

Cytopathic Effects of Viruses Protocols

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Information **History**

In 1954, J. F. Enders proposed classifying viruses into groups of those that caused cell degeneration, those that caused formation of inclusion bodies and cell degeneration, those that caused the formation of multinucleated cells (syncytia), and those that caused no cytopathic effect (CPE) (1). Although the type and rate of CPE formation are still useful in classifying and identifying viruses, better more useful classification systems have since been developed (3).

Purpose

During the time that synthesis of viral components is occurring in the infected cell, the cell undergoes characteristic biochemical and morphological changes. Progression of these changes is most readily observed in cell culture, where infection of cells is more easily synchronized and where the cells can be observed and sampled frequently during the course of infection. Morphological changes in cells caused by viral infection are called cytopathic effects (CPE); the responsible virus is said to be cytopathogenic. The degree of visible damage to cells caused by viral infection varies with type of virus, type of host cells, multiplicity of infection (MOI), and other factors. Some viruses cause very little or no CPE in cells of their natural host. Their presence can be detected visually only by hemadsorption or interference, in which infected cell cultures showing no CPE inhibit the replication of another virus subsequently introduced into the cultures, or in situ by viral antigen or nucleic acid detection. On the other hand, some viruses cause a complete and rapid destruction of the cell monolayer after infection. The microscopic appearance of the CPE caused by some of these cytotoxic viruses may be sufficiently characteristic to allow provisional identification of an unknown virus (2).

Theory

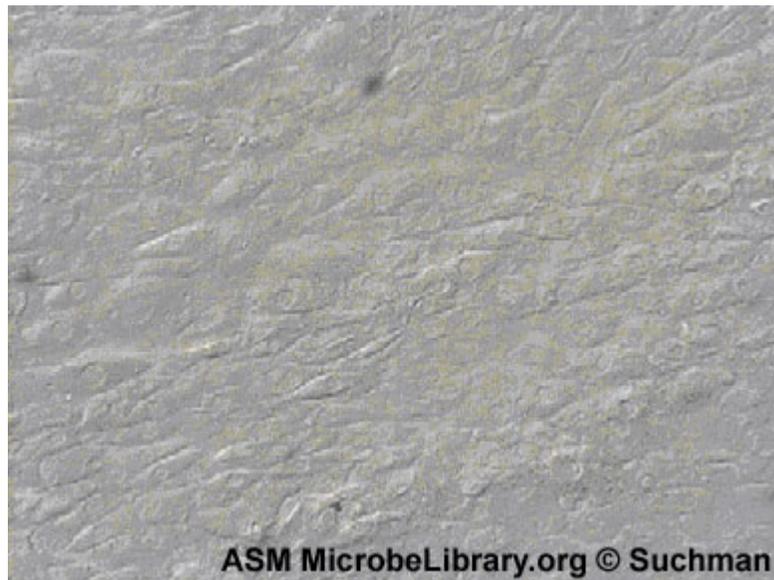
Some CPE can be readily observed in unfixed unstained cells under low power (10X objective with 10X ocular for 100X magnification) of the light microscope, with the condenser down and the iris diaphragm partly closed to obtain the contrast needed for viewing translucent cells. Several types of CPE are distinguishable in living cultures, but fixation and staining of the cells is necessary to see manifestations of viral infection such as inclusion bodies. Recognizing CPE and using it as a

diagnostic tool requires much experience in examining both stained and unstained cultures of many cell types.

Listed below are several general types of CPE. Keep in mind that a given virus may not conform to the norm for its family, or it may produce different CPE in different host cell types. Characteristic CPE is best viewed by daily observation of cultures that have been infected at a low MOI (<0.1). The best knowledge of viral CPE comes from experience. And control uninfected cells should always be observed to distinguish normal cell changes that occur as cells age from cytopathic effect.

