# Preparation of Cell Cultures and Vaccinia Virus Stocks

Because vaccinia virus has a broad host range, there is considerable latitude in the selection of cell lines; those described in the first two basic protocols below have been found to give good results. BS-C-1 cells give the best results for a plaque assay whereas HeLa cells are preferred for preparation of virus stocks. CV-1 cells can be used for both procedures, but they are generally used for transfection (*UNIT 16.17*). Human TK$^-$ 143B cells are used when TK selection is employed (*UNIT 16.17*), but they can be used for transfection as well as for a plaque assay. Table 16.16.1 presents a summary of the uses for specific cell lines.

*NOTE:* Carry out all procedures in this unit using sterile technique, preferably in a tissue culture hood.

*BASIC PROTOCOL* **16.16**

## CULTURE OF MONOLAYER CELLS

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

Frozen ampule of cells (Table 16.16.2): BS-C-1 (ATCC #CCL26), CV-1 (ATCC #CCL70), *or* HuTK$^-$ 143B (ATCC #CRL8303) cells

70% ethanol

✓ Start-up medium (Table 16.16.2): complete MEM-20, complete DMEM-20, *or* complete MEM-20/BrdU

✓ Maintenance medium (Table 16.16.2): complete MEM-10, complete DMEM-10, *or* complete MEM-10/BrdU

✓ Phosphate-buffered saline (PBS; optional)

0.25% trypsin/0.02% EDTA (trypsin/EDTA; Quality Biological #18-112-1), 37°C

25-cm$^2$ and 150-cm$^2$ flasks

Humidified 37°C, 5% $CO_2$ incubator

**Table 16.16.1** Cell Lines Used in Specific Vaccinia Protocols

| Cell line | Use[a] | Procedure |
|---|---|---|
| HeLa S3 | Virus stock preparation | *UNIT 16.16* basic protocol |
| | Purification of vaccinia virus | *UNIT 16.17* support protocol |
| | Amplification of a plaque | *UNIT 16.17* basic protocol |
| BS-C-1 | Plaque assay | *UNIT 16.16* support protocol |
| | XGPRT selection | *UNIT 16.17* basic protocol |
| CV-1 | Transfection | *UNIT 16.17* basic protocol |
| | Virus stock preparation (optional) | *UNIT 16.16* basic protocol |
| | Plaque assay (optional) | *UNIT 16.16* support protocol |
| HuTK$^-$ 143B | TK selection | *UNIT 16.17* basic protocol |
| | Plaque assay (optional) | *UNIT 16.16* support protocol |
| | Transfection (optional) | *UNIT 16.17* basic protocol |

[a]The preferred use(s) for each cell line is listed first; if optional is indicated, the cell line can be used for the indicated procedure but the results may not be as good as those from the preferred cell line.

***Begin culture***

1. Thaw frozen ampule of cells in a 37°C water bath. Sterilize ampule tip with 70% ethanol, break neck, and transfer cells with a pipet into a 25-$cm^2$ flask containing 5 ml of start-up medium. Rotate flask to evenly distribute cells and place overnight in $CO_2$ incubator at 37°C.
2. Aspirate start-up medium and replace with appropriate maintenance medium. Return to $CO_2$ incubator and check daily for confluency. When the cells are a confluent monolayer, trypsinize and transfer to a 150-$cm^2$ flask as in steps 3 to 5. Passage cells when they become confluent (if cells are split 1:20, they reach confluence in ~1 week).

***Maintain culture***

3. Aspirate medium from confluent cell monolayer. Wash cells once with PBS or trypsin/EDTA by covering cells with the solution and pipetting it off (to remove remaining serum from cells).
4. Overlay cells with 37°C trypsin/EDTA using a volume that is just enough to cover monolayer (e.g., 1.5 ml for a 150-$cm^2$ flask). Allow to sit 30 to 40 sec (cells should become detached) and shake flask to completely detach cells. Add 8.5 ml appropriate maintenance medium. Pipet cell suspension up and down several times to disrupt clumps (cells are ready for passage).
5. Remove 0.5 ml of cell suspension and add it to a new 150-$cm^2$ flask containing 30 ml maintenance medium. Rotate flask to evenly distribute cells and place in $CO_2$ incubator at 37°C until cells are confluent (~1 week). Maintain cells by splitting ~1:20 in maintenance medium at approximately weekly intervals.

