Published in final edited form as:

Curr Protoc Microbiol. 2008 November ; APPENDIX: Appendix-4E. doi:
10.1002/9780471729259.mca04es11.

Growth and Maintenance of Vero Cell Lines

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Abstract

Vero cells are derived from the kidney of an African green monkey, and are one of the more commonly used mammalian continuous cell lines in microbiology, and molecular and cell biology research. This unit includes protocols for the growth and maintenance of Vero cell lines in a research laboratory setting.

Keywords

Vero cells; cell culture techniques; cell line

INTRODUCTION

Derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in the 1960s, Vero cells are one of the most common mammalian continuous cell lines used in research. This anchorage-dependent cell line has been used extensively in virology studies, but has also been used in many other applications, including the propagation and study of intracellular bacteria (*e.g.*, *Rickettsia* spp.; UNIT 3A.4) and parasites (*e.g.*, *Neospora*), and assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level. In addition, Vero cells have been licensed in the United States for production of both live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines, and throughout the world Vero cells have been used for the production of a number of other viruses, including Rabies virus, Reovirus and Japanese encephalitis virus. The protocols outlined in this appendix detail procedures for the routine growth and maintenance of Vero cells in a research laboratory setting. There are several lines of Vero cells commercially available (*i.e.*, Vero, Vero 76, Vero E6), but they were all ultimately derived from the same source, and the protocols in this unit can be used with any line of Vero cells.

BASIC PROTOCOL 1

PROPAGATION OF VERO CELL CULTURE FROM FROZEN STOCKS

For long term storage, Vero cells are kept either in liquid nitrogen or at -80°C. This protocol describes how to start growing Vero cells obtained from frozen stock. After recovery from frozen stock, Vero cells usually take 2-3 passages to reach their regular growth rate, and this should be taken into account if planning to use the cells for experiments, infections, *etc.* It is

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important to note that Vero cells are anchorage-dependent cells and therefore cannot be grown in suspension.

Materials

- Vero cell stock, frozen in liquid nitrogen or at -80°C
- Dulbecco's modification of Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), filter sterilized (see recipe)
- 15mL conical tubes, sterile
- 25cm² or 50cm² tissue culture flasks with vented caps, sterile
- Serological pipets, sterile
- 70% ethanol solution (used for decontamination of laminar flow hood and objects brought into the hood)

NOTE: All equipment and solutions coming into contact with cells must be sterile, and proper sterile technique should be used.

NOTE: All cell culture incubations are performed in a humidified 37°C incubator with 5% CO₂.

NOTE: All solutions should be warmed to room temperature or 37°C before contacting cells.

1. Quickly thaw vial (cryovial) of Vero cells by gently swirling in a 37°C water bath.
The water in the water bath is a potential source of contamination for the cells. To reduce the risk of contamination, keep the O-ring and cap of the cryovial out of the water.
2. In a laminar flow hood, decontaminate the vial by spraying with 70% ethanol.
3. Transfer the Vero cell suspension from the cryovial into a 15mL conical tube containing 10mL of DMEM supplemented with FBS.
Frozen cell stocks will contain the cryopreservant dimethyl sulfoxide (DMSO), which can be harmful to the cells. Therefore, after thawing the cells, it is necessary to dilute and remove the DMSO before transferring the cells to tissue culture flasks.
Media other than DMEM can also be used for growing Vero cells. See COMMENTARY for more information.
4. Pellet cells by centrifugation at 200 × g for 5 minutes at room temperature.
5. Remove and discard supernatant; resuspend cells in 5-10mL DMEM supplemented with 10% FBS.
Vero cells recover better after freezing when initiated in a small (25cm² or 50cm²) tissue culture flask. If using a 25cm² flask, resuspend the cells in 5mL media; if using a 50cm² flask, resuspend the cells in 10mL media.
6. Transfer Vero cell suspension to tissue culture flask with vented cap.
7. Incubate flasks in 37°C incubator with 5% CO₂.
8. Monitor cells daily or every other day. Change media every 3-4 days. When cells reach a >90% confluent monolayer, passage cells into new tissue culture flasks (see *BASIC PROTOCOL 2*).

Vero cells recover slowly after freezing; therefore, it may take a week or more before the cells are ready to be passaged. It may take 2 or 3 passages before the Vero cells reach their normal growth rate.