The rate of CPE appearance is also a characteristic that can be used to help identify viruses. In general the rule of thumb is that a virus is considered slow if CPE appears after 4 to 5 days in cultures inoculated at low MOI, and rapid if CPE appears after 1 to 2 days in cultures inoculated at low MOI. It is important to note that at a high MOI all CPE can occur rapidly. So all decisions about rate of CPE appearance should be based on the lowest MOI that produces CPE.

1. Total destruction of the cell monolayer is the most severe form of CPE. All cells in the monolayer rapidly shrink, become dense (pyknosis), and detach from the glass within 72 hours (Fig. 1). This CPE is typical of most enteroviruses.



a.



b.

FIG. 1. Bovine fetal spleen cells (a) uninfected and (b) 2 days postinfection with a high MOI of the bovine enterovirus, a Picornavirus, showing pyknotic shrinking cells observed using the 20X objective (200X total magnification). Note: this shows viral CPE within 24 hours, within 48 hours all of the cells will be dead and detached.

2. Subtotal destruction consists of detachment (death) of some but not all of the cells in the monolayer. Some togaviruses (alphaviruses), some picornaviruses, and some of the paramyxoviruses may cause this type of CPE which can be observed using the 20X objective (Fig. 2).



FIG. 2. Bovine fetal spleen cells 2 days postinfection with a high MOI of the vesicular stomatitis virus, a rhabdovirus, showing subtotal cell destruction; the cells appear pyknotic (observed using the 20X objective).

3. Focal degeneration is characteristic of the herpesviruses and poxviruses. Instead of causing a generalized destruction of the cell monolayer, these viruses produce localized areas (foci) of infection. The focal nature of these lesions is due to direct cell-to-cell transfer of virus

rather than diffusion through the extracellular medium. Cells initially become enlarged, rounded, and refractile (more easily seen), then eventually detach from the growth surface leaving cleared areas surrounded by rounded up cells as the infection spreads concentrically (Fig. 3 and 4). Eventually, the entire monolayer may be involved. Stranding of the cytoplasm (greatly elongated and thin appearance) may be pronounced, and cell fusion may be evident. Note: it is important to view infected cells at a low MOI to determine if cytopathic effect is focal or generalized, at a high MOI all CPE appears to be generalized. Also sometimes it helps to observe cells with the 4X objective (40X total magnification) to determine if CPE is focal or generalized (Fig. 5 through 8).

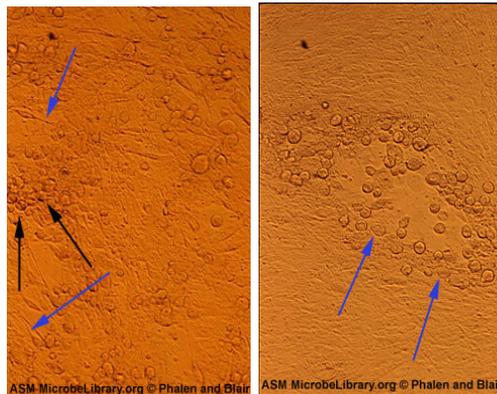


FIG. 3.

FIG. 4.

FIG. 3. Bovine fetal spleen cells 2 days postinfection with a high MOI of the bovine herpesvirus 1, a *Herpesvirus*. Black arrows point to cell rounding in a focal pattern and blue arrows point to cytoplasmic stranding. The cells were observed using the 20X objective (200X total magnification).

FIG. 4. Bovine fetal spleen cells 2 days postinfection with a high MOI of the Orf virus, a *Parapoxvirus*. Blue arrows point to cell rounding in a focal pattern. Cells were observed using the 20X objective (200X total magnification).