## CULTURE OF SUSPENSION CELLS

*BASIC PROTOCOL*

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

HeLa S3 cells (ATCC #CCL2.2)
✓ Complete MEM-10
✓ Complete spinner medium containing 5% horse serum (complete spinner medium-5)
0.25% trypsin/0.02% EDTA (trypsin/EDTA; Quality Biological #18-112-1), 37°C

25-$cm^2$ flasks
Humidified 37°C, 5% $CO_2$ incubator
50-ml centrifuge tubes
Sorvall H-6000A rotor or equivalent
100- or 200-ml vented spinner bottles (Bellco #1965) and caps with filters (Bellco #A523-A59)
Hemacytometer (*UNIT 1.2*)

**Table 16.16.2** Media Used for Growth and Maintenance of Cell Lines

| Cell line | Maintenance medium[a] | Start-up mediuma |
|---|---|---|
| BS-C-1 | Complete MEM-10 | Complete MEM-20 |
| CV-1 | Complete DMEM-10 | Complete DMEM-20 |
| HuTK$^-$ 143B | Complete MEM-10/BrdU | Complete MEM-20/BrdU |
| HeLa S3 | Complete spinner medium-5 | Complete MEM-10 |

[a]See *APPENDIX 1* for recipes.

*Begin culture*

1. Thaw frozen ampule of cells and transfer to a 25-$cm^2$ flask (step 1 first basic protocol), using 5 ml complete MEM-10. Place overnight in $CO_2$ incubator at 37°C.
2. Aspirate medium. Overlay with 0.5 ml of 37°C trypsin/EDTA; let sit 30 to 40 sec (because cells do not attach firmly to flask, do not wash prior to trypsinization).
3. Add 10 ml complete spinner medium-5 and transfer cells to a 50-ml centrifuge tube. Centrifuge 5 min at 1800 × *g* (2500 rpm in a Sorvall H-6000A rotor), room temperature. Suspend cell pellet in 5 ml complete spinner medium-5 by pipetting up and down to disrupt clumps.
4. Add 50 ml complete spinner medium-5 to a 100- or 200-ml vented spinner bottle and transfer cell suspension to this bottle. Remove 1 ml cell suspension and count cells using a hemacytometer. Add complete spinner medium-5 to adjust to 3-4 × $10^5$ cells/ml. Place cells in 37°C incubator without $CO_2$ and stir continuously.

   *The initial high density is used because some cells are not viable.*

5. At daily intervals, count cells and add complete spinner medium-5 to adjust concentration to 3-4 × $10^5$ cells/ml. Return cells to incubator—when cells have grown for 2 successive days, proceed to step 8.

***Maintain culture***

8. Remove 1 ml cell suspension and count cells. When density is 4-5 × $10^5$ cells/ml, dilute cells to 1.5 or 2.5 × $10^5$ cells/ml with complete spinner medium-5 for alternate-day or daily feeding, respectively. Place vented spinner bottle containing cells in 37°C incubator without $CO_2$ and stir continuously. Passage every 1 to 2 days.

