringe.) Microcentrifuge 30 sec at top speed. Resuspend pellet in 200 µl PBS.
9. Lyse cell suspension by three freeze-thaw cycles as follows: freeze in dry ice/ethanol, thaw in 37°C water bath, and vortex.
10. Sonicate as in step 13 of second basic protocol, *UNIT 16.17*.
11. Wet nitrocelluose membrane of proper size by immersing it in water and place in dot-blot apparatus. Add 20 to 100 µl cell lysate (from step 10) to each well. Draw liquid through membrane by applying suction.

12. Cut six pieces of Whatman 3MM filter paper so they are larger than the size of the nitrocellulose membrane. Soak one piece of 3MM paper with 0.5 N NaOH. Lay nitrocellulose membrane (transfer with forceps) on top of the wet 3MM paper and let sit for 1 min. Remove membrane and place on a separate piece of dry 3MM paper for 1 min. Repeat this procedure two times using the same 3MM paper.
13. Repeat step 12 with Whatman 3MM papers soaked in 1 M Tris·Cl, pH 7.5 and then 2× SSC (three times per membrane, respectively). Bake membrane 2 hr at 80°C.
14. Proceed with DNA hybridization as in *UNIT 2.9*, protocol using nitrocellulose membranes.

BASIC PROTOCOL

DETECTION OF VACCINIA DNA USING SOUTHERN BLOT HYBRIDIZATION

The TK gene, into which most foreign genes are inserted in recombinant viruses, is located in the 5.1-kbp *Hin*dIII J fragment. Thus in a recombinant virus, if the inserted gene has no *Hin*dIII sites, this fragment should be increased in size. The 5.1-kbp *Hin*dIII J fragment should be absent, indicating lack of contamination with wild-type vaccinia virus.

Materials (see APPENDIX 1 for items with ✓)

Confluent BS-C-1 cell monolayer (*UNIT 16.16*)
✓ Complete MEM-10 and -5
Recombinant vaccinia virus stock (*UNIT 16.16*)
0.25 mg/ml trypsin (2× crystallized and salt-free, Worthington; filter sterilize and store at –20°C)
✓ Phosphate-buffered saline (PBS)
✓ Low-salt buffer
20 mg/ml proteinase K
Buffered phenol and 1:1 phenol/chloroform (*UNIT 2.1*)
✓ 3 M sodium acetate, pH 6.0
95% and 70% ethanol, ice-cold
TE buffer [10 mM Tris·Cl (pH 7.8)/1 mM EDTA]
*Hin*dIII restriction endonuclease (*UNIT 3.1*)

Hemacytometer (*UNIT 1.2*)
12-well tissue culture dishes
Humidified 37°C, 5% CO_2 incubator

1. Repeat steps 1 to 3 of first basic protocol using a confluent BS-C-1 cell monolayer.
2. Count cells and plate 2.5×10^5 cells/well in 12-well tissue culture dishes (1 ml/well final). Place in CO_2 incubator at 37°C and allow to reach confluency (<24 hr).
3. Just prior to use, mix equal volume of recombinant vaccinia virus stock (~2×10^9 pfu/ml) and 0.25 mg/ml trypsin, and vortex vigorously. Incubate 30 min in 37°C water bath, vortexing 5- to 10-min intervals.

If there are still clumps, chill to 0°C and sonicate 30 sec on ice. Sonication can be repeated several times but the sample should be allowed to cool on ice between sonications.