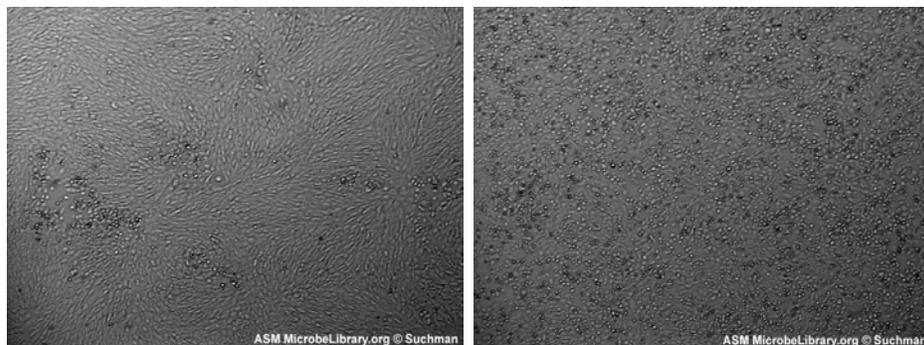


FIG. 5.

FIG. 6.

FIG. 5 and 6. Bovine fetal spleen cells 3 days postinfection with the Orf

virus, a *Parapoxvirus*. Cells show focal cell rounding at (5) a low MOI and (6) high MOI. Cells were observed using a 4X objective (40X total magnification). Note: you can see the CPE is focal at the low MOI, but it appears generalized at the high MOI.

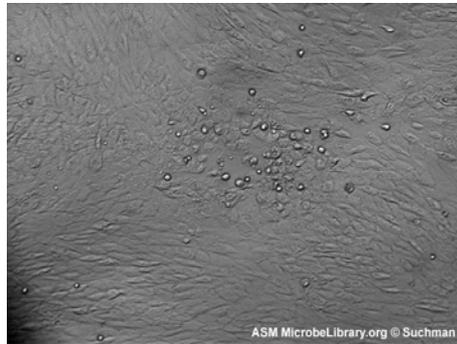


FIG. 7.

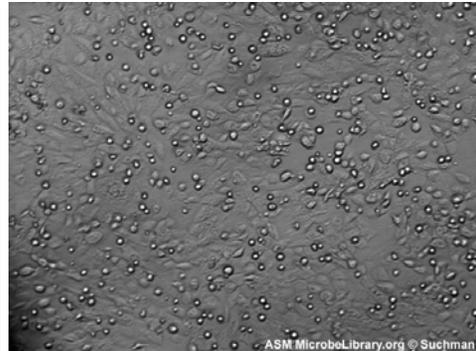


FIG. 8.

FIG. 7 and 8. Bovine fetal spleen cells 3 days postinfection with the Orf virus, a *Parapoxvirus*, showing focal cell rounding at (7) a low MOI and (8) high MOI. Cells were observed using a 10X objective (100X total magnification). Note: it is easier to determine if the CPE is focal using the 4X objective than the 10X objective (Fig. 5).

4. Swelling and clumping of cells before detachment is typical of adenoviruses (Fig. 9). Infected cells greatly enlarge and clump together in "grape-like" clusters.

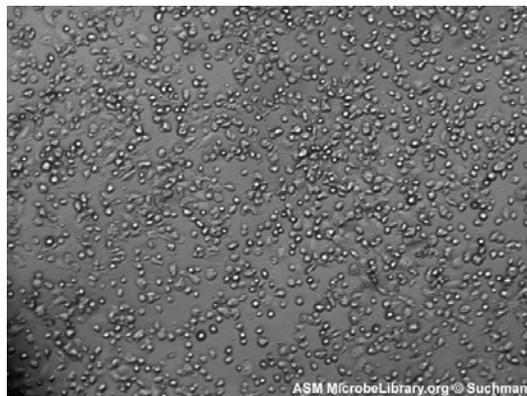


FIG. 9. Bovine fetal spleen cells 4 days postinfection with a high MOI of the bovine adenovirus, an *Adenovirus*, showing cell rounding and small amounts of clumping. Cells were observed using the 10X objective (100X total magnification).