   *Grow and maintain cells in complete spinner medium-5 in vented spinner bottles at 37°C without $CO_2$. Dilute cells with fresh medium at 1- to 2-day intervals to keep density at 1.5-5 × $10^5$ cells/ml (horse serum is used because it is cheaper than FCS and may give less cell clumping).*

## PREPARATION OF A VACCINIA VIRUS STOCK

***BASIC PROTOCOL***

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

HeLa S3 cells from suspension culture (second basic protocol)
✓ Complete MEM-10 and -2.5, 37°C
Vaccinia virus (ATCC #VR1354 or other source)
0.25 mg/ml trypsin (2× crystallized and salt-free, Worthington; filter sterilize and store at −20°C)
Hemacytometer (*UNIT 1.2*)
Sorvall H-6000A rotor or equivalent
150-$cm^2$ tissue culture flask

1. Count HeLa S3 cells from suspension culture. Centrifuge 5 × $10^7$ cells 5 min at 1800 × *g* (2500 rpm in an H-6000A rotor), room temperature.
2. Resuspend cells in 25 ml of 37°C complete MEM-10, dispense in one 150-$cm^2$ flask, and place overnight in a $CO_2$ incubator at 37°C (for infection the following day).

3. Just prior to use, mix an equal volume of vaccinia virus stock (~$2 \times 10^9$ pfu/ml) and 0.25 mg/ml trypsin, and vortex vigorously. Incubate 30 min in 37°C water bath, vortexing at 5- to 10-min intervals.

   *If there are still visible clumps, chill to 0°C and sonicate 30 sec on ice (UNIT 16.17). Sonication can be repeated several times but the sample should be allowed to cool on ice between sonications.*

4. Dilute trypsinized virus in complete MEM-2.5 to 2.5-7.5 $\times 10^7$ pfu/ml. Remove medium from the 150-$cm^2$ flask of HeLa cells and add 2 ml diluted, trypsinized virus (optimal MOI is 1-3 pfu/HeLa cell). Place 2 hr in $CO_2$ incubator at 37°C, rocking flask by hand at 30-min intervals.

5. Overlay cells with 25 ml complete MEM-2.5 and place 3 days in $CO_2$ incubator at 37°C.

6. Detach infected cells from flask by shaking and pour or pipet into sterile plastic screw-cap tube. Centrifuge 5 min at 1800 $\times$ *g*, 5° to 10°C. Resuspend cells in 2 ml complete MEM-2.5 (per 150-$cm^2$ flask) by gently pipetting or vortexing.

7. Lyse cell suspension by three freeze-thaw cycles as follows: freeze in dry ice/ethanol, thaw in 37°C water bath, and vortex.

8. Keep virus stock on ice while dividing it into 0.5- to 2-ml aliquots. Store the aliquots at –70°C (stable for many years).

16.16

*SUPPORT PROTOCOL*

## TITERING A VACCINIA VIRUS STOCK USING A PLAQUE ASSAY

*Additional Materials*

BS-C-1 cells from confluent monolayer culture (first basic protocol)
Virus stock (third basic protocol)
0.1% crystal violet (Sigma #C3886) in 20% ethanol (store indefinitely at room temperature)
6-well 35-mm tissue culture dishes

1. Trypsinize confluent monolayer of BS-C-1 cells as in steps 3 and 4 of first basic protocol. Count cells and seed 6-well tissue culture dishes with $5 \times 10^5$ cells/well in complete MEM-10 (each well should contain 2 ml). Place overnight in $CO_2$ incubator at 37°C to reach confluency.

2. Trypsinize virus stock as in step 3 of third basic protocol.

3. Make nine 10-fold serial dilutions (*UNIT 1.11*) of trypsinized virus in complete MEM-2.5, using a fresh pipet for each dilution.

4. Remove medium from BS-C-1 cells and infect cells in duplicate wells with 0.5 ml of the $10^{-7}$, $10^{-8}$, and $10^{-9}$ dilutions. Place 1 to 2 hr in $CO_2$ incubator at 37°C, rocking dish at 15- to 30-min intervals to keep cells moist.

5. Overlay cells in each well with 2 ml complete MEM-2.5 and place 2 days in $CO_2$ incubator at 37°C.

6. Remove medium and add 0.5 ml of 0.1% crystal violet to each well. Incubate 5 min at room temperature. Aspirate crystal violet and allow wells to dry.

7. 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





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$^-$ 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 × $10^{10}$ pfu of purified virus should be obtained per liter (5 × $10^8$) 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 β-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 × $10^9$ 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