BASIC PROTOCOL 2

MAINTENANCE OF VERO CELL CULTURE

Vero cells are derived from normal kidney cells; because the cells are not transformed, they have not lost their contact inhibition. When these cells reach confluency, they stop growing and start to die; therefore, it is extremely important to monitor Vero cells and to subculture them as they form confluent monolayers. Actively growing Vero cell cultures double approximately every 24 hours (Nahapetian *et al.* 1986). Depending on the number of cells seeded and the flask size, the cells usually need to be passaged 2-3 times per week. This protocol describes a general method for the subculturing of Vero cells in 75cm² tissue culture flasks.

Materials

- Vero cells grown to a confluent monolayer in a 75cm² flask with vented cap
- DPBS without calcium or magnesium, filter-sterilized (see *APPENDIX 2A*)
- 1X trypsin-EDTA in DPBS without calcium or magnesium, filter-sterilized (see recipe)
- DMEM supplemented with 10% heat-inactivated FBS, filter-sterilized (see recipe)
- 75cm² tissue culture flasks with vented caps, sterile
- 15mL conical tubes, sterile
- Serological pipets, sterile

NOTE: All equipment and solutions coming into contact with cells must be sterile, and proper sterile technique should be used.

NOTE: All cell culture incubations are performed in a humidified 37°C incubator with 5% CO₂.

NOTE: All solutions should be warmed to room temperature or 37°C before contacting cells.

1. Remove growth medium from confluent monolayer of Vero cells.
2. Wash cells with 10mL 1X DPBS.
Serum contains trypsin inhibitors, so it is important to rinse off any remaining media with DPBS.
3. Add 5mL of 1X trypsin-EDTA and incubate cells at 37°C for 2-3 minutes, until cells start to streak as they detach from the flask.
Gentle shaking or tapping of the flask may help cells detach.
4. Add 5mL DMEM with 10% FBS to inactivate the trypsin-EDTA.
5. Wash down cells in media, pipetting gently to break up any clumps of cells.
6. Remove cell suspension from flask and transfer to a sterile 15mL conical tube.
7. Centrifuge at $200 \times g$ for 5 minutes at room temperature.

8. Remove and discard supernatant; resuspend cells in 10mL DMEM with 10% FBS.
9. Prepare desired dilution of cells in a total of 12-20mL DMEM with 10% FBS and add to 75cm² cell culture flasks with vented caps.

Dilutions of 1:5 to 1:10 are typical for routine cell culture. See Table A.1 for recommended total medium volumes for growth in various sizes of tissue culture vessels.

10. Incubate flasks in 37°C incubator with 5% CO₂.

Monitor cells daily or every other day. Change media every 3-4 days. When cells reach a >90% confluent monolayer, passage cells again by repeating this protocol.

BASIC PROTOCOL 3

PREPARATION OF FROZEN STOCKS OF VERO CELL CULTURE

Maintenance of frozen stocks is extremely important when culturing cell lines. When actively growing cells will not be needed for an extended period of time (3 weeks or more), keeping frozen stocks allows researchers to discontinue regular subculturing, saving valuable time and money. Also, very importantly, frozen stocks of cells provide a new source of cells should contamination occur during subsequent passages. In order to maintain an inventory of low-subculture Vero cells, frozen stocks should be prepared shortly after initiating cultures from frozen stocks.

Materials

- Vero cells grown to a confluent monolayer in a 75cm² flask with vented cap
- DPBS without calcium or magnesium, filter-sterilized (see *APPENDIX 2A*)
- 1X trypsin-EDTA in DPBS without calcium or magnesium, filter-sterilized (see recipe)
- DMEM supplemented with 20% heat-inactivated FBS, filter-sterilized (see recipe)
- Dimethyl sulfoxide (DMSO)
- Cryovials suitable for freezing at -80°C or liquid nitrogen, sterile
- 15mL conical tubes, sterile
- Serological pipets, sterile

CAUTION: DMSO is hazardous; see *UNIT 1A.3* for guidelines on handling, storage, and disposal.

NOTE: All equipment and solutions coming into contact with cells must be sterile, and proper sterile technique should be used.

NOTE: All solutions should be warmed to room temperature or 37°C before contacting cells.