5. Foamy degeneration (vacuolization) is due to the production of large and/or numerous cytoplasmic vacuoles. Several virus families including certain retroviruses, paramyxoviruses, and flaviviruses (pestiviruses) may cause vacuolization (Fig. 10). Vacuolization is difficult to visualize without staining.



FIG. 10. Giemsa-stained bovine fetal spleen cells 1 day postinfection with the bovine viral diarrhea virus, a *Flavivirus*, showing vacuoles (arrow). Cells were observed with the 40X objective (400X total magnification). For a full description, see Giemsa-Stained Bovine Viral Diarrhea Virus (Flaviviridae)-Infected Bovine Fetal Spleen Cells Showing Cytopathic Effects.

6. Cell fusion (syncytium or polykaryon formation) involves the fusion of the plasma membranes of four or more cells to produce an enlarged cell with four or more nuclei. Small syncytia are readily seen only after staining. Syncytia formation may be the only detectable CPE of some paramyxoviruses; herpesviruses may produce syncytia as well as other forms of CPE (Fig. 11 and 12). Careful viewing of the cytoplasm is required to distinguish cell fusion from cell clumping or clustering, in which plasma membranes remain distinct. Syncytia are much easier to observe when cells have been stained.

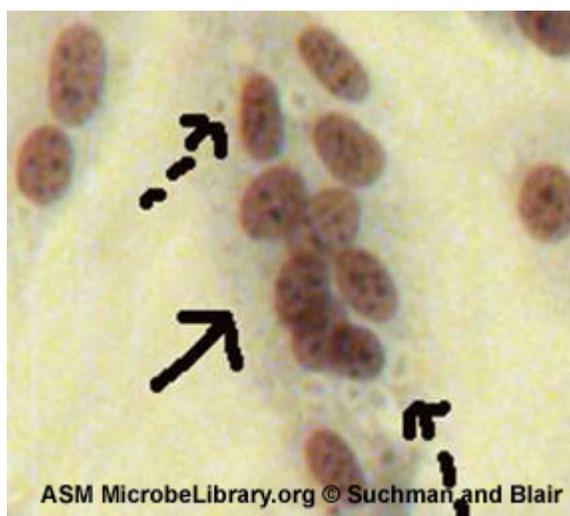


FIG. 11.

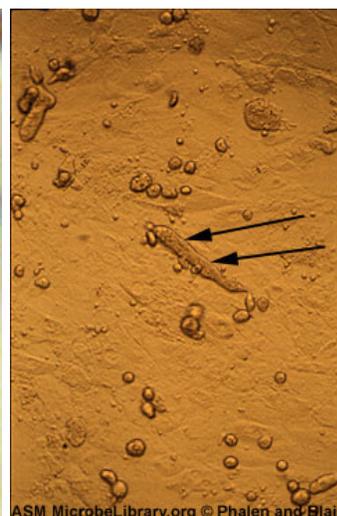


FIG. 12.

FIG. 11. Giemsa-stained bovine fetal spleen cells 4 days postinfection with the bovine respiratory syncytial virus, a *Paramyxovirus*, showing syncytia (arrows) and faint basophilic cytoplasmic inclusion bodies (dashed arrows). Cells were observed with the 20X objective (200X total magnification). For a full description, see Giemsa-Stained Bovine Respiratory Syncytial Virus (Paramyxoviridae)-Infected Bovine Fetal Spleen Cells Showing Cytopathic Effects and Inclusions.

FIG. 12. Bovine fetal spleen cells infected with the parainfluenza virus type 3, a *Paramyxovirus*, showing syncytia (arrows).

7. Inclusion bodies are areas of altered staining in cells, which cannot be seen in live cell cultures. Depending on the causative virus, these inclusions may be single or multiple, large or small, round or irregularly shaped, intranuclear (Fig. 13–15) or intracytoplasmic (Fig. 16), eosinophilic (pink staining) or basophilic (blue-purple staining). Chromatin margination may also be present and causes a thin stained ring to form around the edge of the nucleus (Fig. 17). In most cases, inclusion bodies indicate areas of the cell where viral protein or nucleic acid is being synthesized or where virions are being assembled, but in some cases no virus is present and the inclusion bodies indicate areas of viral scarring.