4. Aspirate medium and infect with an MOI of 10 to 20 pfu/cell in 250 µl complete MEM-5. Place 1 to 2 hr in CO_2 incubator at 37°C, rocking at ~30-min intervals. Overlay with 1 ml complete MEM-5. Place ~24 hr in CO_2 incubator at 37°C.
5. Scrape cells and transfer to microcentrifuge tube. Microcentrifuge 3 min at top speed, room temperature. Aspirate supernatant. Resuspend pellet in 50 µl PBS and vortex.
6. Add 300 µl low-salt buffer and 10 µl of 20 mg/ml proteinase K to microcentrifuge tube. Add cell suspension to this same tube. Vortex and incubate 5 hr to overnight at 37°C.
7. Extract once with equal volume buffered phenol and once with equal volume of 1:1 phenol/chloroform (if solution is viscous, pass through a 25-G needle to shear DNA and reduce viscosity).
8. Add 3 M sodium acetate, pH 6.0, to 0.3 M final and 2.5 vol of 95% ethanol. Place 30 min on dry ice or overnight at −20°C.
9. Microcentrifuge 10 min at top speed, 4°C. Aspirate supernatant. Wash pellet with ice-cold 70% ethanol and air dry. Dissolve in 100 µl TE.
10. Digest 15 µl DNA with *Hin*dIII and analyze by Southern hybridization (*UNIT 2.9*).

DETECTION OF VACCINIA DNA USING PCR

ALTERNATE PROTOCOL

Additional Materials (see APPENDIX 1 for items with ✓)

✓ 1 mM Tris·Cl, pH 9.0

1. Resuspend recombinant virus plaques in 100 µl of 1 mM Tris·Cl, pH 9.0. Carry out three rounds of freeze-thaw cycling and 30 sec of sonication as in steps 9 and 10 of first basic protocol.
2. Extract DNA from 30 µl as in support protocol for isolation of vaccinia DNA in *UNIT 16.17*.
3. Carry out PCR for 50 cycles as in *UNIT 15.1*.

DETECTION OF VACCINIA RNA USING NORTHERN HYBRIDIZATION

BASIC PROTOCOL

Northern blotting is useful for analysis of transcripts directed by vaccinia virus early promoters. Infections are performed in the presence of cycloheximide to inhibit protein synthesis and enhance expression of early genes. Northern blotting is not useful for transcripts made by vaccinia virus late promoters since these yield RNAs that are long and heterogeneous in size. For products of late promoters, the correct RNA 5′ ends can be determined by S1 nuclease protection (*UNIT 4.6*) or primer extension (*UNIT 4.8*). If these methods are used, note that late mRNAs typically have a nontranscribed poly(A) leader of ~35 nucleotides (*UNIT 16.15*).

Materials (see APPENDIX 1 for items with ✓)

Confluent BS-C-1 or CV-1 cell monolayer (*UNIT 16.16*)
✓ Complete MEM-10
✓ Complete DMEM-10
✓ Complete MEM-5 containing 100 µg/ml cycloheximide
Recombinant virus stock (*UNIT 16.16*)
✓ Phosphate-buffered saline (PBS), ice-cold
✓ Northern lysis buffer
Buffered phenol (*UNIT 2.1*)
Chloroform
✓ 3 M sodium acetate, pH 6.0
95% ethanol
✓ RNA buffer
1 U/µl DNase I (RQ1, Promega #M6101; *UNIT 3.12*)

Hemacytometer (*UNIT 1.2*)
100-mm tissue culture dish
Humidified 37°C, 5% CO_2 incubator
Sorvall H-6000A rotor or equivalent

1. Repeat steps 1 to 3 of first basic protocol using confluent BS-C-1 or CV-1 cell monolayer.

   a. For BS-C-1 cells, use complete MEM-10.

   b. For CV-1 cells, use complete DMEM-10.

2. Count cells and plate 3×10^6 cells/100-mm-diameter dish in 5 ml final. Place in CO_2 incubator at 37°C and allow to reach confluency (<24 hr).

3. Remove medium from monolayer and overlay with complete MEM-5/cycloheximide. Incubate 10 min in a CO_2 incubator at 37°C.

4. Repeat step 3 of second basic protocol.

5. Aspirate medium and infect monolayer culture at MOI of 10 pfu/cell in 2 ml complete MEM-5/cycloheximide (if purified virus is used, sonicate 30 sec on ice prior to use as in step 13 of second basic protocol, *UNIT 16.17*). Place 1 hr in CO_2 incubator at 37°C, rocking at 15-min intervals.