1. In laminar flow hood, add 1mL DMSO to 9mL of the supplemented DMEM (for a final concentration of 10% DMSO) in a 15mL conical tube.

DMSO will dissolve cellulose acetate membranes commonly used for filter-sterilization, so it should be added after the DMEM has been supplemented with FBS. The DMSO can be filtered using nylon membrane filters; alternatively, only open the DMSO bottle under sterile conditions in the laminar flow hood.

2. Remove growth medium from confluent monolayer of Vero cells.
3. Wash cells with 10mL 1X DPBS.
Serum contains trypsin inhibitors, so it is important to rinse off any remaining media with DPBS.
4. Add 5mL of 1X trypsin-EDTA and incubate cells at 37°C for 2-3 minutes, until cells start to streak as they detach from the flask.
Gentle shaking or tapping of the flask may help cells detach.
5. Add 5mL DMEM with 20% FBS to inactivate the trypsin-EDTA.
6. Wash down cells in media, pipetting gently to break up any clumps of cells.
7. Remove cell suspension from flask and transfer to a sterile 15mL conical tube.
8. Centrifuge at $200 \times g$ for 5 minutes at room temperature.
9. Remove and discard supernatant; resuspend cells in 10mL DMEM with 20% FBS and 10% DMSO.
The DMSO and FBS help preserve the cells during the freezing and thawing processes, respectively.
10. Aliquot 1mL of resuspended cells into each cryovial.
11. Freeze cells slowly to -80°C, then continue to store the cells at -80°C or in liquid nitrogen (preferred).

It is ideal to freeze the cells with the temperature decreasing at a rate of -1°C per minute. This can be achieved using freezing containers such as the Nalgene Cryo 1°C Freezing Container. Alternatively, the cells can be put at 4°C for several hours, then at -20°C overnight, then at -80°C overnight, and then either kept at -80°C or transferred into liquid nitrogen storage.

REAGENTS AND SOLUTIONS

Dulbecco's modification of Eagle Medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS)—10% or 20% heat-inactivated FBS (see *APPENDIX 2A*)

Bring to desired volume in DMEM

Filter-sterilize

Store at 4°C

To make 500mL DMEM with 10% FBS, add 50mL heat-inactivated FBS to 450mL DMEM.

To make 500mL DMEM with 20% FBS, add 100mL heat-inactivated FBS to 400mL DMEM.

1X trypsin-EDTA in DPBS—Dilute 10X trypsin-EDTA in DPBS (see *APPENDIX 2A*) to obtain the desired volume with a 1X final concentration.

Filter-sterilize

Store at 4°C

To make 100mL, add 10mL 10X trypsin-EDTA to 90mL DPBS.

COMMENTARY

Background Information

Vero cells were originally isolated from the kidney of a normal (*i.e.*, non-diseased) adult African green monkey on March 27, 1962 by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. At its 93rd passage, the cell line was brought to the National Institute of Allergy and Infectious Diseases at the National Institutes of Health in the United States, and was provided to the American Type Culture Collection (ATCC) in 1966. By the end of the 1960s, Vero cell lines were being used across the globe, primarily in virology laboratories.

Vero cells can be purchased from ATCC and the European Collection of Animal Cell Cultures (ECACC) repositories. Commonly used Vero cell lines from the ATCC include CCL-81 (Vero), CRL-1286 (Vero C1008), and CRL-1587 (Vero 76). Commonly used cell lines from the ECACC include 84113001 (Vero), 85020206 (Vero C1008), 88020401 (Vero-WHO), and 85020205 (Vero 76).