Nuclear inclusions:



FIG. 13.

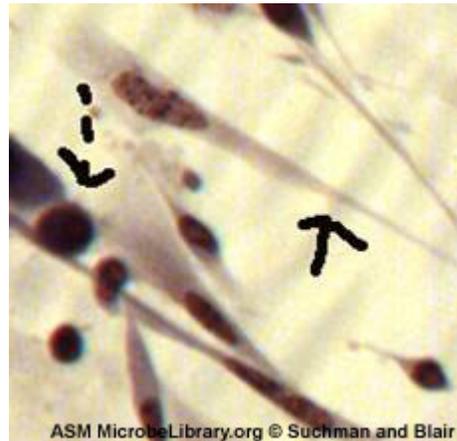


FIG. 14

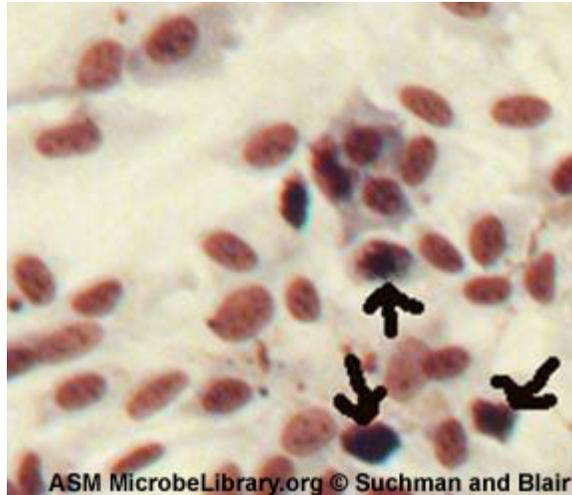


FIG. 15.

FIG. 13. Giemsa-stained bovine fetal spleen cells 3 days postinfection with bovine herpes virus 2, a *Herpesvirus*, showing syncytia and nuclear inclusion bodies (arrow). Cells were observed with the 40X objective (400X total magnification). For a full description, see Giemsa-Stained Bovine Herpes Simplex Virus Type 2 (Herpesviridae)-Infected Bovine Fetal Spleen Cells Showing Cytopathic Effects and Inclusions.

FIG. 14. Giemsa-stained bovine fetal spleen cells 1 day postinfection with bovine herpes virus 1, a *Herpesvirus*, showing cytoplasmic stranding (arrow) and nuclear inclusion bodies (dashed arrow). Cells were observed with the 20X objective (200X total magnification). For a full description, see Giemsa-Stained Bovine Herpes Simplex Virus Type 1 (Herpesviridae)-Infected Bovine Fetal Spleen Cells Showing Cytopathic Effects.

FIG. 15. Giemsa-stained bovine fetal spleen cells 4 days postinfection with bovine adenovirus, an *Adenovirus*, showing rough-edged nuclear inclusion bodies (arrow). Cells were observed with the 20X objective (200X total magnification). For a full description, see Giemsa-Stained Bovine Adenovirus (Adenoviridae)-Infected Bovine Fetal Spleen Cells Showing Inclusions.