6. Overlay with 8 ml complete MEM-5/cycloheximide and incubate 4 hr in CO_2 incubator at 37°C. Place dish on ice. Aspirate medium, overlay with ice-cold PBS, aspirate, and overlay with 5 ml ice-cold PBS.

7. Scrape cells with sterile cell scraper and transfer to centrifuge tube. Rinse plate with an additional 2 ml ice-cold PBS and add to centrifuge tube containing scraped cells. Keep on ice during scraping, centrifugation, and lysis. Centrifuge 3 min at $650 \times g$ (1500 rpm in H-6000A rotor), 4°C. Aspirate supernatant. Dissolve pellet in 1.5 ml northern lysis buffer by vortexing. Let sit 5 min on ice and vortex again.

8. Centrifuge 7 min at $650 \times g$, 4°C, and save supernatant. Split supernatant into two microcentrifuge tubes.

9. Extract once with buffered phenol and once with chloroform (*UNIT 2.1*). Add 3 M sodium acetate, pH 6.0, to 0.3 M final and 4 vol of 95% ethanol. Place 30 min on dry ice or overnight at –20°C.

10. Microcentrifuge 10 min at top speed, 4°C. Dissolve pellet in 0.3 ml RNA buffer. Add 1.5 µl of 1 U/µl DNase I and incubate 10 min at 37°C.
11. Extract once with buffered phenol and once with chloroform (*UNIT 2.1*). Add 3 M sodium acetate, pH 6.0, to 0.3 M final and 2.5 vol of 95% ethanol. Place 30 min on dry ice or overnight at −20°C.
12. Microcentrifuge 10 min at top speed, 4°C. Air dry pellet and resuspend in 20 µl RNA buffer.
13. Proceed with northern hybridization (*UNIT 4.9*).

DETECTION OF EXPRESSED PROTEIN USING IMMUNOBLOTTING

BASIC PROTOCOL

This protocol is usually used as an alternative to DNA dot-blot hybridization for checking plaques.

Materials

PBS with 0.5% Tween-20 (PBS/Tween), with and without 4% BSA
Antibody to foreign protein
[^{125}I]protein A

1. Repeat steps 1 to 11 of first basic protocol.
2. Soak membrane in PBS/Tween containing 4% BSA for ~30 min. Wash once with PBS/Tween by pouring liquid off and replacing it. Dilute antibody to foreign protein 1:50 to 1:5000 in minimal volume of PBS/Tween. Incubate membrane in this solution ≥1 hr at room temperature.
3. Wash approximately five times with excess volume of PBS/Tween. Incubate membrane in minimal volume of PBS/Tween containing 1 µCi of [^{125}I]protein A for 30 min.
4. Wash membrane approximately five times with excess volume of PBS/Tween. Wrap membrane in plastic wrap and expose to X-ray film.

DETECTION OF EXPRESSED PROTEIN USING WESTERN BLOTTING

BASIC PROTOCOL

This method is used to determine if the protein is of the expected size and whether it is secreted from infected cells.

Materials (see APPENDIX 1 for items with ✓)

Confluent BS-C-1 cell monolayer (*UNIT 16.16*)
✓ Complete MEM-5
✓ Cell lysis buffer
Recombinant virus stock
✓ 5× SDS/sample buffer

Hemacytometer (*UNIT 1.2*)
6-well, 35-mm tissue culture dishes
Sorvall H-6000A rotor or equivalent

1. Repeat steps 1 to 3 of first basic protocol using confluent BS-C-1 cell monolayer.

2. Count cells and plate 5×10^5 cells/well in 6-well culture dish (2 ml/well final). Place in CO_2 incubator at 37°C and allow to reach confluency (<24 hr).
3. Repeat step 3 of second basic protocol.
4. Aspirate medium and infect monolayer culture at an MOI of 10 to 30 pfu/cell in 1 ml final complete MEM-5 (if purified virus is used, sonicate 30 sec on ice prior to use as in step 13 of second basic protocol, *UNIT 16.17*. Place 1 to 2 hr in CO_2 incubator at 37°C, rocking at ~30-min intervals.
5. Overlay with 2 ml complete MEM-5 and return 24 to 48 hr to CO_2 incubator at 37°C.