Critical Parameters and Troubleshooting

Mycoplasma contamination—One of the most important issues when growing any cell culture is contamination. To reduce the risk of contamination, always work with the cells in a sterile, laminar flow hood, make sure all equipment and solutions that come into contact with the cells are sterile, and use proper sterile technique when working in the hood. Many researchers will maintain their cells with a low level of antibiotic added to the medium, most commonly a penicillin/streptomycin mixture. This can also help to reduce extraneous bacterial contamination; however, depending on the application for which the Vero cells will be used, adding antibiotics may not be recommended (for example, if the cells are to be infected with bacteria). See Sato and Kan 2001 for a discussion regarding the use of antibiotics in mammalian cell cultures. One of the most common sources of contamination in cell cultures is *Mycoplasma*. These bacteria are very small (< 1µm), and therefore contamination with *Mycoplasma* may not be visible with the naked eye, making these organisms difficult to detect. Several *Mycoplasma* detection kits are commercially available (such as the MycoAlert kit from Lonza, see Mariotti *et al.* 2008), and procedures for detection and treatment of *Mycoplasma* contamination are also outlined in *APPENDIX 3B*. Vero cells should be regularly tested (once per month) for the presence of *Mycoplasma* contamination. If cells are found to be infected, the recommended course of action is to discard the cells and start new cultures from uninfected frozen stocks. There are also several options for treating *Mycoplasma*-infected cells (see Uphoff and Drexler 2002), including commercially available kits.

Culture media—The protocols in this appendix describe the growth and maintenance of Vero cells using DMEM as the culture medium. While DMEM is a very common culture medium, a variety of other media can also be successfully used with Vero cells. See Sato and Kan 2001 for a description of different culture media that can be used with mammalian cell lines.

Depending on the application, it may be desired or necessary to count the number of cells (*i.e.*, if a specific number of cells need to be analyzed, plated, *etc.*) The concentration of cells in suspension (following trypsin treatment) can be determined using a hemacytometer. See Phelan 2007 for a complete protocol for determining the number of viable cells in solution.

When the Vero cells will be used for bacterial infections, the dividing host cells can dilute the infections, which, depending on the system, can confound the analysis of the results. One technique that has been employed to address this issue is gamma irradiation. At appropriate levels, irradiation does not kill the Vero cells, but does prevent cell division, allowing bacterial growth in Vero cells without the complicating factor of host cell division and growth. See Chen *et al.* 1995 and Zamboni *et al.* 2001 for examples of gamma-irradiation of Vero cells prior to infection with *Ehrlichia chaffeensis* and *Coxiella burnetii*, respectively.

Certain applications, such as vaccine production, may require the scaling-up of Vero cell cultures. There are two growth systems used for the scaling-up of anchorage-dependent cell lines: roller bottles and microcarriers. Roller bottles are cylindrical vessels, and the cells grow on the inner surface of the tube. The bottles slowly revolve to continually bathe the cells in growth medium. The surface area available for cell attachment can be even further increased by growing the cells on microcarrier beads. The beads, usually around 0.2mm, can be made of dextran, cellulose, gelatin, glass or silica, and can considerably increase the surface area available for Vero cell growth. See Hegde *et al.* 2008 and Silva *et al.* 2008 for examples of Vero cell growth using roller bottles and microcarriers, respectively, for the production of viral vaccines.

Anticipated Results

When first starting a seed of Vero cells from frozen stock, the cells will usually need a couple of passages before they start growing at their normal rate, which is doubling approximately every 24 hours (Nahapetian *et al.* 1986). After this initial “start-up” period, the cells will proliferate regularly throughout subsequent passages. The use of proper sterile technique when subculturing the cells should result in successful propagation of the Vero cells. An example of Vero cells at around 95% confluency is shown in Figure 1.

Time Considerations

When subculturing Vero cells at a ratio between 1:5 to 1:10, they will generally need to be passaged 2-3 times per week. If splitting the cells at a 1:10 ratio, the medium might need to be changed between subculturing.

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Internet Resources

- <http://www.atcc.org> *The American Type Culture Collection (ATCC) website. The ATCC maintains a cell repository, which includes Vero cells. This site provides additional information regarding the growth and maintenance of Vero cells.*
- <http://www.ecacc.org.uk> *The European Collection of Animal Cell Cultures (ECACC) website. The ECACC maintains a cell repository, which includes Vero cells. This site provides additional information regarding the growth and maintenance of Vero cells.*

Figure 1.
Vero 76 cells (ATCC #CRL-1587), approximately 95% monolayer. These cells will need to be subcultured within one day.

Table A.1

Cell medium volume requirements

Vessel	Total medium volume
25cm ² flask	4-6 mL
50cm ² flask	7-10 mL
75cm ² flask	12-20 mL
150cm ² flask	25-40 mL
96-well plate	100 μ L (per well)
16mm dish	800 μ L
35mm dish	2 mL
60mm dish	8 mL