Cytoplasmic inclusions:

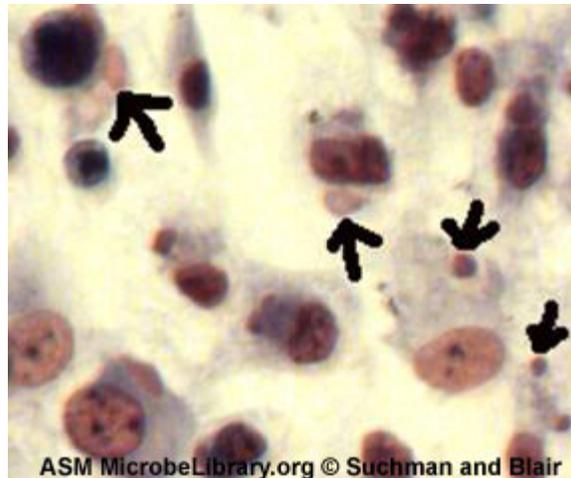


FIG. 16. Giemsa-stained bovine fetal spleen cells 1 day postinfection with Orf virus, a *Poxvirus*, showing pink eosynophilic cytoplasmic inclusion bodies (arrows) and cell swelling near the top of the field. Cells were observed using the 20X objective (200X total magnification). For a full description, see Giemsa-Stained Orf Virus (Poxviridae)-Infected Bovine Fetal Spleen Cells Showing Cytopathic Effects and Inclusions

Note: see Fig. 11 for cytoplasmic inclusions.

Chromatin margination:

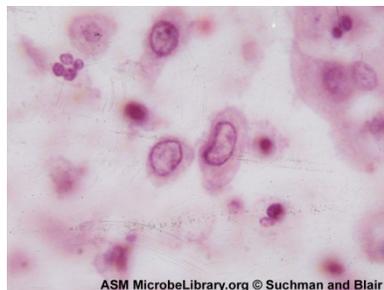


FIG. 17. A Giemsa-stained feline nasal turbinate primary cell culture infected with feline herpes virus-1, a *Herpesvirus*, showing chromatin margination (dark ring around the edge of the nucleus). Cells were observed with the 20X objective (200X total magnification). For a full description, see Feline Herpes Virus Diagnostics.

PROTOCOLS

General procedure for cultured animal cell infection

1. Carefully remove growth medium from cells taking care not to touch the dish to any surfaces to avoid contamination; try to avoid leaving residual medium around edges of wells. Add 1 ml of maintenance medium (fresh medium with 2% serum) to each well.
2. Make 10-fold dilutions of your viruses in dilution tubes, using 1.8 ml of

cold medium plus 0.2 ml of virus stock as diluent. Usually you will want to prepare a wide variety of dilutions of your virus such as 10^{-1} through 10^{-7} .

3. Inoculate 0.1 ml of each dilution into one well of cells, leaving one uninoculated control well for each set of virus dilutions. If you start with the highest (least concentrated) dilution, you may use the same pipette to do all inoculations of the same virus.

4. Incubate the cluster dish in a CO_2 incubator. Observe the dish daily in an inverted microscope to check for development of CPE; note and describe the type of CPE. Be sure to look at the wells that were inoculated with the highest dilutions (lowest MOI), since these will be better indicators of the rate of virus growth, focal versus general CPE, etc.

Giemsa stain for cell cultures

The May-Grunwald-Giemsa staining procedure is commonly used for virus-infected cell cultures and differentially stains acidophilic and basophilic organelles and inclusions. The Giemsa staining procedure described here does not discriminate between acidophilic and basophilic features but has the advantages of speed and simplicity. This procedure allows for observation of CPE such as cell fusion, vacuoles, and inclusions.

Materials

Coverslips with cell culture monolayers, previously infected

Bouin's fixative, Giemsa buffer, Giemsa stain (freshly prepared and filtered)

acetone, acetone:xylene (2:1 and 1:2)

Vials, glass slides, Permount

Procedure

1. Wash coverslips in phosphate-buffered saline.
2. Place coverslips in Bouin's fixative for 10 minutes.
3. Rinse coverslips in three changes of Giemsa buffer, 5 minutes each. The yellow color should be completely gone.
4. Immerse coverslips in Giemsa stain for 30 minutes or more until nuclei are stained well. You can remove the coverslips from the stain, rinse once in Giemsa buffer, and check stain with a regular or inverted microscope. Coverslips can then be returned to the stain if color isn't deep enough. (Note: if you have time, staining works much better if coverslips remain in the stain for 1 hour. Staining can be done in 30 minutes, in the interest of time, but inclusions will be fainter.)
5. Rinse coverslips briefly in Giemsa buffer.
6. Rinse coverslips in water.
7. Soak coverslips in acetone for 15 seconds.