   Secreted proteins can also be analyzed. However, it is important to infect and overlay cells with medium that does not require addition of serum or to use only 1% serum since a high concentration of serum proteins interferes with gel analysis. Concentrate medium using a microconcentrator or by precipitation.

6. Scrape cells and transfer to centrifuge tube. Centrifuge 5 min at 1800 × *g* (2500 rpm in H-6000A rotor), 5° to 10°C. Aspirate supernatant. Resuspend cell pellet in 200 µl cell lysis buffer and transfer to microcentrifuge tube. Vortex and let sit 10 min on ice.

   Cell lysis buffer contains nonionic detergent only; use SDS-containing buffer (see UNIT 16.11) if necessary.

7. Microcentrifuge 10 min at top speed, 4°C. Separate supernatant (cytoplasm) and pellet (nuclei).
8. To 20 µl supernatant, add 5 µl of 5× SDS/sample buffer and heat 5 min at 95°C. Dissolve pellet in 200 µl of 1× SDS/sample buffer and heat 5 min at 95°C. Proceed with protocol for immunoblotting (*UNIT 10.8*).

BASIC PROTOCOL

DETECTION OF EXPRESSED PROTEIN USING IMMUNOPRECIPITATION

When strong late promoters are used, the expressed recombinant protein can usually be detected by polyacrylamide gel electrophoresis following labeling with radioactive amino acids. Immunoprecipitation of the labeled proteins can be used to increase sensitivity and specificity when early or late promoters are used.

Materials (see APPENDIX 1 for items with ✓)

- ✓ Complete MEM-5
- Complete methionine- *or* cysteine-free MEM-5 containing dialyzed FCS (see *APPENDIX 1* for dialyzed FCS)
- 1000 Ci/mmol [^{35}S]methionine (e.g., Amersham #SJ1515) *or* 600 Ci/mmol [^{35}S]cysteine (e.g., Amersham #SJ232)
- ✓ Phosphate-buffered saline (PBS)
- ✓ Cell lysis buffer

1. Follow steps 1 to 4 of immunooblotting basic protocol.
2. Overlay with 2 ml complete MEM-5 and return to incubator. For an early promoter, carry out labeling 1-6 hr postinfection. For a late

promoter, labeling should be done 4-20 hr postinfection. With a compound early/late promoter, either time may be used.

3. At appropriate time after infection, aspirate medium and replace with 1 ml complete methionine- or cysteine-free MEM-5 (dialyzed) containing 25 to 50 µCi of 1000 Ci/mmol [^{35}S]methionine or 600 Ci/mmol [^{35}S]cysteine and 35 µl complete MEM-5. Place 2 to 3 hr to overnight in CO_2 incubator at 37°C.
4. Add 0.15 ml complete MEM-5 as chase. Return 1 hr to CO_2 incubator at 37°C. Remove medium and save if expressed protein is secretory (if medium is analyzed, remove any free cells by microcentrifuging 3 min).
5. Overlay cells with 1 ml PBS, scrape, and transfer to microcentrifuge tube. Microcentrifuge 3 min at top speed, room temperature. Aspirate supernatant.
6. Suspend cells in 200 µl cell lysis buffer. Vortex and place 10 min on ice. Microcentrifuge 10 min at top speed, 4°C. Remove supernatant and proceed with immunoprecipitation beginning at the supernatant step(s) in *UNIT 10.16*.

Reference: Zhang and Moss, 1991.

Contributors: Patricia L. Earl and Bernard Moss