8. Soak coverslips in acetone for 15 seconds.
9. Soak coverslips in acetone-xylene (2: 1) for 30 seconds.
10. Soak coverslips in acetone-xylene (1: 2) for 30 seconds.
11. Soak coverslips in xylene for 10 minutes.
12. Mount in permount, cell side down.
13. Examine the coverslips; look for CPE, inclusion bodies, syncytia, vacuoles, etc.

Bouin's fixative

Picric acid (saturated aqueous)	75 ml
Formalin (40% aqueous formaldehyde)	25 ml
Glacial acetic acid	5 ml

The May-Grunwald-Giemsa

Procedure

1. Place coverslips in methanol for 15 minutes.
2. Stain coverslips in May-Grunwald for 5 minutes.
3. Stain coverslips in Giemsa for 10 minutes.
4. Rinse coverslips in buffer pH 6.8
5. Rinse coverslips in a solution of 50% buffer 6.8 and 50% acetone.
6. Soak coverslips in acetone for 15 seconds.
7. Soak coverslips in acetone for 15 seconds.
8. Soak coverslips in xylene 15 seconds.
9. Soak coverslips in xylene 15 seconds.
10. Mount in permount, cell side down.
11. Examine the coverslips; look for CPE, inclusion bodies, syncytia, vacuoles, etc.

Notes: staining times and concentrations of working solutions may be adjusted as necessary. The staining solutions should be made fresh daily.

May-Grunwald stain working solution

190 ml of Sorenson's pH 6.8 buffer
25 ml of stock May-Grunwald solution

Giemsa buffer stain solution

190 ml of Sorenson's pH 6.8 buffer
25 ml of stock Giemsa solution

Note: stain should be diluted fresh daily.

Phosphate buffer (Sorenson's buffer) pH 6.8

5X Giemsa buffer (with preservative)

pH 6.5 to 6.6

Na_2HPO_4 19.10 g

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 30.90 g

Place salts in a 1-liter volumetric flask. Add about 400 ml of distilled water.

Dissolve the salts. Add 100 ml of 95% EtOH. Remove stir bar. Add water to mark.

Stock solutions:

0.2M dibasic sodium phosphate (dbsp) 1 liter

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (MW = 178.05) 35.61 g

or

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (MW = 268.07) 53.65 g

or

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (MW = 358.14) 71.64 g

+ ddH₂O to make 1 liter

0.2M monobasic sodium phosphate (mbsp) 1 liter

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (MW = 138.01) 27.6 g

or

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (MW = 156.03) 31.21 g

+ ddH₂O to make 1 liter

Working buffer: 0.1M 100 ml

Mix 24.5 ml of 0.2M dibasic sodium phosphate with 25.5 ml of monobasic sodium phosphate. Dilute to 100 ml with ddH₂O or dilute 1:1 with fixative.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

COMMENTS AND TIPS

This section is to evolve as feedback on the protocol is discussed at ASMCUE. Please contact the project manager for further information.

REFERENCES

1. **Enders, J. F.** 1954. Cytopathology of virus infections. *Annu. Rev. Microbiol.* 8:473–502.
2. **Fenner, F., B. R. McAuslan, C. A. Mims, J. Sambrook, and D. White.** 1973. *The biology of animal viruses, the student* 2nd ed. Academic Press, New York, NY.
3. **Knipes, D. M., C. E. Samuel, and P. Palese.** 2001. Virus-host cell interactions, p.134–136. *In* D. M. Knipes and P. M. Howley (ed.), *Fields virology*, 4th ed. Lippincott Williams and Wilkins, Philadelphia, PA.

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